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A multi-parameter approach to measurement of spontaneous myogenic contractions in human stomach: Utilization to assess potential modulators of myogenic contractions

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ABSTRACT

Electrical slow waves, generated by interstitial cells of Cajal (ICC), cause spontaneous contractions of human stomach. Software was developed to measure muscle tone and eleven different parameters defining these contractions in human stomach, displaying data as radar plots. A pilot study assessed the effects of potential modulators, selected from among compounds known to influence ICC activity; n = 4–7 each concentration tested/compound. Human distal stomach (corpus-antrum) muscle strips were suspended in tissue baths for measuring myogenic (non-neuronal) contractions in the presence of tetrodotoxin (10^{-6} M). *Initial characterization:* Contractions (amplitude 4 ± 0.4mN, frequency 3 ± 0.1 min^{-1}, n = 49) were unchanged by ω-conotoxin GVIA (10^{-7} M) or indomethacin (10^{-6} M) but abolished by nifedipine (10^{-4} M). Carbachol (10^{-7} M) increased contraction rate and amplitude; 10^{-6}-10^{-5} M increased tone and caused large, irregular contractions. *$[Ca^{2+}]_i$ modulators*: Ryanodine (10^{-5}-10^{-4} M) increased muscle tone accompanied by inhibition of myogenic contractions. Xestospongin-C (10^{-6} M; IP_3 channel inhibitor) had no effects. SERCA pump inhibitors, 2-APB and cycloplazonic acid (10^{-5}-10^{-4} M) increased tone and myogenic contraction amplitude before abolishing contractions; thapsigargin was weakly active. *CaCC blockers*: MONNA and CaCCinh-A01 had little-or-no effects on tone but reduced myogenic contractions; MONNA (10^{-4} M) was more effective, reducing amplitude (77.8 ± 15.2%) and frequency. *$Ca_V3.1/3.2/3.3$ channel block*: Mibefradil reduced tone and myogenic contraction amplitude (pIC_{50} 4.8 ± 0.9). *Inward-rectifying K^+-channel inhibitor*: E-4031 (10^{-4} M) increased contraction duration (17.4 ± 5.8%). Conclusions: (1) Measurement of multiple parameters of myogenic contractions identified subtle differences between compounds, (2) only E-4031 and CaCC blockers influenced myogenic contractions, not muscle tone, (3) studies are needed with compounds with known and/or improved selectivity/potency for human targets affecting ICC functions.

Abbreviations: $[Ca^{2+}]_i$, intracellular calcium; CaCC, calcium-activated chloride channel, sometimes known as the ANO1 channel; ICC, interstitial cells of Cajal; IP_3, inositol 1,4,5-trisphosphate; K_V, voltage-dependent potassium channel; Na_V, voltage-dependent sodium channel.; mACh, muscarinic ACh receptor; pIC_{50}, negative $logarithm_{10}$ of the half-maximal inhibitory concentration; RyR, ryanodine receptor; SERCA, sarcoplasmic-endoplasmic Ca^{2+}-ATPase; TRP, transient receptor potential channel; TTX, tetrodotoxin.

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1. Introduction

A dominant region of pacemaker cells at the greater curvature of human stomach (mid-to-upper corpus) generates slow waves of electrical activity that propagate in circumferential bands towards the antrum [1]. These are the interstitial cells of Cajal (ICC), which spontaneously depolarise and propagate slow waves via anastomosing networks of other ICCs, which in human stomach are within the plane of the myenteric plexus and found within the circular and longitudinal muscle and between the septa surrounding the fascicles of circular muscle [2–4]. The ICC together with platelet-derived growth factor receptor-α + cells, electrically couple with smooth muscle cells to conduct the slow waves into the muscle, generating phasic contractions and facilitating nerve-muscle signalling [5,6]. In other areas of the gastrointestinal tract, at least in mice, the enteric nervous system may play a more dominant role over the ICC [7].

In human stomach, the pacemaker function of the ICC may become damaged, perhaps because of a reduction in ICC numbers or impairment of their function, leading to disrupted patterns of electrical slow wave activity, irregular and retrograde movements of the stomach, delayed gastric emptying, redistribution of gastric contents, and promotion of reflux and symptoms such as nausea [8,9]. Gastroparesis, for example, is associated with irregular gastric electrical activity [1] and reduced numbers of ICC, in addition to immune and macrophage infiltrate, damaged myenteric neurons, pyloric fibrosis [10] and delayed gastric emptying [11]. It is important, therefore, to look for potential therapeutics which can selectively modulate ICC functions for treatment of gastric disorders associated with nausea.

In cell-based assays and in animal tissues, different compounds with an ability to modulate ICC functions can influence slow wave electrical activity; some have also been shown to modulate the spontaneous, phasic contractions of gastric or intestinal muscle from rodents (e.g., mouse, guinea-pig [6]). However, these compounds have not been evaluated for any ability to modulate the spontaneous contractions of the human stomach. This is an important omission because gastric functions of rodents differ markedly from humans, including gross differences in stomach anatomy and physiology, possibly associated with loss of ability to vomit [12]. Further, the larger, thicker human stomach musculature needs a greater distribution and inter-connectivity of ICC to propagate electrical activity to the different regions of the muscle, which could not otherwise regenerate the depolarisation provided by the ICC [4,13]. The actions of compounds which modulate spontaneous contractions of the rodent stomach must therefore be translated to the human stomach.

Well-established methods exist to study the functions of compounds on movements of the human stomach, with most examining their ability to change muscle tone and/ or influence responses to exogenous agents or nerve stimulation [14]. One study has shown a temporal relationship between spontaneous, phasic muscle contractions of human isolated stomach, resistant to inhibition of neuronal action potentials by tetrodotoxin (TTX), and the occurrence of slow wave electrical activity thought to originate from the ICC (as measured by intracellular muscle recording [4]). In the present study, software was developed to measure eleven different parameters of these contractions in human isolated stomach, together with muscle tone, before visualisimg the data as radar plots. Using this technique, the spontaneous contractions were further characterised in the presence of TTX (hereafter referred to as non-neuronally-mediated or 'myogenic contractions'), and a pilot study conducted to assess the effects of potential modulators of the contractions. These included compounds reported to influence ICC functions by (1) reducing intracellular Ca^{2+} availability (by blocking pacemaker current initiated by Ca^{2+} release from IP_3 [inositol 1,4,5-trisphosphate] and possibly ryanodine receptor operated stores, and by blocking the SERCA [sarcoplasmic-endoplasmic Ca^{2+}-ATPase] pump responsible for sequestration of Ca^{2+} from the cytosol into organelles during recovery from cell excitation), (2) blocking the opening of calcium-activated chloride channels (CaCC) and (3), modulating key ion channels involved in ICC depolarisation [6].

It was important to recognise that the compounds under investigation often have unknown affinity and unknown selectivity for their human targets, may be non-selective in their activity in other species, and may sometimes affect the same molecular target in different cell systems. Thus, the purpose was not to investigate ICC mechanisms but to identify compounds from among those influencing ICC function, which modulate myogenic contractions of the human stomach without affecting muscle tone.

2. Materials and methods

2.1. Human stomach

Stomach tissue was obtained from human donors (8 male, 41 female, age 20–67 [median 45] years, median Body Mass Index 46) undergoing vertical sleeve gastrectomy (removing stomach ~2 cm distal to the gastro-esophageal junction, ~8 cm proximal to the pylorus and ~$^2/_3^{rd}$ of the width between the curvatures). Supplementary Table 1 summarizes patient anthropometric measurements, medications and comorbidities. Local ethical approval (REC-15/LO/2127) and written informed consent was obtained from all donors. Sections ~5 cm^2 were cut ~5 cm from the distal corpus-antrum region (referred to as distal stomach), excluding areas damaged during surgery, and transported to the laboratory in Krebs-Henseleit solution (mM: NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, D-glucose 11.1, $CaCl_2$ 2.5) pre-gassed with 95% O_2/5% CO_2.

The mucosa was removed by blunt dissection and a maximum of eight muscle strips (5x20mm) cut perpendicular to the greater curvature and parallel to circular muscle fibres. Detailed methods for collecting and minimising variation when using human GI tissue have been described [14]. Tissues were used after overnight (15–18 h) storage at 4ºC in pre-oxygenated Krebs-Henseleit solution. The spontaneous, electrically-evoked and agonist-evoked contractions have been demonstrated to be unaffected by this storage [see 14 and 15, and supporting references cited within].

2.2. Functional studies

Muscle strips were mounted in 10 ml tissue baths containing Krebs-Henseleit solution at 37 °C, bubbled with 5% CO_2 in O_2 and connected to isometric force transducers (MLT201/D AD Instruments, UK) using cotton sutures. After 15 min tissues were stretched to apply a tension of 20mN and allowed to equilibrate for 2.5 h with renewal of Krebs-Henseleit solution at 20 min intervals. Changes in baseline muscle tone were recorded in milliNewtons (mN) using an AcqKnowledge v3.8.1 data acquisition system (BIOPAC Systems, USA) on a personal computer (Dell, www.dell.com/uk).

After recording uniform contractions for 60 min, muscle strips were incubated with tetrodotoxin (TTX) 10^{-6} M, atropine (ATR) 10^{-6} M and L-NAME 3×10^{-4} M, followed by application of a test compound or their vehicle for 60 min, either as a single concentration per muscle strip or in cumulatively increasing concentrations. In some experiments, strips were treated with only TTX 10^{-6} M before assessing the effect of a test compound.

2.3. Data analysis and statistics

Donor tissues were used as they became available, without regard to donor sex, age or BMI, but the availability of patient details enabled some retrospective analysis of patient characteristics (see Results). Randomisation was further achieved by use of different tissue baths and recording equipment when repeating experiments with the same concentration of a particular compound. During the experiment, the experimenter was not blinded to the identity of the compound or its

concentration. Muscle strips from among those cut from the same stomach specimen, which did not exhibit spontaneous contractions or in which the contractions were < 0.9 mN, were excluded. Group size (N = 5–7 tissue donors) for investigating each concentration of each potential modulator of myogenic contractions were consistent with those used in comparable published studies with human stomach [4,15].

To complete the construction of concentration-response curves, low concentrations of compounds that were without consistent activity were examined using small numbers of tissue donors (n = 3–4); these were not statistically analysed.

Recorded spontaneous contractions were quantified automatically using custom software: https://github.com/agharibans/GISMCA. The parameters measured were: change in baseline muscle tone (mN), amplitude of spontaneous contractions (mN), number of contractions/ minute (c.min^{-1}) during steady-state, total contraction time (s), time for contractions to peak and decay (s), rates for contraction development and decay (mN s^{-1}) and area under the contraction and of the rising and decay phases (mN.s). These were visualised together as radar plots using OriginPro, v2020 (OriginLab Corporation, USA) (Fig. 1). Since the magnitude of response to a test compound may depend upon the baseline values, the changes in contraction parameters caused by application of a test compound were compared to the baseline values in that experiment, before the first addition of the test compound.

Data were analyzed using GraphPad Prism 7.0 for Windows (GraphPad, USA) and expressed as the mean ± standard error of the mean (mean ± SEM) or median; n values are numbers of patients.

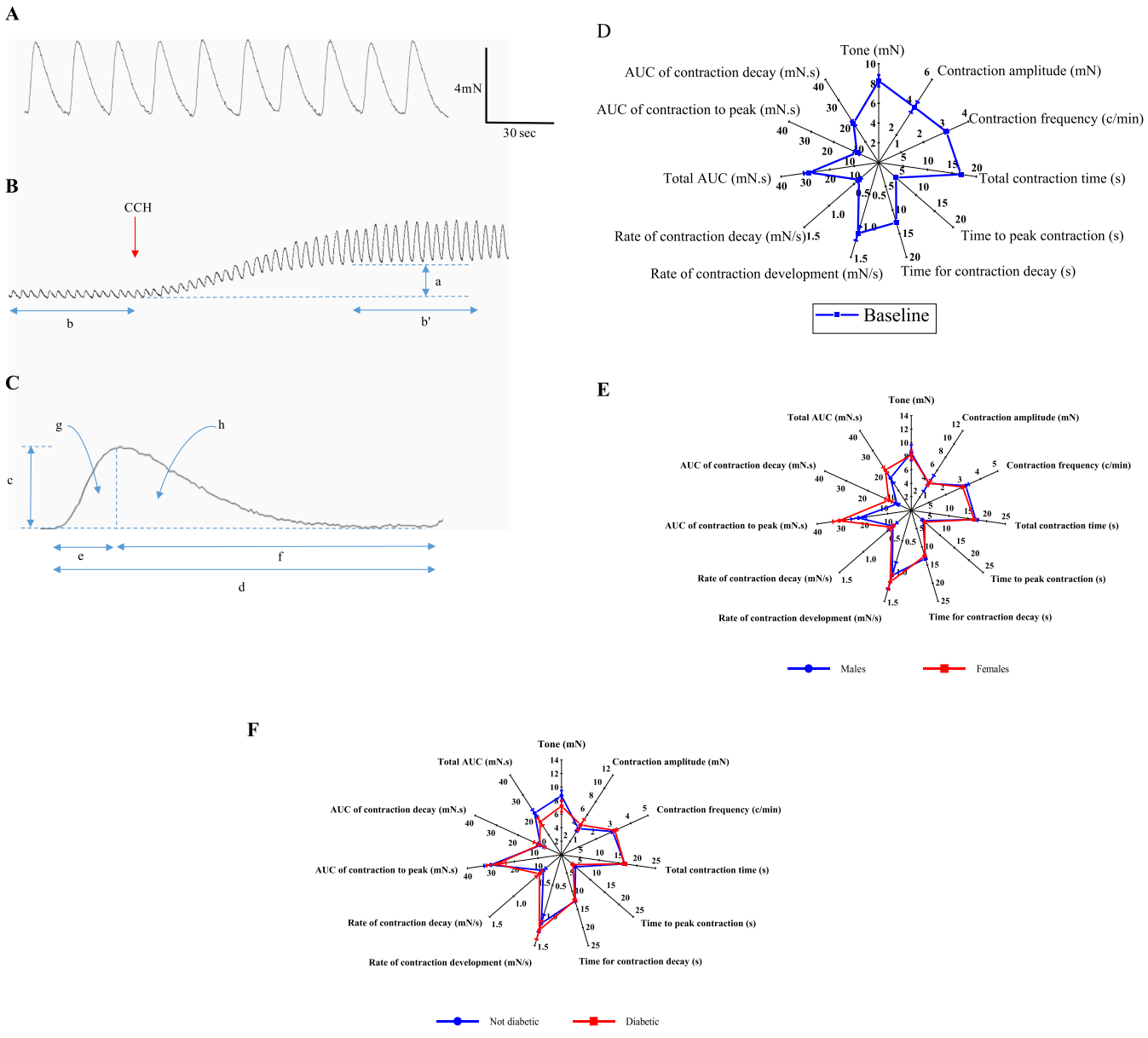


Fig. 1. Panel A: Baseline spontaneous contractions of human isolated distal stomach circular muscle. Panel B: Change in muscle tone (mN), and frequency of spontaneous contractions (number of contractions/ minute; c min-1) before, b and after, b', application of a compound e.g Carbachol (CCH) usually (see Methods for detail) measured over a 5 min steady-state period. Panel C: Components of a spontaneous contraction wave where c = Contraction amplitude (mN), d = Total contraction time (s), e and f = Time for contraction to peak and decay (s), g and h = Area under the curve for contraction to peak and decay (mN.s). Sum of g and h = Total area under curve of the contraction (mN.s), mean change in c over e and c over f = Rate of contraction development and decay (mN.s-1). Panel (D) Radar plot illustrating parameters measured in the absence of manipulation by added compounds. Each point represents the mean and standard error of mean (S.E.M.) of a one randomly selected stomach strip per donor, n = 49 donors. Panels (E) and (F) Radar plots illustrating parameters measured in the absence of any test molecule/ blocker from (E) males (n = 8) and females (n = 41) and (F) people with (n = 15) and without (n = 34) diagnosis of diabetes. Data mean ± S.E.M.

Student's *t*-tests compared data from within a given stomach (paired) or between stomachs from different donors (unpaired), respectively. Only one muscle strip was used from a donor for a given experiment. The declared group size is the number of independent values obtained from different donors and statistical analysis was undertaken only where each group size was at least n = 5; the probability $P < 0.05$ was considered statistically significant.

2.4. Materials

All drugs were freshly prepared prior to use. ATR (atropine sulphate), CCH (carbamylcholine chloride), L-NAME (N_ω-nitro-L-arginine methyl ester hydrochloride) (Sigma-Aldrich, UK) and tetrodotoxin (Tocris, UK) were each dissolved in distilled water. ω-conotoxin GVIA, nifedipine (dimethyl 2,6-dimethyl-4-(2-nitrophenyl)− 1,4-dihydropyridine-3,5-dicarboxylate), indomethacin (2-[1-(4-chlorobenzoyl)− 5-methoxy-2-methylindol-3-yl]acetic acid), E-4031 (N-[4-[1-[2-(6-

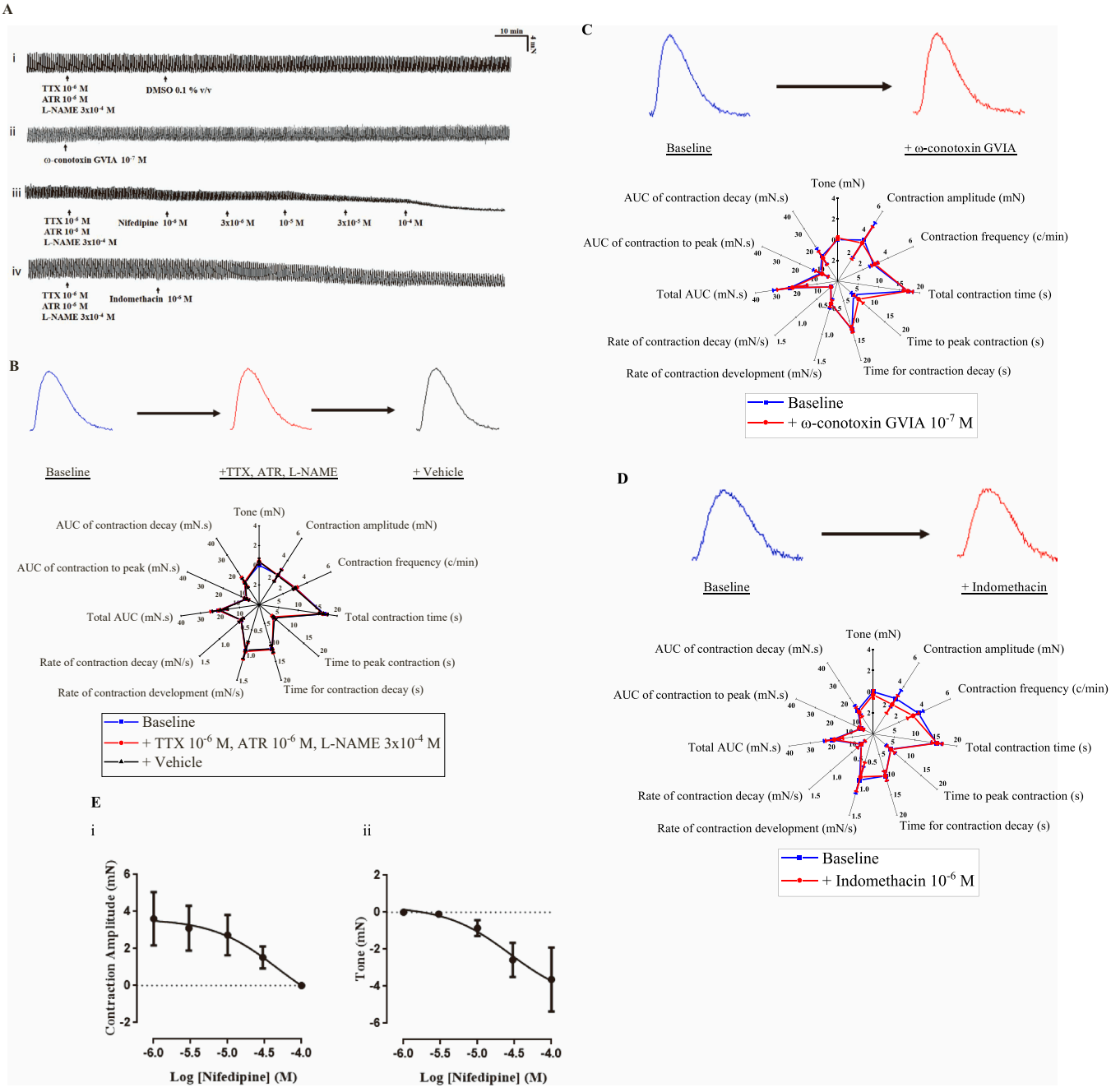


Fig. 2. Myogenic origin of spontaneous contractions of human isolated distal stomach circular muscle. Panel A: Experimental records illustrating the contractions in the absence and presence of different drugs or their vehicle (DMSO). Panels B-D show the changes in contraction parameters given as a Radar plot, before and after a test substance or its vehicle; a representative contraction in the absence and presence of test molecules is shown above each plot. Panel E shows the inhibition of (i) myogenic contraction amplitude and (ii) muscle tone caused by increasing concentrations of nifedipine. All treatments except the addition of DMSO (vehicle) and ω-conotoxin GVIA were applied 30 min after incubation with tetrodotoxin (TTX), L-NAME and atropine (ATR), and changes caused by addition of a test substance were compared with the baseline values immediately prior to its addition. Each data point represents the mean with standard error of mean. N = 7 vehicle (DMSO), n = 5 ω-conotoxin GVIA 10^{-7} M, n = 5 nifedipine, n = 9 indomethacin 10^{-6} M. * $P < 0.05$ versus control (*t*-tests).

methylpyridin-2-yl)ethyl]piperidine-4-carbonyl]phenyl]methanesulfonamide), MONNA (N-((4-methoxy)− 2-naphthyl)− 5-nitroanthranilic acid), CaCCinh-A01 (6-(1,1-Dimethylethyl)− 2-[(2-furanylcarbonyl)amino]− 4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylic acid), xestospongin C ((1 R,4aR,11 R,12aS,13 S,16aS,23 R,24aS)-Eicosahydro-5 H,17 H-1,23:11,13-diethano-2 H,14 H-[1,11]dioxacycloeicosino[2,3-b:12,13-b']dipyridine), 2-APB (2-di(phenyl)boranyloxyethanamine), CPA (cyclopiazonic acid; (6aR,11aS,11bR)-rel-10-Acetyl-2,6,6a,7,11a,11b-hexahydro-7,7-dimethyl-9 H-pyrrolo[1',2':2,3]isoindolo[4,5,6-cd]indol-9-one) (Tocris, UK), thapsigargin (3 S,3aR,4 S,6 S,6aR,7 S,8 S,9bS)− 6-(acetyloxy)− 4-(butanoyloxy)− 3,3a-dihydroxy-3,6,9-trimethyl-8-{[(2Z)− 2-methylbut-2-enoyl]oxy}− 2-oxo-2 H,3 H,3aH,4 H,5 H,6 H,6aH,7 H,8 H,9bH-azuleno[4,5-*b*]furan-7-yl octanoate), ryanodine (1 H-Pyrrole-2-carboxylic acid, (3 S,4 R,4aR,6 S,7 S,8 R,8aS,8bR,9 S,9aS)-dodecahydro-4,6,7,8a,8b,9a-hexahydroxy-3,6a,9-trimethyl-7-(1-methylethyl)− 6,9-methanobenzo[1,2]pentaleno[1,6-bc]furan-8-yl ester) and mibefradil ([(1 S,2 S)− 2-[2-[3-(1 H-benzimidazol-2-yl)propyl-methylamino]ethyl]− 6-fluoro-1-propan-2-yl-3,4-dihydro-1 H-naphthalen-2-yl]2-methoxyacetate) (Aobious, USA) were each dissolved in DMSO. All reagents (Sigma-Aldrich, UK) for preparing the Krebs-Henseleit solution were dissolved in distilled water.

3. Results

3.1. Characterization of myogenic contractions

Regular spontaneous contractions occurred at 3.0 ± 0.1 contractions.min^{-1} with an amplitude of 4.0 ± 0.4 mN (n = 49). Each developed rapidly and decayed significantly more slowly at respectively, 1.1 ± 0.1 and 0.4 ± 0.0 mN.s^{-1} ($P < 0.0001$; n = 49; Fig. 1). Neither the occurrence of spontaneous contractions nor the contraction waveform was dependent on donor's sex, age or diagnosis of diabetes (Fig. 1E, F). The presence of TTX (10^{-6} M), ATR (10^{-6} M) and L-NAME (3×10^{-4} M) for 60 min did not affect the contractions (n = 7, $P > 0.05$ each; Fig. 2A, B). These contractions were also unaffected by 30 min incubation with ω-conotoxin GVIA (CTX) 10^{-7} M (n = 5; a concentration that blocked

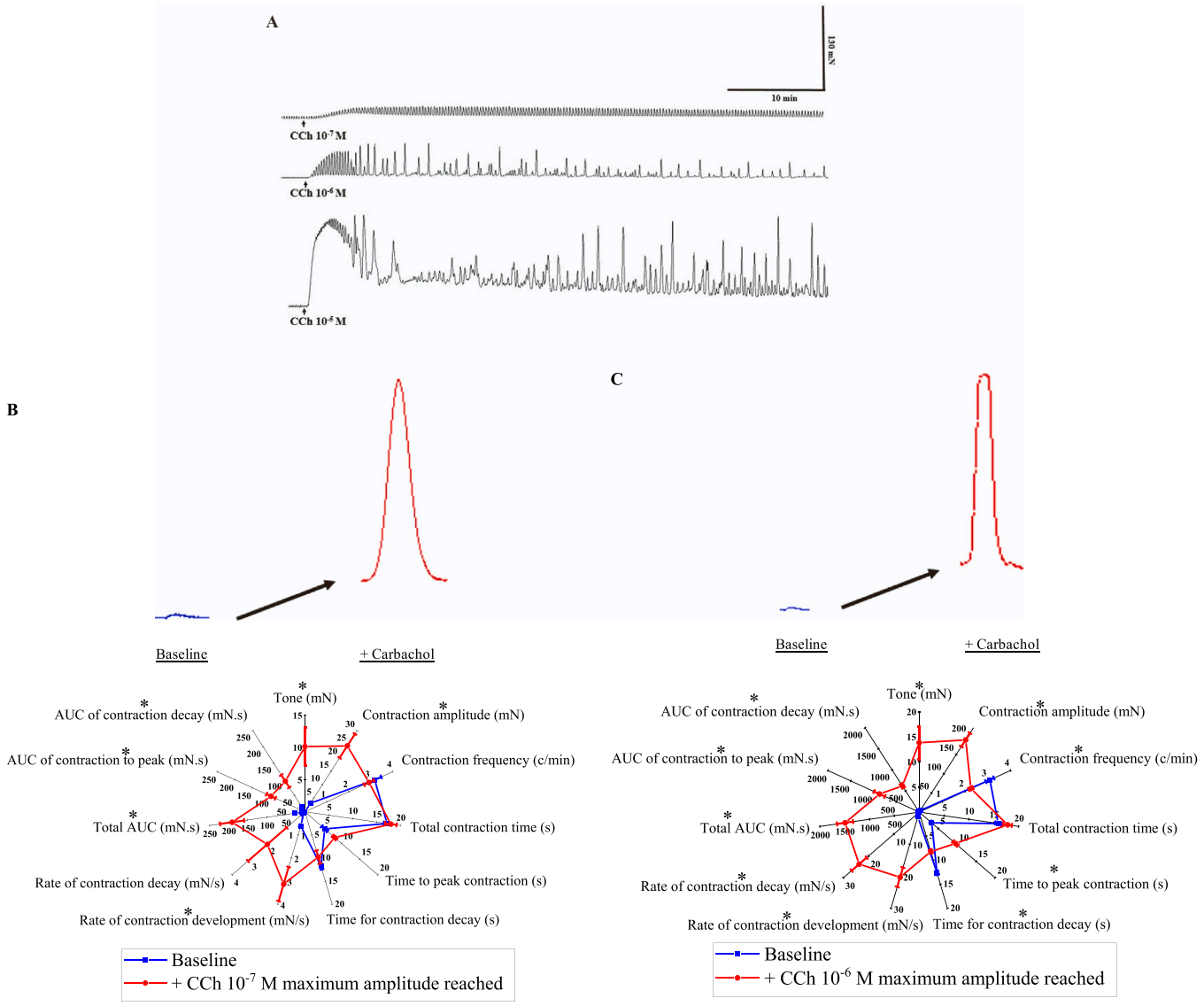


Fig. 3. Effects of carbachol on myogenic contractions of human isolated distal stomach. All experiments were obtained in the presence of tetrodotoxin (10^{-6} M). Panels A and B: Experimental records illustrating the responses to carbachol 10^{-7} M and 10^{-6} M (n = 5 each) measured when the maximum increase in myogenic contraction amplitude was achieved and compared to the baseline values before addition of carbachol. A representative contraction in the absence and presence of test molecules is shown above each radar plot. For each plot, the points represent the mean of tissues studied with vertical lines show standard error of mean. * $P \leq 0.05$ versus control (*t*-tests). Note the difference in Y-axis calibration from pervious figures.

neuronally-mediated responses in human colon [16]) (Fig. 2A, C). Therefore, subsequent experiments were conducted in the presence of TTX (10^{-6} M) and except for the experiments with carbachol (see below), ATR (10^{-6} M) and L-NAME (3×10^{-4} M). Under these conditions the myogenic contractions were stable during the following 1 h period (Fig. 1B). Indomethacin 10^{-6} M for 30 min did not significantly affect baseline tone or any contraction parameter, although the amplitude of contraction tended to reduce after 1 h (from 3.0 ± 0.7 mN to 2.5 ± 0.7 mN, n = 9, P = 0.08; Fig. 2A, D). Nifedipine (10^{-6}-10^{-4} M) inhibited the contractions in a concentration dependent manner (pIC_{50} 4.3 ± 0.8, n = 5; Fig. 2A, E), abolishing the contractions and causing a small fall in muscle tone (by −3.6 ± 1.7 mN, a reduction of 23.6 ± 6.8%) at the highest concentration tested (10^{-4} M; Fig. 2A, E). Finally, the application of carbachol (CCH) 10^{-7} M (evaluated in the presence of TTX 10^{-6} M; different concentrations added individually with 60 min contact time) caused a slow increase in muscle tone (reaching maximum 421.2 ± 97.9 s after administration) and increased myogenic contraction amplitude (maximum after 1249 ± 343.3 s) with unchanged frequency but increased rate of contraction development (n = 5, Fig. 3A, B). Higher concentrations of CCH (10^{-6} and 10^{-5} M; n = 5 each) increased muscle tone more rapidly (reaching maximum respectively, 165.7 ± 15.8 s and 151.9 ± 15.5 s after administration) and as this declined during continued presence of carbachol, myogenic contraction amplitude increased (maximum 365.9 ± 44.7 s with 10^{-6} M) before declining in frequency (Fig. 3A, C), or at 10^{-5} M, were of irregular amplitude and declining frequency (Fig. 3A).

Compounds reported to influence mechanisms of ICC activity were investigated for their ability to modulate myogenic activity. Since their affinities or potencies and their selectivity of action for their human targets are mostly unknown, full concentration-response curves were constructed by applying single concentrations to individual muscle strips. The effective concentrations were compared with what is known about their primary action and any non-selective actions from studies with cell-based preparations or in animal tissue. For a summary of the pharmacological properties of the ligands investigated, see Table 1.

3.2. Ryanodine channel block

Ryanodine (10^{-4} M) increased muscle tone, sustained over > 60 min, during which myogenic contractions were abolished or inhibited (Fig. 4A, B); 10^{-5} and 10^{-6} M (n = 6 each; data not shown) had no consistent effects.

3.3. Xestospongin C; An inhibitor of inositol 1,4,5-trisphosphate (IP_3) receptor-gated channels

Xestospongin C (10^{-6} M) had no consistent activity on muscle tone or myogenic contractions (n = 6; Fig. 4A, C), even at higher (3×10^{-6} M) and lower (10^{-7} M) concentrations (n = 5 each, data not shown).

3.4. Compounds which inhibit the sarcoplasmic-endoplasmic Ca^{2+}-ATPase (SERCA) pump

2-APB (10^{-4} M) caused a small, short-lived increase in muscle tone (in 5 of 6 experiments) with progressive abolition of myogenic contractions (99.2 ± 0.8% inhibition of amplitude; n = 6; Fig. 4A, D). Exploratory experiments indicated that lower concentrations (10^{-6} and 10^{-5} M) of 2-APB were without consistent effect (n = 4 each concentration, data not shown). CPA (10^{-4} M), rapidly and markedly increased muscle tone and amplitude of myogenic contractions, followed by sustained, total abolition of contractions as the increased muscle tone faded (100% inhibition of amplitude; n = 6; Fig. 4A, E); exploratory experiments indicated that lower concentrations (10^{-6}, 10^{-5} M, n = 4, data not shown) were without consistent activity on muscle tone or myogenic activity. Further exploratory experiments suggested that thapsigargin (10^{-6}, 10^{-5} and 10^{-4} M, n = 4 each) increased muscle tone but had no

Table 1
Properties of ligands used to investigate the effects of modulating ICC function on human gastric myogenic activity.

Compound	Significant activity or relevant receptor expression	Reference
Reduction of intracellular Ca^{2+} availability		
Ryanodine	• Acts at Ry_{1-3} receptor ion channels on intracellular Ca^{2+} storage/release organelles in animal and human tissues. • Low concentrations (nM to μM) lock Ry receptors in open state (increasing Ca^{2+} release), whereas high concentrations (>10^{-4} M) can block the channels	[46]
Xestospongin C	• Cell membrane-permeable inhibitor of IP_3 receptor-1-gated channel function (preventing intracellular Ca^{2+} release) in different animal cell-based assays • Low concentrations (IC_{50} 358 nM) block IP_3-induced Ca^{2+} release in endoplasmic-reticulum vesicles of rabbit cerebellum without interacting with the IP_3-binding site • Higher concentrations (20–100 μM) needed to block IP_3-induced Ca^{2+} release in permeabilized cells from embryonic rat aorta, demonstrating similar potency as an inhibitor of SERCA pump	[47,48]
2-APB	• Inhibits IP_3 receptor-mediated functions and the SERCA pump (100 μM) in HeLa cells, rat myocytes and mouse intestine. • Decreased mitochondrial calcium uptake, activated calcium permeable cation channels, inhibited voltage-gated potassium currents in various non-human preparations. • Human/ Mouse TRPV/M/C channel modulator (pEC_{50} >10^{-6} M)	[31,49,50]
Cycloplazonic acid (CPA)	• SERCA pump inhibitor in rabbit and other animal preparations. • Depolarized mouse smooth muscle cells and hyperpolarized cultured ICC. • The ability to increase muscle tone in mice may be due to raised intracellular Ca^{2+} concentration	[24,31,51]
Thapsigargin	• SERCA pump inhibitor (50–100 nM in COS cells) • TRPV1 vanilloid receptor inhibitor (μM concentrations in CHO cells)	[52,53]
Block of opening of calcium-activated chloride channels (CaCC)		
MONNA and CaCCinhA01	• At least four human CaCC splice variants exist; the pharmacological consequences are unknown but in the mouse GI tract, splice variants may explain regional differences in potency of channel blockers. • MONNA blocks human CaCC channel in cultured cells (IC_{50} 1.27x10^{-6}M; maximum ~10^{-4} M). • In immortal cell lines, CaCCinh-A01 blocks human CaCC channels (IC_{50} 3.9 μM; maximum ~10^{-5} M. • No evidence for non-selective interference by CaCCinh-A01 10^{-4} M of agonist-induced calcium signalling in HT-29 cells. • MONNA and CaCCinh-A01 (10^{-7}-10^{-5} M) relaxed noradrenaline or U46619–induced constriction of mouse vascular smooth muscle cells (VSMC) even in Cl^--free environment, and MONNA hyperpolarized VSMC membranes suggesting ability to directly open a potassium channel. • CaCCinh-A01 may reduce $K_{Ca}1.1$ channel activity and lower intracellular Ca2 +	[37,38,47,54–56]
Modulation of ion channels involved in the electrical pacemaker potential of the ICC		
E-4031	• In cell-based preparations low concentrations blocked the human inward-rectifying voltage gated K^+ channel $K_V11.1$ (IC_{50}	[57]

(continued on next page)

Table 1 (*continued*)

Compound	Significant activity or relevant receptor expression	Reference
Mibefradil	7.7x10^{-9}M; maximum block ~3 ×10^{-7} M) (also known as the ether-à-go-go-related gene or ERG K^+ channel) • Blocked recombinant human Ca_V3.1/3.2/3.3 (T-type) channels at ≤ 1 μM, with 10–15-fold selectivity over Ca_V 1.2 (L-type) and Ca_V 21/2/3 (P/Q, N-, R-type) channels • Blocked recombinant human Na_V1.5 Na^+ channel (tetrodotoxin-resistant) at ≤ 10^{-6} M	[58,59]

ICC: interstitial cells of Cajal; IP_3: inositol 1,4,5-trisphosphate; TRP: transient receptor potential channel; Ry receptor: ryanodine receptor; SERCA: sarcoplasmic-endoplasmic Ca2 + -ATPase; CaCC: calcium activated chloride channel, sometimes known as the ANO1 channel; KV: voltage-dependent potassium channel; NaV: voltage-dependent sodium channel.

effect on myogenic contractions (although at 10^{-4} M myogenic contraction amplitude tended to be reduced; Fig. 4A, F); lower concentrations appeared to have no consistent effects (10^{-7}, 10^{-8} M, n = 3 each, data not shown).

3.5. Compounds which block the CaCC channel

MONNA 10^{-4} M had no consistent effects on baseline muscle tension, whereas CaCCinh-A01 10^{-4} M sometimes caused a small, short-lived increase in tension (in 4 of 6 experiments) which declined and fell below the baseline tone level during the 60 min observation period and before the changes in spontaneous contractions were analysed. Both inhibited or tended to inhibit myogenic contractions with slow onset of activity (Fig. 5A). MONNA (10^{-4} M) appeared more effective, reducing amplitude and frequency of contractions by, respectively 77.8 ± 15.2% and 80.6 ± 12.5% (n = 7; Fig. 5B); exploratory experiments indicated that lower concentrations (10^{-6}, 10^{-5} M) were without effect (n = 4 each; data not shown). CaCCinh-A01 (10^{-4} M) tended to reduce all parameters of contraction, but only the effects on amplitude and rate of contraction and decay were statistically significant (inhibition by respectively, 47.3 ± 16.7%, 50.2 ± 15.2%, 52.8 ± 13.2%; n = 6; Fig. 5C); exploratory experiments indicated that lower concentrations (10^{-6}, 10^{-5} M) were without activity (n = 4 each; data not shown).

3.6. Mibefradil: A Ca_V3.1/3.2/3.3 channel blocker

The Ca_V3.1/3.2/3.3 channel blocker mibefradil (10^{-5}, 10^{-4} M) caused a small reduction in muscle tone (by 1.25 ± 0.1 mN and to 1.9 ± 0.5 mN, n = 5 and 6 respectively) and concentration-dependently inhibited myogenic contractions (pIC_{50} 4.8 ± 0.9; Fig. 6A). At the submaximally-effective concentration of 10^{-5} M (66.4 ± 3.8% inhibition of amplitude of contractions; n = 5), the rate of contraction development and decay were reduced, but the frequency was unchanged (Fig. 6B). At 10^{-4} M, contractions were abolished (Fig. 6A; n = 5).

3.7. E-4031: An inhibitor of the inward-rectifying voltage gated K^+ channel K_V11.1

E-4031 (10^{-4} M), an inhibitor of the inward-rectifying voltage gated K^+ channel K_V11.1, caused a small, statistically significant increase in the total time of contraction (from 17.9 ± 1.2 s to 20.9 ± 1.5 s, representing an increase of 17.4 ± 5.8%, P < 0.05; n = 6). There were no consistent effects on muscle tone, contraction frequency or rate of contraction and decay and although the amplitude and the AUC of the contractions tended to increase, this was not statistically significant (Fig. 6A, C). Lower concentrations of E-4031 (10^{-6}, 10^{-5} M) were without consistent activity (n = 5 each; data not shown).

4. Discussion

Rhee et al. [4] suggested that spontaneous contractions of human stomach are initiated by the ICC (as in human colon [17]). This study confirmed that these contractions of the stomach were unaffected by blocking neuronal activity, using a cocktail of TTX, atropine and L-NAME. Although not studied specifically, the inability of TTX to affect muscle tone or myogenic contractions in this region of human stomach contrasts with the increased tone and frequency of spontaneous contractions observed in human isolated distal colon, following application of TTX [18]; this difference points to the existence of regional differences in tonic neural control of gastrointestinal motility. The present study also confirmed the ability of nifedipine to inhibit the myogenic contractions of the stomach. This reflects the inhibition of muscle contractions but not slow wave electrical activity by Ca_V1 channel inhibitors in human gastric antrum and animal GI preparations [4,19,20]. In addition, the myogenic contractions were inconsistently affected by indomethacin. Previously, indomethacin had been found to cause a small reduction of slow wave frequency in human antrum [4], suggesting prostanoids contributed at least partly, to the high frequency of slow waves reported in that study (~8–9 cycles.min^{-1}). In the present study, baseline contraction frequency was 3.0 min^{-1}, consistent with the frequency of electrical activity and contractions *in vivo* (e.g., [1,21]). Low concentrations of carbachol (an acetylcholinesterase-resistant agonist at muscarinic ACh (mACh) and at higher concentrations (>10^{-5} M), nicotinic ACh receptors [22]) contracted the human stomach, increased the rate of myogenic contraction development and also increased the contraction amplitude, leaving the overall frequency of contractions unchanged. In other studies, application of ACh to human antrum (in the absence of TTX) increased muscle tone and both the frequency and amplitude of spontaneous contractions [23] and in the stomach of mice, a species in which the ICC (and smooth muscle) express functionally active mACh receptors (M_2, M_3) [24–26] carbachol caused depolarization and increased slow wave frequency, not amplitude [27]. The reasons for these variations, between different experimental conditions and species, are unclear but complicated by the existence of mACh receptors on both the muscle (likely to influence amplitude of contractions) and the ICC (likely to influence the frequency of contractions).

4.1. Compounds modulating ICC functions

A role for ryanodine receptor-operated stores in propagating Ca^{2+} waves by the ICC in mouse intestine remains contentious [6,28]. In human intestine, ryanodine 10^{-5} M did not affect pacemaker electrical activity [29]. In the present experiments with human stomach, ryanodine 10^{-4} M increased muscle tone then inhibited spontaneous contractions. It is not clear if these different actions reflect non-selectivity of action or different activities in muscle and/or the ICC, locking ryanodine channels open (increasing Ca^{2+} release from sarcoplasmic reticulum) or blocking at higher concentrations (Table 1).

The inability of xestospongin-C to affect myogenic contractions (at concentrations active in cell-based systems; Table 1) contrasts with considerable evidence for a role of IP_3 receptors in mouse ICC functions [30]. This could be explained by poor tissue penetration or species differences. Indeed, for most modulators of ICC functions, little is understood about their actions in humans. Nevertheless, in mouse intestine similar concentrations did not inhibit myogenic contractions under conditions in which tissue penetration was proven [31] and did not reduce the occurrence of slow waves in this tissue [24] or in guinea-pig ileum [32].

Inhibition of SERCA pumps by 2-APB and CPA may explain the progressive abolition of myogenic contractions of the human stomach, also reported with similar concentrations in mouse intestine [24,31,33], an activity consistent with abolition of electrical slow waves in human [29] and mouse intestine [24,31]. In the human stomach 2-APB also

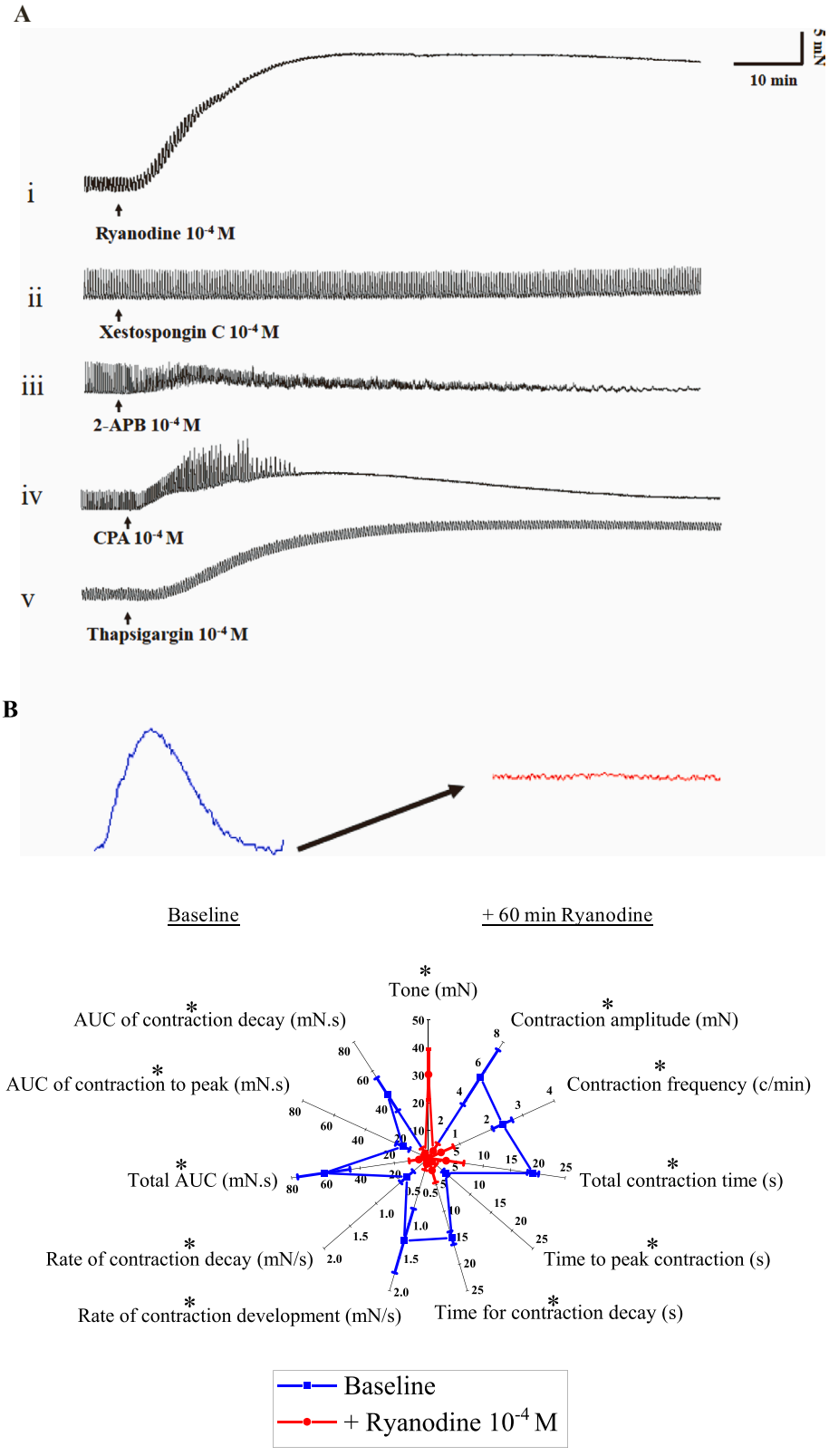


Fig. 4. Effect of modulators of intracellular calcium on myogenic contractions of human isolated distal stomach circular muscle. All experiments were obtained in the presence of tetrodotoxin (10^{-6} M), atropine (10^{-6} M) and L-NAME (3×10^{-4} M). Panel A: Experimental records illustrating the responses to different modulators. Panels B-F show the changes produced in the myogenic contraction parameters in the presence of xestospongin C (n = 5), ryanodine (n = 6), 2-APB (n = 6), CPA (n = 6) and thapsigargin (n = 4 each), following 60 min incubation and compared to the baseline values before addition of the test compound. A representative contraction in the absence and presence of test molecules is shown above each radar plot. For each plot, the points represent the mean of the tissues studied with vertical lines showing standard error of mean. * $P \leq 0.05$ versus control (t-tests).

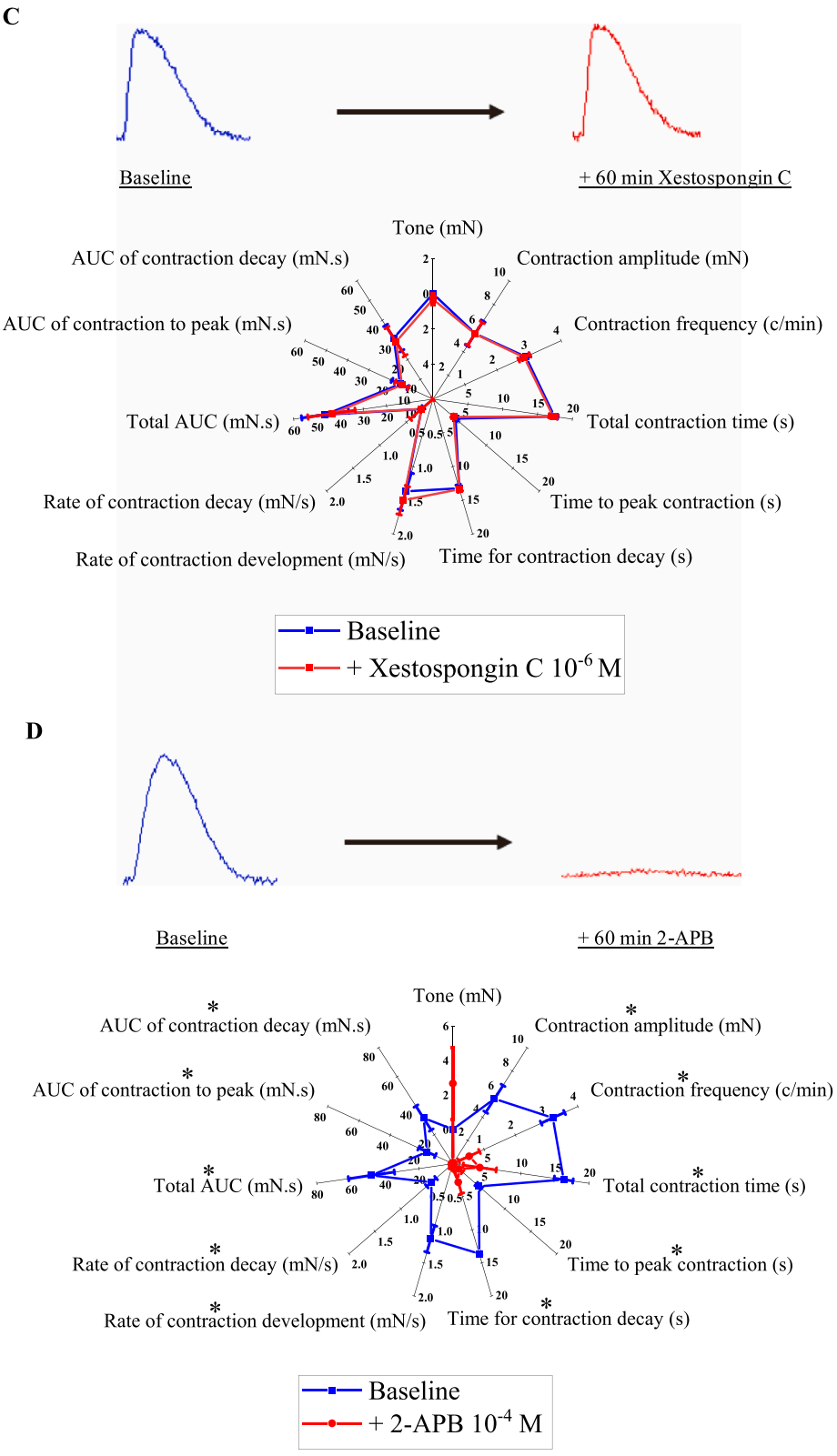
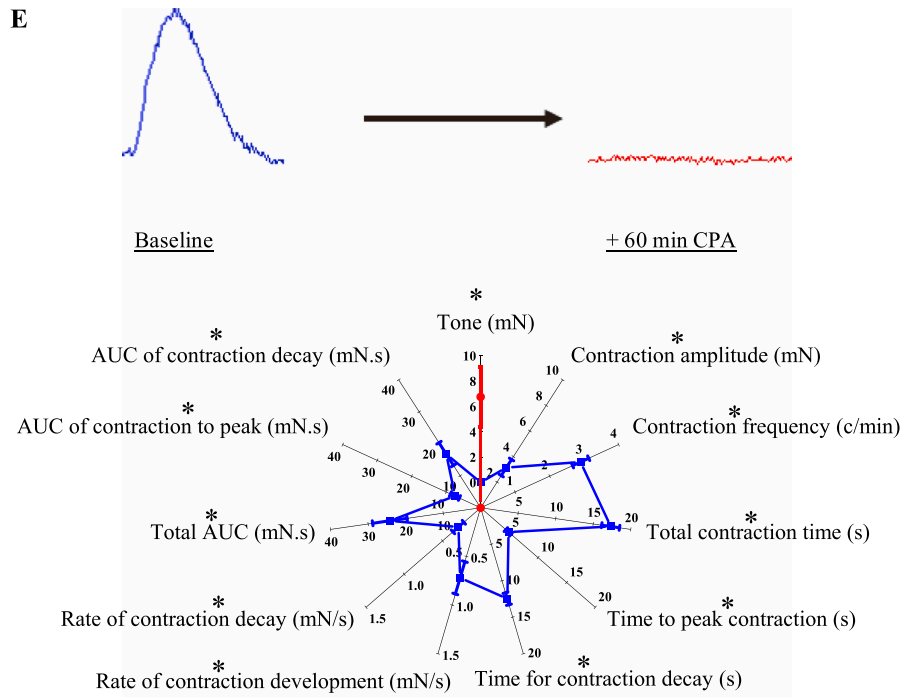


Fig. 4. (*continued*).

initially caused a short-lived increase in muscle tone, and CPA markedly increased muscle tone and myogenic contraction amplitude prior to inhibition of myogenic contractions. In exploratory experiments with thapsigargin (SERCA pump blocker; Table 1) nM concentrations were without effect whereas higher concentrations increased muscle tone without consistently affecting myogenic contractions. Notably, the activity of this compound was less effective in intact mouse intestine, compared to that in cell-based assays [31]. The mechanisms by which these compounds cause contraction and the reasons for differences are unclear, but seem likely to involve an ability to directly cause smooth



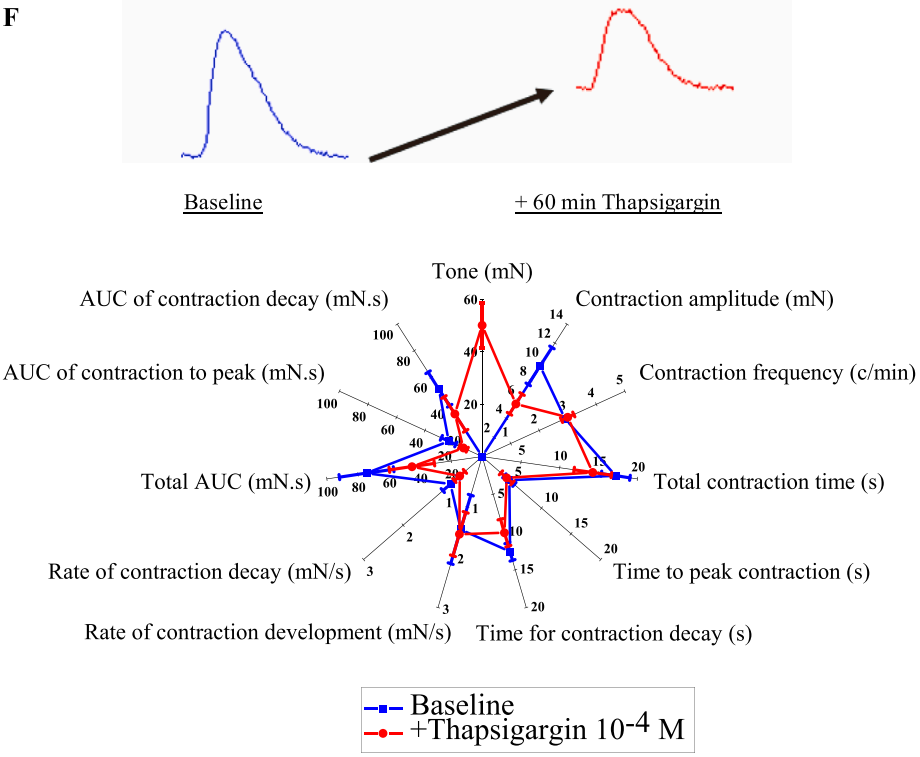


Fig. 4. (*continued*).

muscle depolarisation by elevating cytoplasmic Ca^{2+}via inhibition of uptake into sarcoplasmic reticulum [24]. Interestingly, CPA did not affect human lower oesophageal sphincter tone [34] and in mouse cultured ICCs, CPA 50 μM increased slow wave frequency [35].

CaCC channels are expressed by human GI, ICC [3]. Mice lacking the channel cannot generate electrical slow waves (e.g., [36], and slow waves in mouse gastric antrum and intestine were inhibited by CaCCinh-A01 (near abolition at respectively, 5, 30 μM); CaCC splice variants may explain the regional differences [37]. It might, therefore, seem surprising that CaCC blockers did not also fully inhibit the

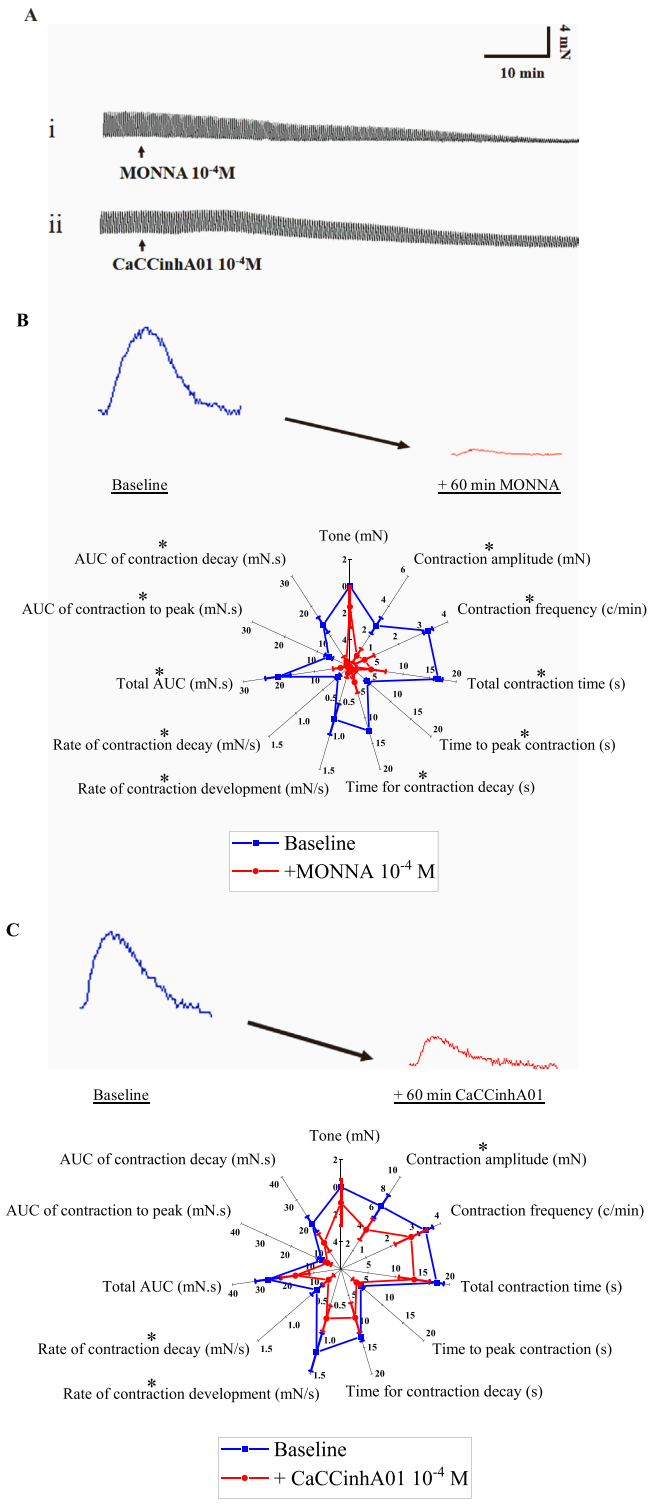


Fig. 5. Effects of the CaCC channel blockers MONNA and CaCCinhAO1 on myogenic contractions of human isolated distal stomach circular muscle. All experiments were obtained in the presence of tetrodotoxin (10^{-6} M), atropine (10^{-6} M) and L-NAME (3×10^{-4} M). Panel A: Experimental records illustrating the responses to CaCC channel blockers. Panels B and C show the changes in the myogenic contraction parameters in the presence of MONNA (n = 7) and CaCCinhAO1 (n = 6), following 60 min incubation and compared to the baseline values before addition of the test compound. A representative contraction in the absence and presence of test molecules is shown above each radar plot. For each plot, the points represent the mean of the tissues studied with vertical lines showing standard error of mean. * $P \leq 0.05$ versus control (*t*-tests).

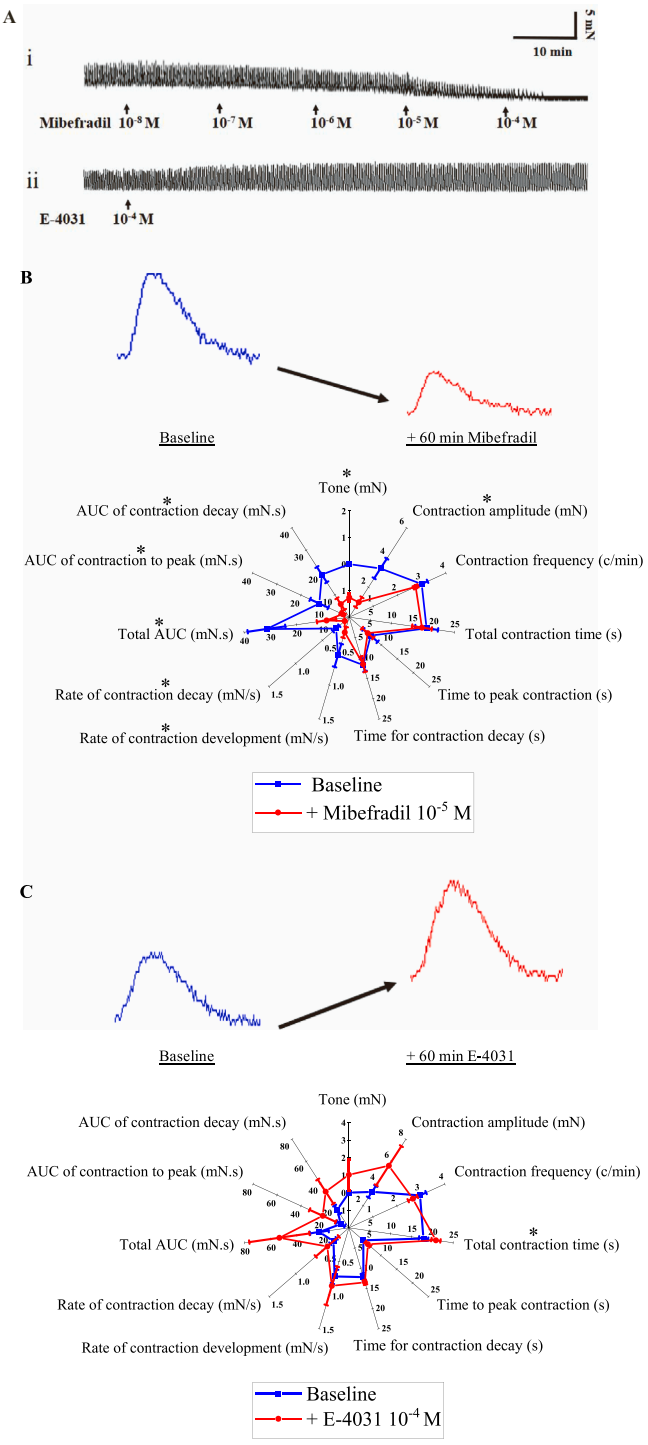


Fig. 6. Effect of modulators of slow wave electrical activity on myogenic contractions of human isolated distal stomach circular muscle. All experiments were obtained in the presence of tetrodotoxin (10^{-6} M), atropine (10^{-6} M) and L-NAME (3×10^{-4} M). Panel A: Experimental records illustrating the responses to (i) mibefradil and (ii) E-4031. Panels B and C show the changes in the myogenic contraction parameters in the presence of mibefradil 10–5 M (n = 5) and E-4031 10–4 M (n = 6), following 60 min incubation and compared to the baseline values before addition of the test compound. A representative contraction in the absence and presence of test molecules is shown above each radar plot. For each plot, the points represent the mean of the tissues studied with vertical lines showing standard error of mean. * $P < 0.05$ versus values before application of mibefradil or E-4031(*t*-tests).

myogenic contractions of the human stomach. Indeed, the contraction frequency and amplitude were greatly reduced by MONNA 10^{-4} M, a concentration which unlike nifedipine, did not affect muscle tone and is reported to maximally block the channel in cultured cells of human origin (10^{-4} M; Table 1). However, only partial inhibition was observed with CaCCinhA01 10^{-4} M, a concentration sometimes causing small reductions in tone and higher than the concentration which was maximally-effective in cultured cells of human origin (10^{-5} M) (Table 1). It is possible that both compounds only poorly reached the site(s) of action within the thicker human stomach strip, explaining their slow onset of activity, the need for relatively high concentrations, and the apparently incomplete activity of CaCCinhA01 (in intact tissues, the effective concentrations are often higher than in cell-based assays; see [15] for example). However, both compounds are known to exert additional, non-selective actions (affecting calcium availability and/ or influencing potassium channel activities; Table 1), complicating interpretation of the present data. Further experiments are needed to determine if MONNA and CaCCinhA01 can inhibit submaximally-effective agonist-induced muscle contractions, although it should be noted that such activity was not apparent for CaCCinh-A01 10^{-4} M when measuring calcium signalling in HT-29 cells [38].

The $Ca_V3.1/3.2/3.3$ blocker mibefradil inhibited the amplitude of myogenic contractions (along with their rate of contraction and decay), and reduced tone. Similar concentrations blocked Ca^{2+}-influx into human intestinal pacemaker cells [29] whereas lower concentrations blocked human recombinant receptors (Table 1) and Ca^{2+}-influx into mouse ileum ICC [39]. Inhibition of myogenic contractions in human stomach is consistent with a role for $Ca_V3.1/3.2/3.3$ channels in the upstroke of electrical pacemaker potential in ICC [40]. The fall in muscle tone may be attributed to Ca_V3 channels within muscle cells [41] but at the effective concentrations it is difficult to exclude $Ca_V1.2$ block (Table 1). Further experiments are needed with more selective Ca_V3 channel blockers such as Z944 [42].

The inward-rectifying K^+-channel inhibitor E-4031 (100 μM) increased myogenic contraction duration. In cell-based preparations lower concentrations blocked human $K_V11.1$ channel current with higher concentrations potentially blocking other K^+ channels (Table 1). In mouse intestine E-4031 (1 μM) blocked slow rectifying K^+ channels in the ICC, thought to be $K_V11.1$, reducing frequency and increasing slow wave duration, leading to increased muscle excitability [43]. In smooth muscle of opossum oesophagus, E-4031 inhibited recovery from slow wave depolarisation, reducing frequency and causing muscle depolarisation [44]. These observations suggest a minor role for $K_V11.1$ in regulating human stomach myogenic contractions, but at concentrations greater than those effective in mice or cell-based preparations.

5. Conclusion and limitations

The purpose of this study was not to investigate the functions of the ICC but to conduct a study to determine if it was possible to identify compounds that influence myogenic contractions of the human stomach, without simultaneously changing muscle tone. For this purpose, a method was devised to examine multiple parameters of the myogenic contractions since it was possible that tested compounds might affect different parameters of the contractions. A clear limitation is that the selectivity of action of the compounds and sometimes their affinity or activity at the human targets are poorly characterised. For this reason, a range of different concentrations were investigated, and the results compared with what is known about the activity of the compounds in cell-based assays and animal tissues. A further limitation is that the mucosa was removed, potentially removing non-neuronal sources of ACh that operate with ICC to regulate mouse stomach motility [45].

None of the compounds investigated inhibited myogenic contraction frequency without also inhibiting myogenic contraction amplitude and/ or affecting muscle tone. Several increased or reduced muscle tension at the same or different concentrations which modulated myogenic contractions. This suggests additional activity at the same target in muscle (e.g., Ca_V, possibly ryanodine and CCH acting at mACh receptors), or reflects the known non-selectivity of action (e.g., the SERCA pump inhibitors, with marked differences in activity and efficacy between different compounds within this class).

Examples from two compound classes modulated the myogenic contractions of human stomach without changing muscle tone; each was observed at high concentrations. In one example, E-4031 (an inward-rectifying K^+-channel inhibitor but with unclear potency for the human channel) increased the duration of the myogenic contractions. Experiments are now needed to determine the potential of this channel to influence human stomach motility, by examining the actions of E-4031 (or other inhibitors of the channel) on myogenic contractions in parallel with measurements of activity at the inward-rectifying K^+-channel in human stomach. The second example was provided by the CaCC blockers MONNA, which inhibited myogenic contraction amplitude and frequency, and CaCCinh-A01, with similar but less effective activity. Experiments are now required to determine if the high concentrations needed for efficacy reflects a poor ability of these CaCC blockers to penetrate the muscle strip and/or a non-selective activity, the latter, if correct, would then suggest a less critical role for CaCC channels in the link between slow wave electrical activity and myogenic contractions of human stomach.

We conclude that there is a need to evaluate additional compounds as potential modulators of the myogenic contractions of human stomach, which have greater potency and selectivity of activity at ICC functions (e,g, Ca_V3 blockers). Such studies should utilise the type of multiple-parameter analysis described here to ensure full characterisation of the motility effects of the pharmacological agents, necessary to understand the consequences of ICC modulation on gastric functions in health and disease.

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CRediT authorship contribution statement

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Declaration of Competing Interest

MS, M-AK, RM, EC, AP, WC, MA, JL and PLRA declare no potential conflict of interest. GJS has received research funding from Takeda Pharmaceuticals and advises BIOMass. GO'G and AG are Directors in Alimetry Ltd. and receive funding from the NZ Health Research Council.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2022.106247.

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