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Muscarinic regulation of pacemaker frequency in murine gastric interstitial cells of Cajal

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Peristaltic contractions in the stomach are regulated by the spread of electrical slow waves from the corpus to the pylorus. Gastric slow waves are generated and propagated by the interstitial cells of Cajal (ICC). All regions distal to the dominant pacemaker area in the corpus are capable of generating slow waves, but orderly gastric peristalsis depends upon a frequency gradient in which the corpus pacemaker frequency exceeds the antral frequency. Cholinergic, muscarinic stimulation enhances pacemaker frequency. We investigated this phenomenon using intact murine gastric muscles and cultured ICC. Acetylcholine (ACh) increased the frequency of slow waves in antrum and corpus muscles. The increase was significantly greater in the antrum. ACh and carbachol (CCh) increased the pacemaker currents in cultured ICC. At high doses of CCh, transient pacemaker currents fused into sustained inward currents that persisted for the duration of stimulation. The effects of CCh were blocked by low doses of the M_3 receptor antagonist 1-dimethyl-4-diphenylacetoxypiperidinium. Frequency enhancement by CCh was not affected by forskolin, but the phospholipase C inhibitor U-73122 inhibited both the increase in frequency and the development of tonic inward currents. 2-Aminoethyldiphenyl borate also blocked the chronotropic responses to CCh. Inhibitors of protein kinase C did not block responses to CCh. These studies show that mice are an excellent model for studying mechanisms that regulate gastric slow-wave frequency. CCh, apparently via production of inositol 1,4,5-trisphosphate, accelerates the frequency of pacemaker activity. High concentrations of CCh may block the entrainment of pacemaker currents, resulting in a tonic inward current.

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Gastric peristaltic waves originate near the greater curvature of the corpus and spread towards the pylorus (Kelly & Code, 1971). These events are important in the mixing and trituration of ingested food. Peristaltic contractions are timed by the occurrence of electrical slow waves, and depend upon the orderly propagation of slow waves from corpus to pylorus (see Szurszewski, 1987). Each region of the stomach distal to the orad corpus is capable of generating spontaneous electrical slow waves, but there is an intrinsic frequency gradient from the proximal to the distal stomach in which slow waves occur at a higher frequency in the proximal stomach (e.g. 3.7 cycles min^{-1} in the human corpus) than in the distal stomach (1.4 cycles min^{-1} in the mid-antrum; El-Sharkawy *et al.* 1978, but see also Kelly & Code, 1971; Sarna *et al.* 1972, 1976). The corpus pacemaker is dominant because slow waves are generated at the highest frequency in this region. Active propagation of slow waves from the corpus entrains more distal pacemakers because there is time for a corpus slow wave to propagate to the antrum and activate the pacemaker mechanism before it discharges spontaneously (Kelly & Code, 1971; Sarna *et al.* 1972).

Disruption in the gastric slow-wave frequency gradient can lead to failure of the normal corpus-to-pylorus propagation of slow waves and interfere with gastric emptying. For example, if the antral slow-wave frequency rises, entrainment by the corpus pacemaker may fail because antral events may occur before events can propagate from the corpus. Under these conditions, both regions manifest pacemaker activity, but 'functional uncoupling' can occur between gastric regions due to disruption in the proximal-to-distal frequency gradient. There are numerous reports in the literature linking gastric motility disorders, dyspepsia, gastroparesis, chronic nausea and vomiting to defects in slow-wave frequency and propagation and the development of ectopic pacemaker activity in the distal stomach (e.g. You & Chey, 1984; Chen *et al.* 1995; Ördög *et al.* 2000; Koch, 2001; Owyang & Hasler, 2002). Thus, regulation of slow-wave frequency, particularly by antral pacemakers, is an important issue in normal and abnormal gastric motility. Numerous conditions, agonists and biological stimuli have been shown to elicit gastric dysrhythmias and ectopic pacemaking (e.g. Kim *et al.* 1987; Sanders, 1984; Owyang & Hasler, 2002), but at

present there is no explanation as to why such a variety of stimuli elicit gastric dysrhythmias or why some patients are more prone to these defects than the normal population.

Electrical pacemaker activity in the stomach results from spontaneous inward currents generated by the interstitial cells of Cajal (ICC; see Ördög *et al.* 1999; Dickens *et al.* 1999). We have developed a preparation of cultured gastric ICC and used these cells to study the mechanism underlying gastric pacemaking and how prostaglandins and cyclic nucleotides affect gastric slow-wave frequency (Kim *et al.* 2002). Excitatory hormones and neurotransmitters, such as gastrin, cholecystokin, noradrenaline and acetylcholine (ACh) also profoundly affect antral slow-wave frequency (e.g. El-Sharkawy & Szurszewski, 1978). These compounds are released during the postprandial period, but at present little is known about how these agonists regulate pacemaker frequency. In the study presented here, we have confirmed the chronotropic effect of cholinergic stimulation in the murine stomach and studied cholinergic regulation of pacemaker frequency in cultured ICC from the murine antrum. We have also investigated the receptors and second-messenger coupling that regulates pacemaker current frequency during muscarinic stimulation.

METHODS

Animals

Balb/C mice (0–30 days old) of either sex were anaesthetized with CO_2 and killed by cervical dislocation. Their stomachs were removed, opened along the lesser curvature and the luminal contents were washed away with Krebs-Ringer bicarbonate solution (KRB). Mice were maintained and the experiments performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all protocols were approved by the Institutional Animal Use and Care Committee at the University of Nevada, Reno.

Electrophysiology of intact muscles

Antrum and corpus muscles were pinned, with the circular muscle facing upward, onto the surface of a dish coated with silicone elastomer (Sylgard 184; Dow Corning, Midland, MI, USA) and perfused with oxygenated KRB warmed to 37.5 ± 0.5 °C. Circular muscle cells were impaled with glass microelectrodes filled with 3 M KCl with resistances of 40–100 MΩ. Transmembrane potential was measured with a standard microelectrode amplifier (Duo 773; WPI, Sarasota, FL, USA), displayed on an oscilloscope (HM 205-3, HAMEG, Frankfurt am Main, Germany) and recorded simultaneously on videotape (VTR Model 420 M, A.R. Vetter, Rebersburg, PA, USA) and chart paper (RS 3200, Gould, Cleveland, OH, USA).

Isolation and culturing of ICC

Small strips of antral muscle were prepared and equilibrated in Ca^{2+}-free Hanks' solution for 10 min. Cells were dispersed from these strips, as described previously (Koh *et al.* 1998), with an enzyme solution containing: collagenase (Worthington Type II, 1.3 mg ml^{-1}), bovine serum albumin (Sigma, St Louis, MO, USA, 2 mg ml^{-1}), trypsin inhibitor (Sigma, 2 mg ml^{-1}) and ATP (0.27 mg ml^{-1}). Cells were plated onto sterile glass coverslips coated with murine collagen (2.5 μg ml^{-1}, Falcon/BD) in 35 mm culture dishes. The cells were cultured at 37 °C in a 95 % O_2–5 % CO_2 incubator in smooth muscle growth medium (Clonetics, San Diego, CA, USA) supplemented with 2 % antibiotic–antimycotic (Gibco, Grand Island, NY, USA) and murine stem cell factor (5 ng ml^{-1}, Sigma).

Patch-clamp experiments

The whole-cell configuration of the patch-clamp technique was used to record inward currents (voltage clamp) from cultured ICC (after 2–3 days in culture). Typically, recordings were made from small clusters of ICC (< 10 cells) because, as reported previously in studies of intestinal and gastric ICC, the spontaneous inward currents from small groups of cells are more robust and more regular than from single cells (Koh *et al.* 1998; Kim *et al.* 2002). Currents were amplified with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA, USA) and digitized with a 12 bit A/D converter (Axon Instruments). Recording, storage and analyses were performed with Axoscope software (Axon Instruments). All recordings were performed at 29 °C.

The cells were bathed in a solution containing (mM): KCl 5, NaCl 135, $CaCl_2$ 2, glucose 10, $MgCl_2$ 1.2 and Hepes 10; adjusted to pH 7.4 with Tris. The pipette solution contained (mM): KCl 140, $MgCl_2$ 5, K_2ATP 2.7, Na_2GTP 0.1, creatine phosphate disodium salt 2.5, Hepes 5 and EGTA 0.1; adjusted to pH 7.2 with Tris.

Solutions and drugs

The standard KRB solution used in studies of intact muscles included (mM): NaCl 118.5, KCl 4.5, $MgCl_2$ 1.2, $NaHCO_3$ 23.8, KH_2PO_4 1.2, dextrose 11.0 and $CaCl_2$ 2.4. The pH of the KRB was 7.3–7.4 when bubbled with 97 % O_2–3 % CO_2 at 37 ± 0.5 °C. ACh chloride, carbachol (carbamylcholine chloride, CCh), MCN-A-343, pilocarpine hydrochloride, 1,1-dimethyl-4-diphenylacetoxypiperidinium (4-DAMP), himbacine hydrochloride, methoctramine tetrahydrochloride, pirenzepine dihydrochloride, chelerythrine chloride and calphostin C were purchased from Sigma. Forskolin (FSK), U 73122, U 73343, GF 109203x, 4-α-phorbol and phorbol 12,13-dibutyrate (PDBu) were purchased from Calbiochem (San Diego, CA, USA). Himbacine and FSK were dissolved in ethanol. Chelerythrine, calphostin C, GF 109203x, PDBu and 4-α-phorbol were dissolved in DMSO. Other drugs tested were dissolved in distilled water. The final concentration of DMSO or ethanol was less than 0.1 %, and neither DMSO nor ethanol had effects at this concentration. All drugs tested were applied via bath perfusion for 10–20 min.

Statistical analyses

Data are expressed as means ± S.E.M. In patch-clamp experiments, differences in the data were evaluated by ANOVA or Student's *t* test. The *n* values reported in the text refer to the number of cells used in patch-clamp experiments or the number of muscle strips used in intracellular electrophysiological experiments. In intact muscle experiments, SigmaStat Statistical Software for Windows version 2.03 (SPSS Science, Chicago, IL, USA) was used for statistical analyses. The frequency of slow waves was calculated from the mean interevent interval for the particular recording. Before performing tests of significance, data were examined for normality and equal variance to determine whether parametric or non-parametric tests should be employed. Unpaired and paired Student's *t* test, rank-sum test, signed-rank test and Kruskal-Wallis one-way ANOVA followed by all-pairwise multiple comparison (Tukey test) were used for statistical comparisons. A probability value of $P < 0.05$ was used as a cut-off for statistical significance in all statistical procedures.

RESULTS

ACh increased slow-wave frequency in intact gastric muscles

Previous studies have shown that exogenous ACh or release of ACh from cholinergic neurons increases the frequency of electrical slow waves in gastric muscles of dog (Szurszewski, 1975) and guinea-pig (Hirst *et al.* 2002). We first tested exogenous ACh on intact murine gastric muscles to confirm that cholinergic stimulation has positive chronotropic effects on slow waves in this preparation. Intracellular electrophysiological recordings were performed on muscles of the gastric corpus ($n = 8$) and antrum ($n = 12$). The effects of ACh on resting membrane potential (RMP, taken as the most negative potential during the slow-wave cycle) and slow-wave frequency were recorded using concentrations of 10^{-6} M (corpus: $n = 4$; antrum: $n = 7$) and 10^{-5} M (corpus: $n = 4$; antrum: $n = 5$). The effects of these two concentrations on slow-wave frequency were statistically indistinguishable according to Student's t tests and the data were therefore pooled. In the corpus, RMP averaged -62 ± 2 mV and slow-wave frequency was 6.05 ± 0.18 cycles min^{-1}. ACh significantly depolarized RMP to -58 ± 3 mV ($P = 0.008$; signed-rank test) and increased slow-wave frequency to 6.99 0.27 cycles min^{-1} ($P = 0.018$; paired t test). Under control conditions RMP was -60 ± 2 mV in antrum muscles. This was not significantly different from the RMP recorded in the corpus (one-way ANOVA). Slow-wave frequency, however, was significantly slower in antral muscles than in the corpus (e.g. 2.54 ± 0.38 cycles min^{-1}, $P < 0.001$; one-way ANOVA). ACh significantly depolarized the RMP of antral muscles to -51 ± 2 mV ($P < 0.001$; paired t test) and increased slow-wave frequency to 4.17 ± 0.44 cycles min^{-1} ($P < 0.001$; paired t test). The degree of depolarization (ΔRMP) elicited by ACh tended to be greater in the antrum (9 ± 2 mV) than in the corpus (5 ± 1 mV), but the difference did not reach statistical significance (Student's t test).

The ACh-induced increase in slow-wave frequency (expressed as (maximum slow-wave frequency/control frequency) $\times$ 100) was significantly greater in the antrum (201.2 ± 26.6 %) than in the corpus (116.1 ± 5.1 %), as assessed by rank-sum test. Finally, the peak frequency of antral slow waves during ACh administration was still significantly slower than corpus slow waves ($P < 0.001$; one-way ANOVA; see data above). The effects of ACh on gastric slow waves are illustrated in Fig. 1.

CCh, a muscarinic agonist, had essentially the same effects as ACh. For example, CCh (10^{-6} M) depolarized the RMP of antral smooth muscle from -68 ± 3 to -38 ± 5 mV ($n = 5$, $P < 0.001$) and increased slow-wave frequency from 3.8 ± 0.5 to 7.1 ± 0.5 cycles min^{-1} ($P < 0.001$). These effects were not blocked by tetrodotoxin (TTX, 10^{-6} M; Fig. 1*C*). Pretreatment of muscles with 4-DAMP (10^{-7} M) reduced CCh-induced depolarizations (i.e. from -65 ± 1 to -61 ± 2 mV ($n = 7$, $P > 0.05$) and blocked the chronotropic effects of CCh (i.e. from 4.4 ± 0.2 to 4.5 ± 0.2 cycles min^{-1}; $P > 0.05$; Fig. 1*D*).

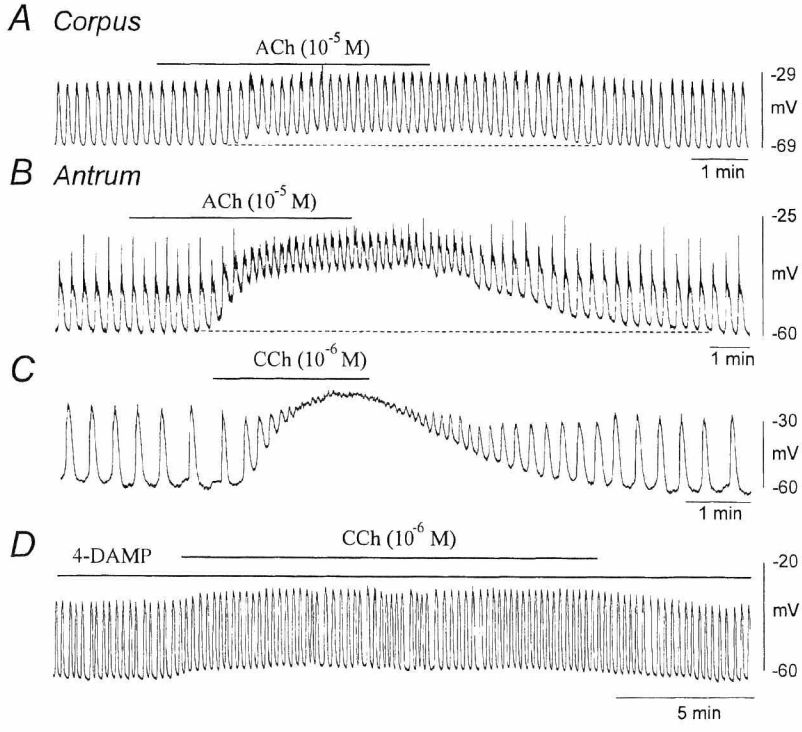

Figure 1. Effects of acetylcholine (ACh) and carbachol (CCh) on electrical slow waves in the murine stomach

Intracellular electrophysiological recordings were performed in the circular muscle layer of gastric corpus (*A*) and antrum (*B*) tunica muscularis. ACh (10^{-5} M) was perfused during the period indicated by the horizontal bars. ACh caused tonic depolarization of the resting membrane potential (maximum transmembrane potential between slow waves) and increased slow-wave frequency. *C*, CCh had similar effects to those of ACh on an antral muscle strip. CCh increased the frequency of slow waves and depolarized the resting membrane potential. This experiment was performed in the presence of tetrodotoxin (10^{-6} M), suggesting that the effects were mediated at postjunctional muscarinic receptors. This was also demonstrated by pretreating muscles with 1-dimethyl-4-diphenylacetoxypiperidinium (4-DAMP, 10^{-7} M; *D*). This antagonist greatly reduced the depolarization response and blocked the chronotropic effects of CCh.

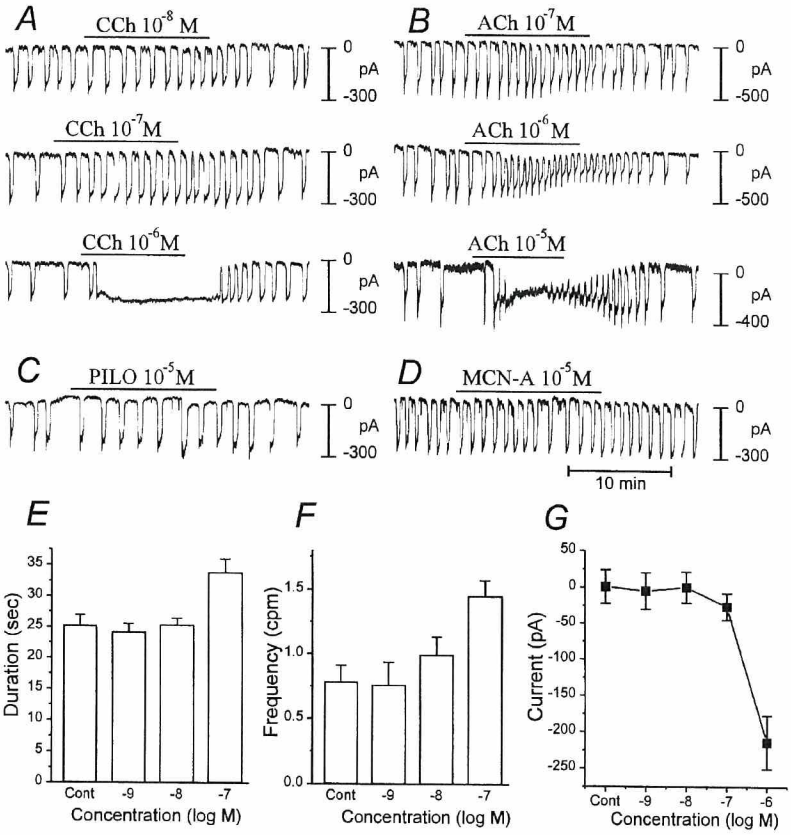

Figure 2. Effects of cholinergic stimulation on pacemaker currents in interstitial cells of Cajal (ICC)

CCh (*A*) and ACh (*B*) caused concentration-dependent increases in pacemaker current frequency. At higher concentrations (e.g. 10^{-6} M for CCh and 10^{-5} M for ACh) tonic inward currents were elicited that persisted for the duration of stimulation. Pilocarpine (PILO, *C*) and MCN-A (*D*) did not mimic the effects of ACh and CCh. *E*–*G*, summary of the effects of CCh on pacemaker current duration, frequency and basal current.

Effect of cholinergic stimulation on pacemaker activity

To understand how cholinergic stimulation affects pacemaker frequency, we conducted voltage-clamp experiments on cultured ICC isolated from the gastric antrum. The basic properties of spontaneous pacemaker currents from murine antral ICC have been described previously (Kim *et al.* 2002). We examined the concentration–response relationship for the effects of CCh, a non-selective

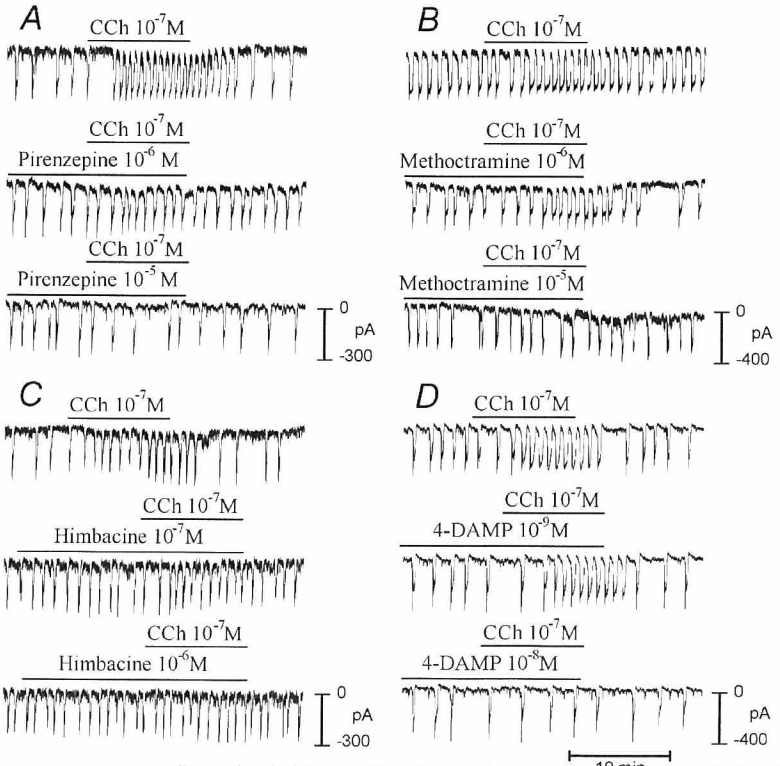

Figure 3. Effects of muscarinic antagonists on responses to CCh

Each panel shows a control response to CCh (10^{-7} M) and then a repeat of the exposure to CCh in the presence of a muscarinic blocker. Relatively high concentrations of pirenzepine (10^{-5} M; *A*), methoctramine (10^{-5} M; *B*) and himbacine (10^{-7} M; *C*) blocked the frequency-enhancing effects of CCh on pacemaker currents. Low concentrations of 4-DAMP (10^{-8} M; *D*) blocked the chronotropic effects of CCh.

muscarinic receptor agonist, on antral pacemaker currents. The frequency, duration and amplitude of spontaneous pacemaker currents were 0.8 ± 0.1 min^{-1}, 25.2 ± 1.8 s and 228 ± 42 pA, respectively, at a holding potential of 60 mV. The resting current was 0.8 ± 23 pA ($n = 6$). CCh (10^{-9}–10^{-8} M) did not significantly change the duration, frequency or amplitude of pacemaker currents (Fig. 2*A*). CCh (10^{-7} M) increased the frequency and duration of pacemaker currents (e.g. 1.5 ± 0.1 min^{-1} and 33.8 ± 2.2 s, $n = 6$; $P < 0.01$); however, resting current and amplitude of the pacemaker currents were not significantly changed. A higher concentration of CCh (10^{-6} M) induced a sustained inward current (the resting current, 214 ± 37 pA at −60 mV, $n = 6$; $P < 0.01$) and blocked the pacemaker currents. These experiments are summarized in Fig. 2*E*–*G*.

We also tested the effects of ACh on pacemaker currents. In these experiments, the frequency and duration of pacemaker currents were 1.3 ± 0.1 min^{-1} and 21 ± 2.3 s, respectively ($n = 4$), under control conditions at a holding potential of −60 mV. ACh (10^{-7} M) increased the frequency (1.5 ± 0.3 min^{-1}) and duration (23 ± 5 s) of the pacemaker currents. At 10^{-6} M, ACh induced a net inward current (50 ± 19 pA at −60 mV, $n = 4$; $P < 0.05$) and increased the frequency and duration of pacemaker currents (1.7 ± 0.1 min^{-1} and 28.5 ± 2.1 s, respectively, $n = 4$). At 10^{-5} M, ACh showed similar effects to CCh at 10^{-6} M (Fig. 2*B*).

Pilocarpine (10^{-5} M), an M_1 muscarinic receptor-selective agonist, and MCN-A343 (10^{-5} M), an M_1 and M_4-selective agonist, did not significantly affect the duration, frequency or amplitude of pacemaker currents ($n = 4$ each; Fig. 2*C* and *D*).

Effects of muscarinic antagonists on the frequency of pacemaker currents

We tested the effects of muscarinic antagonists on the enhancement of the pacemaker current frequency elicited by CCh (10^{-7} M). Under control conditions (i.e. −60 mV holding potential), the frequency, resting current and amplitude of pacemaker currents were 0.8 ± 0.1 min^{-1}, 25.6 ± 15 pA and 410 ± 55 pA, respectively. Application of CCh (10^{-7} M) increased the pacemaker frequency (1.4 ± 0.1 min^{-1}, $n = 16$, $P < 0.01$). Pretreatment with pirenzepine (10^{-6} M), a selective M_1 antagonist, did not change the frequency or amplitude of pacemaker currents; however, the increase in frequency observed in response to CCh (10^{-7} M) was reduced by pirenzepine (10^{-6} M; i.e. 49 ± 7.8 % inhibition, $n = 4$, $P < 0.05$). A high concentration of pirenzepine (10^{-5} M) had no direct effect on spontaneous frequency, but blocked the increase in frequency caused by CCh (Fig. 3*A*, $n = 4$). We also tested the effect of methoctramine, a selective M_2 antagonist, himbacine, a selective M_2 and M_4 antagonist, and 4-DAMP, a selective M_3 antagonist, on the frequency of pacemaker currents. Pretreatment with methoctramine (10^{-6} M) did not inhibit the effects of CCh. However, higher concentrations of methoctramine (i.e. 10^{-5} M) completely inhibited the effects of CCh on frequency (Fig. 3*B*, $n = 3$). Pretreatment with himbacine (up to 10^{-6} M) did not inhibit the effects of CCh on frequency (Fig. 3*C*). The effects of 4-DAMP were more potent than those of the other muscarinic antagonists tested. 4-DAMP (10^{-9} M) did not inhibit the effects of CCh, but the frequency effects of CCh

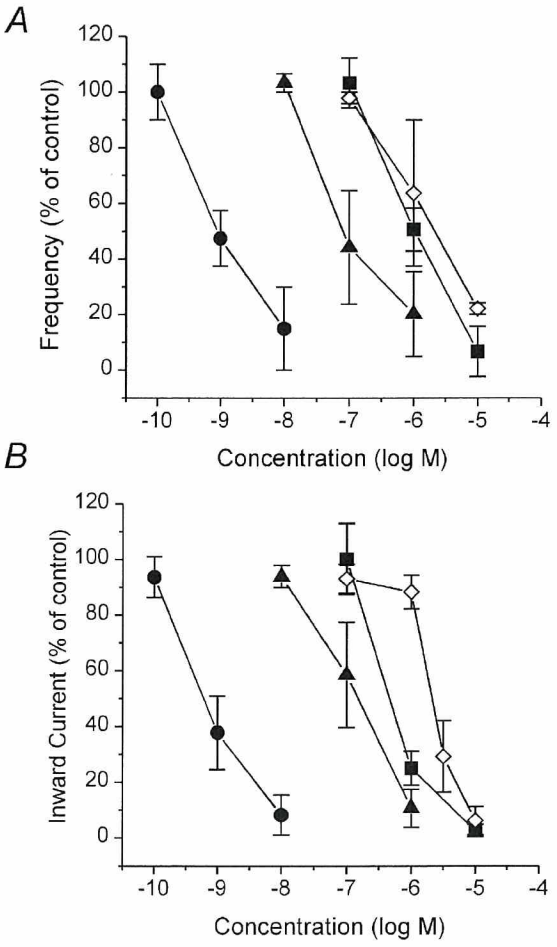

Figure 4. Concentration–response curves showing the effects of various muscarinic antagonists

A, the effects of antagonists on the chronotropic effects of CCh. Data are normalized to the increase in frequency produced by a control exposure to CCh, and show the effects of 4-DAMP (filled circles), himbacine (filled triangles), pirenzepine (filled squares) and methoctramine (open diamonds) as a function of concentration. *B*, concentration–response curves showing the effects of various muscarinic antagonists on the tonic inward current elicited by CCh. Data are normalized to the current elicited by an initial exposure to CCh (10^{-6} M), and show the effects of 4-DAMP (filled circles), himbacine (filled triangles), pirenzepine (filled squares) and methoctramine (open diamonds) as a function of concentration. The efficacy of 4-DAMP was at least two orders of magnitude greater than any of the other agonists on both the chronotropic response and the development of tonic inward current, suggesting that these responses were mediated via M_3 receptors.

Table 1. Order of antagonist potency (IC_{50}) on the effects of carbachol (CCh)

Frequency (CCh, 10^{-7} M)	4-DAMP (9.0)	>>	Himbacine (7.1)	>	Pirenzepine (6.0)	> Methoctramine (5.7)
Inward current (CCh, 10^{-6} M)	4-DAMP (9.2)	>>	Himbacine (6.8)	>	Pirenzepine (6.4)	> Methoctramine (5.6)
pK_B values for M_3 receptors*	4-DAMP (8.9–9.3)	>>	Himbacine (6.9–7.4)	>	Pirenzepine (6.7–7.1)	> Methoctramine (6.3–6.9)

Concentrations (−log M) are given in parentheses. *pK_B values for the antagonists used were obtained from Caulfield & Birdsall (1998). 4-DAMP, 1,1-dimethyl-4-diphenylacetoxypiperidinium.

were completely blocked by 4-DAMP (10^{-8} M) ($n = 4$; Fig. 3*D*). Concentration–response curves showing the relative potency of the muscarinic antagonists on the chronotropic effects of CCh are shown in Fig. 4*A*.

Effects of muscarinic antagonists on the sustained inward current induced by CCh

We also examined the effects of muscarinic antagonists on the sustained inward currents activated by CCh (10^{-6} M). In these experiments CCh (10^{-6} M) induced an inward current averaging 284 ± 28 pA ($n = 20$). Pretreatment with pirenzepine (up to 10^{-7} M) did not affect the inward current activated by CCh. However, high concentrations of pirenzepine (e.g. 10^{-5} and 10^{-6} M) inhibited the activation of inward currents induced by CCh (Fig. 5*A*). Methoctramine (10^{-6} M) did not inhibit the activation of inward currents by CCh (11.8 ± 6.0 % inhibition compared to CCh-induced inward current, $n = 5$; $P > 0.05$), however a higher concentration of methoctramine (e.g. 10^{-5} M) inhibited the development of the sustained inward current recorded in response to CCh (93.4 ± 5 % inhibition, Fig. 5*B*). Himbacine showed a similar effect to pirenzepine. At 10^{-6} M, himbacine inhibited the activation of inward currents by CCh (90.3 ± 6.8 % inhibition, $n = 4$, Fig. 5*C*). It should be noted that when high concentrations of these blockers were used, instead of activating sustained inward currents, CCh increased the frequency of spontaneous pacemaker currents (see Fig. 5*A–C*). Pretreatment with 4-DAMP (10^{-9} M) inhibited the sustained inward current induced by CCh by 62.2 ± 13 % ($n = 4$). At 10^{-8} M, 4-DAMP nearly abolished the effects of CCh on the sustained inward current (92.8 ± 7.2 % inhibition, $n = 4$, Fig. 5*D*). Concentration–response curves showing the relative potency of the muscarinic antagonists are shown in Fig. 4*B*. The data were fitted with a Boltzmann equation, and the IC_{50} values for pirenzepine, methoctramine, himbacine and 4-DAMP are shown in Table 1. On the basis of the antagonists used, it appears that the chronotropic effects of muscarinic stimulation are due to the activation of M_3 receptors.

Intracellular mechanism underlying the effects of muscarinic activation on frequency and inward currents

Activation of M_2 and M_4 receptors decreases intracellular cAMP production (Ehlert *et al.* 1997). Stimulating cAMP production with FSK (10^{-8} M) profoundly lowers pacemaker current frequency (Kim *et al.* 2002), but in the present study this did not block the frequency-enhancing effects of CCh (10^{-7} M) or the sustained inward current elicited by CCh (10^{-6} M; Fig. 6). These data suggest that the enhancement of cAMP due to stimulation by FSK does not interfere with responses to CCh, and M_2 and/or M_4 receptors do not mediate these responses.

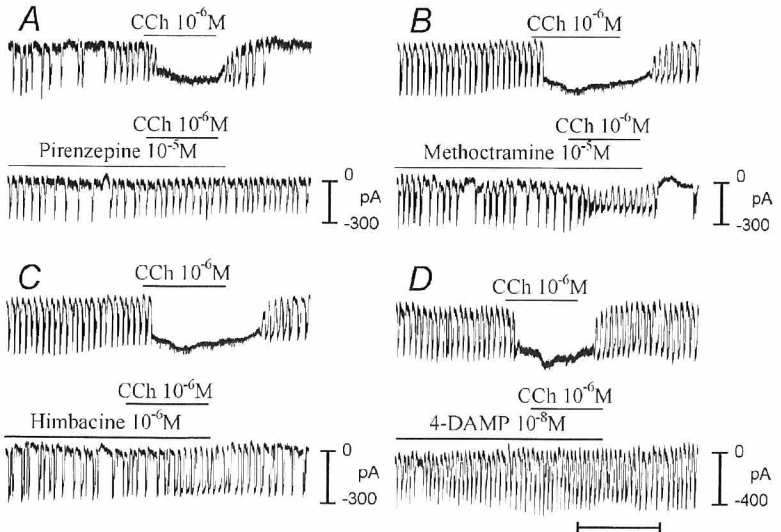

Figure 5. Effects of muscarinic antagonists on tonic currents elicited by CCh

Each panel shows a control response to CCh (10^{-6} M) and then a repeat of the exposure to CCh in the presence of a muscarinic blocker. Relatively high concentrations of pirenzepine (10^{-5} M; *A*), methoctramine (10^{-5} M; *B*) and himbacine (10^{-6} M; *C*) blocked the tonic inward current elicited by stimulation with CCh. Low concentrations of 4-DAMP (10^{-8} M) blocked the chronotropic effects of CCh (*D*). Note that while the tonic inward currents are blocked by the muscarinic agonists in *A–C*, there is still an increase in frequency in response to CCh.

Muscarinic activation of M_3 receptors stimulates phospholipase C (PLC) and generates inositol 1,4,5-trisphosphate (IP_3) and diacylgycerol (see Felder, 1998). The latter is a potent activator of protein kinase C (PKC). We tested the effects of a PLC inhibitor, U-73122 (3×10^{-6} M). This compound blocked pacemaker currents and inhibited the sustained inward current induced by CCh (10^{-6} M; Fig. 7, $n = 4$). The inactive isoform, U-73343 (3×10^{-6} M), had no effect on spontaneous pacemaker currents or on the activation of sustained inward current by CCh (10^{-6} M; Fig. 7).

Previous reports have shown that pacemaker currents in ICC are dependent upon IP_3 receptor (IP_3R)-dependent Ca^{2+} release. Therefore, we tested the effects of 2-aminoethyldiphenyl borate (2-APB) on the effects of CCh. Pretreatment of cells with 2-APB (3×10^{-5} M) completely inhibited spontaneous pacemaker currents. In the presence of 2-APB, CCh did not restore pacemaker currents and failed to induce sustained inward current (Fig. 7, $n = 4$). These data suggest that the increase in pacemaker current frequency and development of sustained inward currents due to muscarinic receptor activation by CCh are mediated through the PLC pathway and IP_3 production.

We also tested the involvement of PKC on the effects elicited by CCh. Treatment with PDBu, an activator of PKC, mimicked the CCh response to some extent. For example, application of PDBu (10^{-7} M) increased the frequency of pacemaker currents (from 0.8 ± 0.1 to 1.4 ± 0.2 min^{-1}; $P < 0.05$, $n = 5$) and induced a sustained inward current (88 ± 40 pA) at a holding potential of -60 mV (Fig. 8*A*). 4-α-Phorbol (10^{-7} M), an inactive analogue of PDBu, had no effect on pacemaker current frequency or basal holding current. Pretreatment of cells with chelerythrine (50×10^{-6} M; $n = 7$), calphostin C (5×10^{-7} M; $n = 3$) or GF 109203x (10^{-6} M; $n = 3$) did not affect the generation of spontaneous pacemaker currents, and in the presence of these PKC inhibitors, the effects of CCh on pacemaker current frequency and activation of net inward current were unaffected (Fig. 8*C* and *E*). It should also be noted that chelerythrine did not block the effects of PDBu (data not shown), suggesting that these effects were independent of the activation of PKC enzymes.

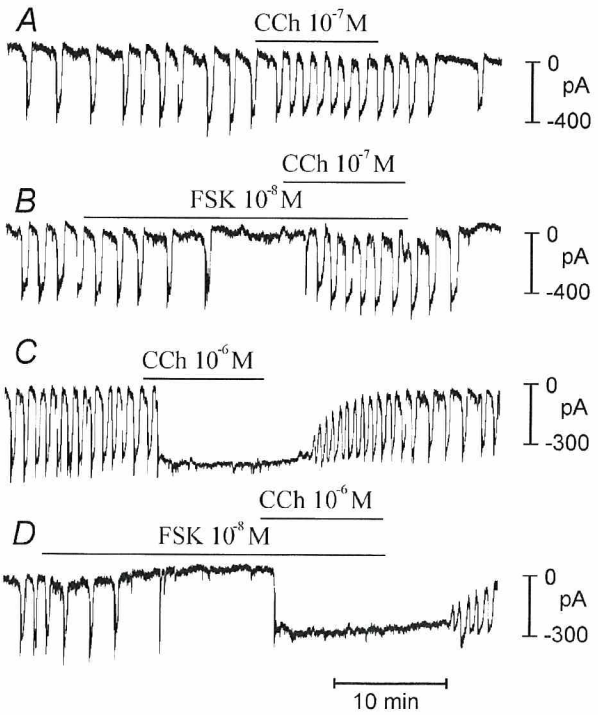

Figure 6. Effects of CCh were not antagonized by forskolin (FSK)

A, the increase in the pacemaker frequency in response to CCh (10^{-7} M). *B*, the increase in frequency caused by CCh was not inhibited by pretreatment of the tissue with FSK (10^{-8} M), which is known to increase levels of cAMP and slow pacemaker frequency (see Kim *et al.* 2002). *C*, the tonic inward current elicited by CCh (10^{-6} M). This response was also not inhibited by pretreatment with FSK (*D*).

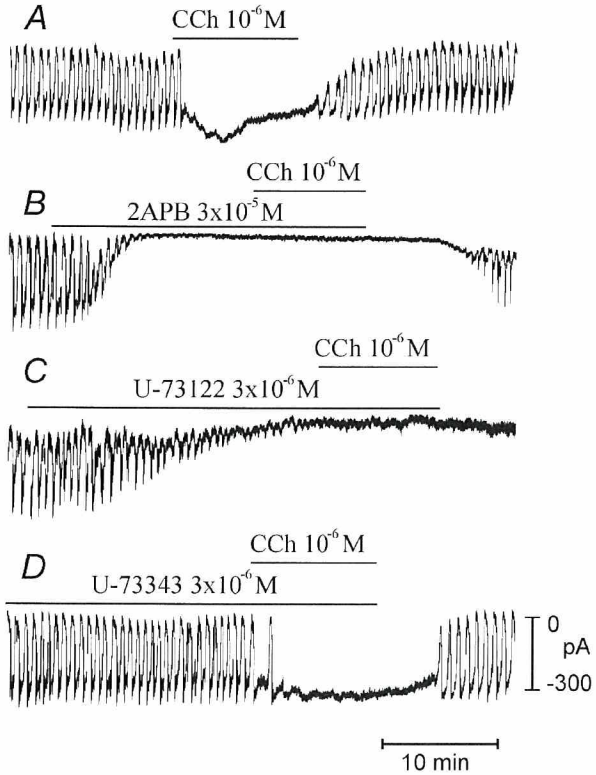

Figure 7. 2-Aminoethyldiphenyl borate (2-APB) and U-73122 inhibit responses to CCh

A, the tonic inward current elicited by CCh (10^{-6} M). Pretreatment with 2-APB (3×10^{-5} M) completely inhibited pacemaker currents and blocked both the chronotropic effects of CCh and the tonic inward current elicited by 10^{-6} M CCh (*B*). The same effects were noted with U-73122 (3×10^{-6} M; *C*). The inactive analogue, U73343 (3×10^{-6} M, which has no effect on phospholipase C, did not affect the response to CCh (*D*).

DISCUSSION

Cholinergic stimulation of antral muscles has been shown to enhance the intrinsic slow-wave frequency of slow-wave pacemakers (Szurszewski, 1975) or to elicit premature slow waves in the intact stomach (Sarna & Daniel, 1975; Daniel & Sarna, 1976; Hirst *et al.* 2002). We have confirmed the chronotropic effects of cholinergic stimulation in the murine corpus and antrum. We found that cholinergic stimulation increased slow-wave frequency in both regions of the stomach; however, the chronotropic effect was greatest in antral muscles. The chronotropic effects of cholinergic stimulation are also manifest in ICC isolated from antral muscles. There was a concentration-dependent increase in pacemaker current frequency in these cells in response to muscarinic stimulation. Using the order of potencies and relative antagonist affinities for muscarinic receptor antagonists (see Caulfield & Birdsall, 1998), our results suggest that the chronotropic effects of CCh and tonic inward currents elicited by higher concentrations of CCh were mediated by M_3 receptors (See Table 1). M_3 receptors are coupled through G_q/G_{11} to PLC and production of IP_3 and diacylglycerol (e.g. Felder, 1998). The former is a direct stimulus of the primary pacemaker mechanism (i.e. via IP_3R-dependent Ca^{2+} release), and the latter is an activator of PKC that has, as yet, undetermined effects on the pacemaker mechanism. The effects of CCh were blocked by an inhibitor of PLC, but not by PKC inhibitors. Our data suggest that the ongoing activity of PLC in ICC is required for spontaneous activity, and enhanced production of IP_3 due to muscarinic stimulation may be responsible for the chronotropic effects. This hypothesis is consistent with the model for slow-wave generation that we described recently (see Sanders *et al.* 2000).

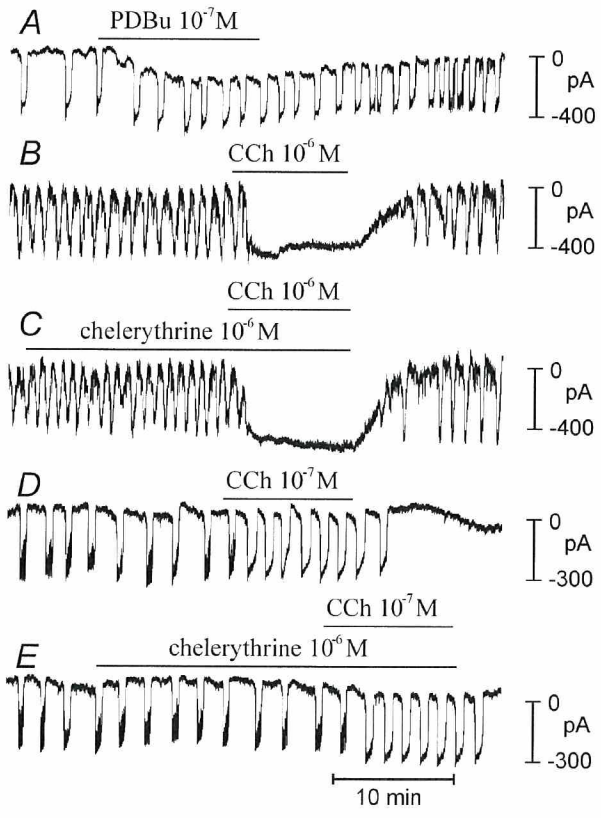

Figure 8. Effects of protein kinase C (PKC) and its blocker, chelerythrine, on pacemaker current

Although activation of PKC by infusion of phorbol 12,13-dibutyrate (PDBu; a potent activator of PKC) had similar effects to CCh on pacemaker frequency and activation of tonic inward current (*A*), a blocker of PKC, chelerythrine (10^{-6} M), did not inhibit either the chronotropic effect stimulated by CCh (10^{-7} M; *B* and *C*) or the tonic inward current elicited by CCh (10^{-6} M; *D* and *E*).

Pacemaker currents in ICC are initiated by the release of Ca^{2+} from IP_3R-operated stores (Suzuki *et al.* 2000; van Helden *et al.* 2000, Ward *et al.* 2000*b*). Ca^{2+} release events occur spontaneously in ICC, and this is likely to be due to basal levels of IP_3 and cytoplasmic Ca^{2+} and the intrinsic, excitable properties of IP_3Rs (Berridge, 1993). Animals lacking type 1 IP_3Rs are incapable of generating slow waves, suggesting that this isoform is central to the pacemaker mechanism (Suzuki *et al.* 2000). IP_3 activates type 1 IP_3Rs by relieving Ca^{2+} inhibition of the receptors (Mak *et al.* 1998). M_3 activation, by increasing IP_3 production, should increase the open probability of IP_3Rs, phase advance the occurrence of pacemaker events and reduce the cycling time between pacemaker events. These changes would increase pacemaker frequency. Thus, muscarinic stimulation, apparently via IP_3 production, enhances pacemaker frequency. We hypothesize that other agonists with receptors coupled to stimulation of PLC may also enhance pacemaker frequency in the gastric antrum.

Previous studies of gastric and small intestinal pacemaker currents in ICC have demonstrated both positive and negative regulation of pacemaker frequency (Koh *et al.* 2000; Kim *et al.* 2002). A central theme in these studies is that enhancement of cyclic nucleotides depresses pacemaker frequency. Similar conclusions were reached in microelectrode studies of intact gastric muscles (Ozaki *et al.* 1992; Tsugeno *et al.* 1995) and other rhythmic smooth muscles (e.g. von der Weid *et al.* 2001). A previous study also identified positive chronotropic regulation of gastric pacemaker frequency via prostaglandin stimulation of the E-prostanoid$_3$ (EP_3) receptors (Kim *et al.* 2002). The second-messenger coupling of EP_3 receptors is, in some cases, coupled through $G_{\alpha i}$ to the inhibition of adenylyl cyclase (e.g. Namba *et al.* 1993), and it is possible that bidirectional regulation of gastric slow-wave frequency occurs via cAMP production. EP_3 receptors have also been linked to PLC stimulation via a pertussis toxin-insensitive pathway (non-$G_{\alpha i}$; see Asboth *et al.* 1996). Thus, it is possible that two mechanisms serve to enhance pacemaker frequency in antral ICC: IP_3-dependent positive chronotropic effects, as suggested by the present study, and

suppression of cAMP production. Both cholinergic stimulation and the effects of prostaglandin E_2 (via EP_3 receptors) could be mediated by elevations in IP_3 levels.

Many previous studies have shown that cholinergic stimulation activates a non-selective cation conductance in isolated gastrointestinal smooth muscle cells (e.g. Inoue & Isenberg, 1990; Vogalis & Sanders, 1990; Sims, 1992; Kang *et al.* 2001). In the present study, the chronotropic effects of muscarinic stimulation were observed in the absence of significant changes in peak current amplitude during pacemaker events or increases in basal current. These observations suggest that the frequency effect is not due to the activation of the muscarinic-dependent non-selective current observed in smooth muscle cells, but rather occurs by stimulation of the ongoing pacemaker mechanism. A sustained inward current was activated by the higher concentrations of CCh and ACh tested, and this current persisted for the length of the stimulus. Pre-treatment with U-73122 and 2-APB, compounds that block aspects of the pacemaker mechanism, blocked pacemaker currents, the chronotropic effects of CCh and the sustained inward current observed in response to CCh. These findings suggest that the sustained current is due to a similar mechanism to that underlying the pacemaker current. One caveat to this is that there are reports that 2-APB can exert effects on conductances such as the Ca^{2+} release-activated current (I_{CRAC}) and store-operated channels (SOCs) independently of its actions on IP_3Rs (Prakriya & Lewis, 2001; Tesfai *et al.* 2001). The study of Tesfai *et al.* (2001) concluded that 2-APB can inhibit SOCs through a mechanism involving binding of 2-APB to channel proteins or to a regulatory protein.

The increase in pacemaker frequency observed at lower concentrations of muscarinic agonists gave way to a tonic inward current with higher levels of stimulation. The development of a sustained current appeared to represent fusion of rapid pacemaker currents. One explanation of how this might occur is as follows: the phasic pacemaker currents we measure in small networks of ICC are likely to result from entrainment of currents from multiple pacemaker sites throughout the ICC network. When frequency rises, entrainment may fail. Each pacemaker site may be 'functionally uncoupled' and begin to cycle independently. This could lead to a sustained inward current as multiple pacemaker sites generate currents out of phase.

PDBu (but not 4-α-phorbol) also increased pacemaker current frequency and activated a sustained inward current in ICC. Thus, we considered whether PKC might be involved in the chronotropic effects of muscarinic stimulation. Chelerythrine, calphostin C and GF 109203x did not block either effect, however, suggesting that the effects of PDBu and muscarinic stimulation are independent of PKC activation.

Our data suggest that ACh, the major excitatory neurotransmitter released from enteric motor neurons, has powerful positive chronotropic effects on gastric pacemaker cells. The increase in slow-wave frequency is greater in the antrum than in the corpus in the mouse. The larger chronotropic effect in antral muscles could result from the simple fact that antral pacemakers normally operate at lower frequencies and, therefore, antral ICC may have a greater capacity for positive chronotropic regulation. Previous studies have shown that ACh released from enteric motor neurons is directed primarily at intramuscular ICC (IC-IM; Ward *et al.* 2000*a*) or 'septal' ICC that lie between muscle bundles (IC-SEP; see Horiguchi *et al.* 2001). In the dog, IC-IM or IC-SEP have intrinsic pacemaker capability. Hirst and colleagues (2002) have recently suggested that neural regulation of slow-wave frequency is mediated by intramuscular ICC in the guinea-pig stomach. So it is possible that the normally dominant pacemaker cells (IC-MY) in the stomach (Horiguchi *et al.* 2001) become subservient to entrainment by intramuscular pacemaker activity during neural release of ACh. An interesting prediction arising from the current study is that postprandial activation of cholinergic motor neurons should enhance gastric slow-wave frequency. This may be a subtle increase in the intact stomachs of normal individuals, as corpus pacemakers maintain dominance and the corpus frequency only increased by 16 % in response to ACh stimulation. Since the chronotropic capacity of antral pacemakers appears to be much greater than in the corpus, activation of cholinergic motor neurons may inherently tend to challenge the proximal-to-distal slow-wave frequency gradient. This may be why ingestion of meals tends to be a stimulus for arrhythmias in some human patients. Collapse of the proximal-to-distal frequency gradient could lead to antral tachygastria if there is unequal activation of cholinergic motor neurons, and firing of excitatory motor nerves to the antral region becomes greater relative to firing of nerves to the corpus. Indeed, it has been shown that selective stimulation of the anterior nerves of Latarjet, which innervate the distal stomach, can elicit premature slow waves that are not produced by propagation from the corpus and are functionally uncoupled from the dominant pacemaker (Sarna & Daniel, 1975). Central misprogramming of postprandial vagal excitatory inputs during the cephalic and gastric phases of digestion might lead to such a condition.

In summary, the question of gastric dysrhythmias has eluded mechanistic explanation and effective therapies for many years (see Owyang & Hasler, 2002). Our data show that the chronotropic effects of cholinergic stimulation are an intrinsic property of ICC, and suggest that any stimulus that raises IP_3 production has positive chronotropic effects on gastric ICC by decreasing the cycling time of the

pacemaker mechanism. Use of ICC cultured from various regions of the stomach and from animal models of motility disorders in future studies may reveal why gastric pacemakers go awry under some circumstances and disrupt the proximal-to-antral slow-wave frequency gradient.

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