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# CHOLINERGIC STIMULATION ACTIVATES A NON-SELECTIVE CATION CURRENT IN CANINE PYLORIC CIRCULAR MUSCLE CELLS

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## SUMMARY

1. Cholinergic stimulation of circular muscle from the canine pyloric sphincter results in excitatory junction potentials and an increase in slow-wave frequency. Experiments were performed on isolated pyloric muscle cells to determine the effects of acetylcholine on membrane conductance and voltage-dependent ionic currents.

2. Acetylcholine depolarized circular muscle cells and increased membrane conductance. Under voltage clamp, these effects were associated with the development of an inward current.

3. The ACh-dependent current ($I_{ACh}$) reversed at about $-20$ mV and was about equally selective for potassium and sodium. Changes in the chloride gradient had no effect on the reversal potential of $I_{ACh}$.

4. The response to ACh was blocked by atropine suggesting that the response was mediated by muscarinic receptors. $I_{ACh}$ could not be elicited in the presence of ions normally used to block potassium currents (e.g. bath-applied $TEA^+$ and replacement of $K_i^+$ with $Cs_i^+$).

5. In some cells single-channel openings could be resolved in response to ACh. These channels had a slope conductance of 30 pS, and open probability increased with depolarization.

6. Acetylcholine had little or no effect on voltage-dependent $Ca^{2+}$ currents, and increased voltage-dependent outward currents. The latter effect may have been due to increased release of $Ca^{2+}$ from internal stores.

7. The non-selective cationic current elicited by ACh can explain the excitatory junction potentials in pyloric muscle cells that are generated by transmural nerve stimulation and may also explain the chronotropic effects of ACh on slow waves.

## INTRODUCTION

Gastrointestinal motility is regulated by neural, humoral and myogenic mechanisms (see Szurszewski, 1987). The neurotransmitter acetylcholine (ACh) is an important mediator in the neural control excitation of gastrointestinal smooth muscle. The ionic mechanisms underlying muscarinic responses of mammalian gastrointestinal smooth muscles appear to vary from preparation to preparation. In guinea-pig ileum and rabbit jejunum, ACh caused an increase in membrane conductance. This effect was mediated by an increase in permeability to $Na^+$ and $K^+$

(Inoue, Kitamura & Kuriyama, 1979; Benham, Bolton & Lang, 1985). In toad gastric muscle cells muscarinic stimulation decreased membrane conductance by suppressing an 'M-current', a specific, $Ca^{2+}$-independent $K^+$ conductance (Sims, Singer & Walsh, 1985). ACh also increased voltage-dependent $Ca^{2+}$ current in toad cells (Clapp, Vivaudou, Walsh & Singer, 1987). We have previously reported that ACh shifts the voltage dependence of $Ca^{2+}$-activated $K^+$ channels to more positive levels in canine colonic myocytes (Cole, Carl & Sanders, 1989). This effect may underlie the prolongation of slow waves by ACh.

In previous studies we have investigated the organization of electrical activity and the innervation of the canine pyloric musculature (Sanders & Vogalis, 1989; Vogalis & Sanders, 1989*a*). We found that the canine pylorus is innervated by intrinsic, cholinergic nerves which can elicit suprathreshold excitatory junction potentials (EJPs) in the smooth muscle. Cholinergic stimulation also increased the frequency and duration of slow waves and increased the force of contractions. EJPs and other effects elicited by neural stimulation or application of exogenous acetylcholine were blocked by atropine, indicating that cholinergic excitatory responses are mediated by muscarinic receptor stimulation, as reported in other gastrointestinal smooth muscle preparations (see Bolton, 1979).

In the present study we investigated the ionic mechanism of cholinergic excitation in isolated canine pyloric smooth muscle cells. The patch-clamp technique was used to allow voltage- and current-clamp studies.

## METHODS

### *Preparation of single smooth muscle cells from pyloric circular muscle*

Mongrel dogs of either sex were anaesthetized with sodium pentobarbitone (30 mg/kg). The abdomen was opened by mid-line incision and the stomach, including a short segment of the duodenum, was removed and placed in pre-oxygenated Krebs–bicarbonate (KRB) solution (see Sanders & Vogalis, 1989). The pyloric ring was dissected from the stomach and pinned out in a dissecting dish containing KRB at room temperature. After removing the mucosa, a strip of muscle was dissected from the sphincteric region parallel to the long axis of the circular layer. This muscle strip was pinned on its side and the longitudinal muscle layer and the remaining submucosa were removed by sharp dissection. The circular muscle was cut into small pieces (1–2 $mm^3$) which were transferred into a dish containing pre-oxygenated, $Ca^{2+}$-free Hanks' solution of the following composition (mM): $Na^+$, 140·5; $K^+$, 5·8; $Cl^-$, 130·3; $HCO_3^-$, 15·5; $HPO_4^{2-}$, 0·336; $H_2PO_4^-$, 0·4; dextrose, 10; sucrose, 2·9. After equilibration with 97% $O_2$–3% $CO_2$ this solution had a pH of 7·4 at room temperature. The muscle pieces were transferred to a test-tube containing dispersion medium of the following composition (per millilitre of Hanks' $Ca^{2+}$-free solution): 1 mg collagenase CLS II (151 U, Worthington Biochemical, NJ, USA), 1·5 mg bovine serum albumin (fatty acid free; Sigma), 2 mg trypsin inhibitor (Sigma), 0·11 mg $Na_2ATP$ (Sigma). The muscle pieces were incubated in dispersion medium at 37 °C for 30–40 min with continuous agitation. The supernatant was aspirated and the remaining pieces of tissue were resuspended in $Ca^{2+}$-free Hanks' solution. After 10–15 min in this solution, gentle trituration released single relaxed smooth muscle cells (200–300 $\mu$m in length and 5–10 $\mu$m in diameter). Aliquots (five to ten drops) of this solution were placed in 2 ml of growth medium (M119, GIBCO). Cells were plated into plastic culture dishes and incubated at 37 °C (90% humidity and 95% $O_2$–5% $CO_2$) for 1 h. The cells were either used immediately or stored at 4 °C for up to 6 h.

### *Whole-cell recording*

Culture dishes containing isolated cells were placed on the stage of an inverted microscope (Nikon Diaphot) and superfused with normal HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2′-ethane-sulphonic acid) perfusion saline (normal solution, NS) containing (mM): $Na^+$, 130; $K^+$, 5·8; $Cl^-$,

135; $HCO_3^-$, 4·16; $HPO_4^{2-}$, 0·336; $H_2PO_4^{2-}$, 0·44; $Ca^{2+}$, 1·8; $Mg^{2+}$, 0·9; $SO_4^{2-}$, 0·8; dextrose, 10; sucrose, 2·9; HEPES, 10. Solution pH was adjusted to 7·4 with NaOH.

Patch pipettes were pulled from borosilicate glass tubing (Sutter BF-150-117-15) using a Sutter micropipette puller (Model P80-PC) and fire-polished to about 2–4 MΩ. The standard pipette solution (potassium gluconate) contained (mM): potassium gluconate, 110; KCl, 20; $MgCl_2$, 1·5; $K_2ATP$, 2·5; $Na_2ATP$, 2·5; $Na_2GTP$, 0·5; phosphocreatine, 5; HEPES, 5; EGTA, 1. The pH was adjusted to 7·2 with KOH and filtered before use. The pipette solution for use in recording inward calcium currents contained (mM): caesium aspartate, 110; caesium chloride, 30; $K_2ATP$, 2·5; $Na_2ATP$, 2·5; phosphocreatine, 5; $MgCl_2$, 1·5; HEPES, 10; EGTA, 1; $Na_2GTP$, 0·5. The pH was adjusted to 7·2 with CsOH, and the solution was filtered, and stored frozen.

The patch pipettes were positioned above the smooth muscle cells using a Narashige hydraulic manipulator (MO-103), and the pipette potential nulled. Gigaseals were obtained by applying negative pressure to the pipette after contact with the smooth muscle plasma membrane. Access to the cell interior was gained by negative pressure or by application of a brief voltage pulse. Voltage commands were delivered by an Axopatch 1A patch-clamp amplifier (Axon Instruments). Voltage clamp test pulse protocols were delivered and membrane current responses were recorded by means of a 12-bit A/D converter (Labmaster, Scientific Solutions) interfaced to an AT-type computer running pClamp software (Axon Instruments). Currents were recorded at a sampling rate of 10 and 20 kHz and records were low-pass filtered at 5 kHz. All voltage measurements recorded using the potassium gluconate pipette solution were corrected for a 10 mV liquid junction potential. Voltage measurements using caesium aspartate-filled pipettes were corrected from a 5 mV liquid junction potential. Data analysis was performed with pClamp software and hard copies were made with a digital plotter (Hewlett Packard, 7475A) running pClamp software and Sigmaplot graphics software (Jandel, Scientific). Averaged data are expressed as the mean ± S.E.M., where $n$ represents the number of cells. All recordings were made at room temperature (22–25 °C).

*Drugs*

The following drugs were used: ethyleneglycol-bis-(β-aminoethylether)*N,N,N',N'*-tetraacetic acid (EGTA; Sigma), tetraethylammonium chloride (TEA; Sigma), nifedipine (Boehringer, Mannheim); acetylcholine chloride (Sigma); atropine sulphate (Sigma). Nifedipine was dissolved in 100% ethanol in a stock solution of $10^{-2}$ M and stored at 4 °C until the day of use. Stock solutions of acetylcholine chloride ($10^{-1}$ M) were prepared using distilled water and stored in 1 ml vials which were kept frozen until the day of use.

## RESULTS

### *Effect of acetylcholine on membrane potential and membrane conductance*

The resting membrane potential of pyloric cells dialysed with standard pipette solution (potassium gluconate) ranged from −30 to −55 mV. In current clamp, membrane potential was maintained at between −60 and −70 mV injection of constant hyperpolarizing current in order to simulate the resting potential of cells *in situ*. Acetylcholine (ACh), added to the perfusing solution at final concentrations ranging from $10^{-5}$ to $10^{-3}$ M, caused depolarization. This depolarization was associated with an increase in membrane conductance (Fig. 1*A*). In four cells tested under current clamp, ACh depolarized the cells to an average potential of −43 ± 2 mV and membrane resistance was reduced to approximately 40% to the pre-agonist value. These observations suggest that ACh activates a conductance that facilitates inward current and has a reversal potential positive to −60 mV.

In voltage-clamp mode cells were held at a holding potential ($V_h$) of −65 mV. This potential required inward holding currents in the range of 10–30 pA. Application of ACh increased the holding current in a dose-dependent manner: ACh ($10^{-5}$ M) increased inward holding current by 10 ± 2 pA ($n = 7$); ACh ($10^{-4}$ M) increased inward holding current by an average of 24 ± 6·5 pA ($n = 9$). An example of this

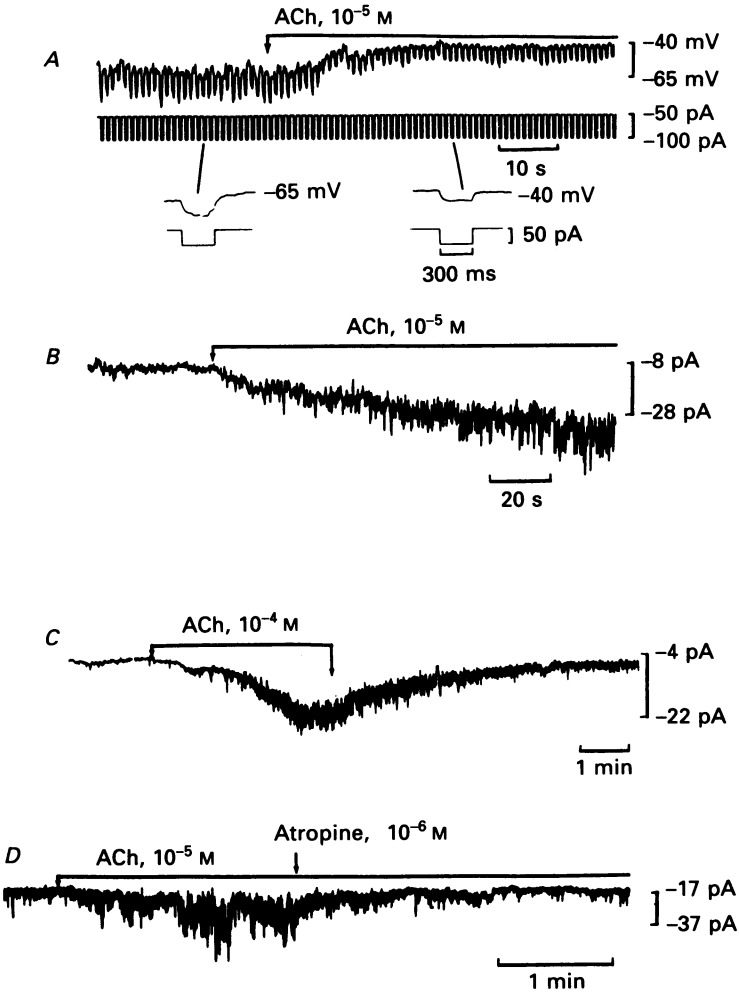


Fig. 1. Effect of acetylcholine (ACh) on membrane potential and conductance. Panel *A* shows effects of ACh in current clamp experiment on membrane potential (top trace). Membrane potential was adjusted to −65 mV and constant current hyperpolarizing pulses (lower trace) were applied. Application of ACh ($10^{-5}$ M) depolarized the cell and decreased membrane resistance as indicated by a decrease in the amplitude of the electrotonic potentials. Insets show electrotonic potentials before and during ACh at an expanded sweep speed. Panel *B* shows the effect of ACh under voltage clamp. At a holding potential of −65 mV, exposure to ACh ($10^{-5}$ M) caused an increase in inward holding current. This was associated with a significant increase in noise in the current trace. The current elicited by ACh ($I_{ACh}$) was reversible upon wash-out of the ACh (panel *C*). Panel *D* shows another cell exposed to ACh. After $I_{ACh}$ was elicited, atropine was added. This caused reversal of the ACh effect.

response is shown in Fig. 1*B*. At higher concentrations ($10^{-3}$ M), ACh caused a large increase in the holding current (> 100 pA), but desensitization rapidly developed and the effect diminished over a 1–2 min period. Desensitization was less rapid at concentrations of $10^{-4}$ M or less. The agonist-induced increase in holding current was reversible upon wash-out of ACh (Fig. 1*C*). Responses to subsequent doses of ACh

were, however, markedly depressed. The increase in resting conductance activated by ACh was blocked by atropine ($10^{-6}$ M) in the three cells tested, indicating that the response is due to stimulation of muscarinic receptors (Fig. 1*D*). The increase in holding current in response to ACh was associated with a large increase in noise in the current trace, suggesting an increase in channel activity.

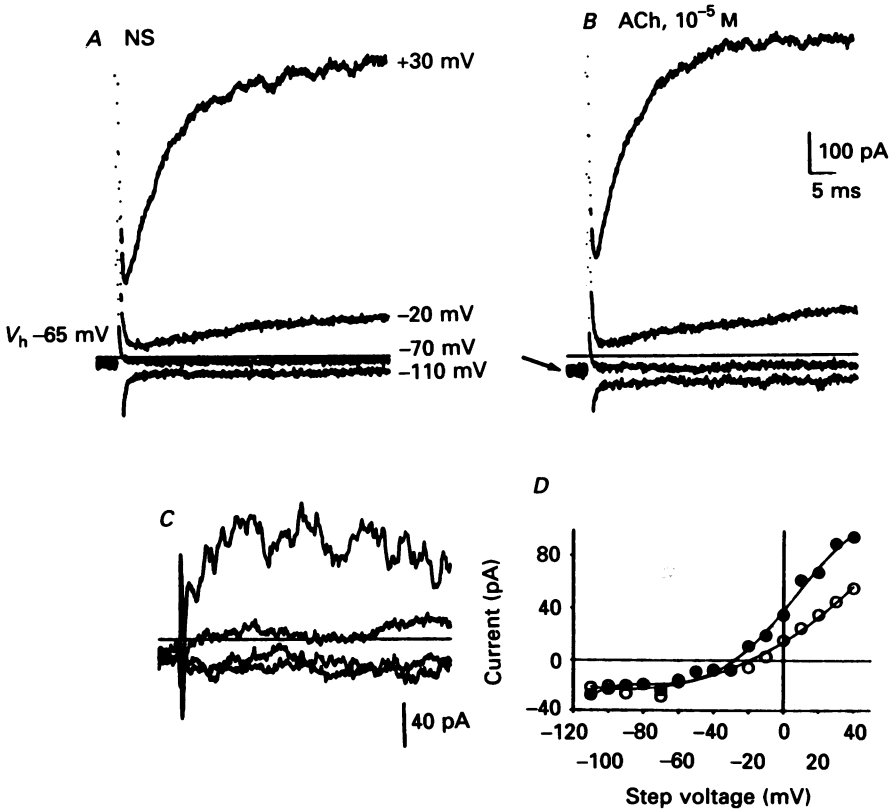


Fig. 2. Current–voltage (*I–V*) relationship of acetylcholine-activated current ($I_{ACh}$). Panel *A* shows currents evoked by steps from a holding potential ($V_h$) of −65 mV to several test potentials ranging from −110 to +30 mV. Panel *B* shows a repeat of the same series of test potentials during exposure to ACh ($10^{-4}$ M). The horizontal line in each panel denotes the zero current level. Note the increase in inward holding current in response to ACh (see arrow). Currents evoked in the presence of ACh (panel *B*) were digitally subtracted from these evoked in normal solutions (NS; panel *A*) to obtain 'difference currents' (panel *C*). Panel *D* is a plot of the *I–V* relationship of the difference current induced by ACh ($I_{ACh}$). Average currents at 5–10 ms after the onset of the test pulse (○, early) and after a steady-state current was attained (approximately 50 ms) (●, late) are plotted. $I_{ACh}$ reversed at about −20 mV. ACh also increased the time-dependent outward current at potentials positive to −30 mV. All recordings were performed in the presence of nifedipine ($10^{-6}$ M). Time calibration in panel *B* refers to all traces.

*Reversal potential of ACh-activated current*

The increase in membrane conductance and depolarization caused by ACh suggests activation of channels that facilitated inward current. To study the selectivity of the ACh-dependent current, experiments were performed to determine

the reversal potential of the current. The current–voltage relationship of ACh-induced current was obtained by a subtraction method: membrane currents evoked in normal solution (NS) were subtracted from membrane currents evoked in the presence of ACh at each test potential (Fig. 2*A* and *B*). The protocol consisted of

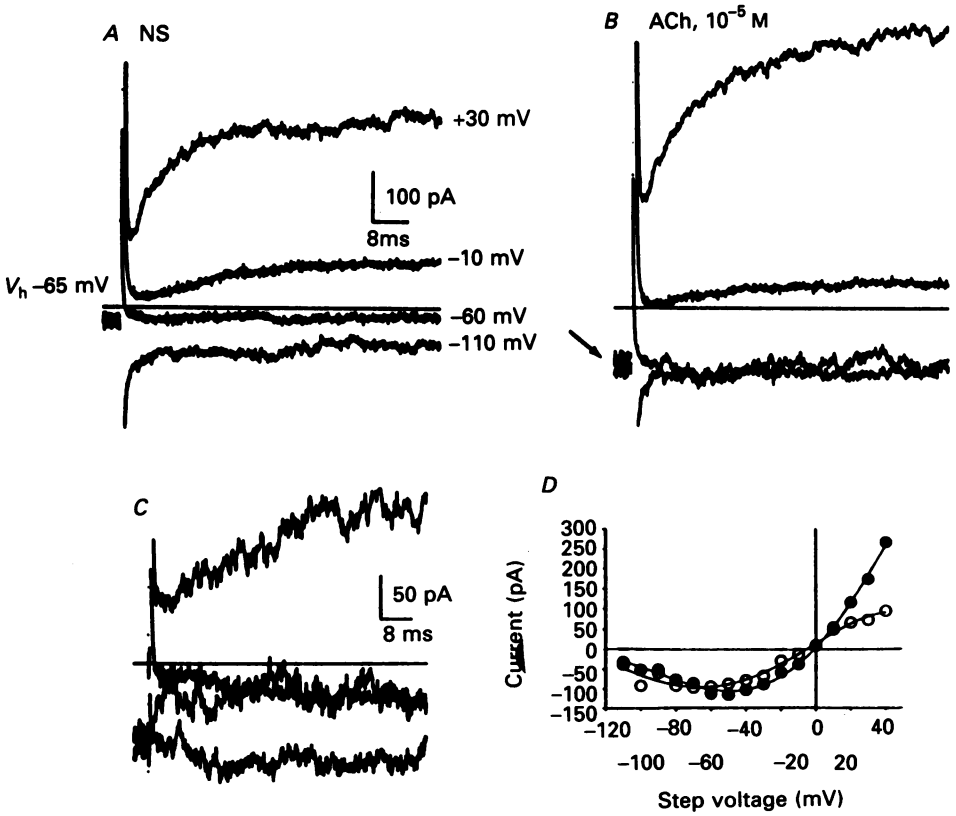


Fig. 3. In some cells $I_{ACh}$ displayed voltage-dependent activation in the range of resting potential. Figure show current responses before and during ACh (panels *A* and *B*) using similar experimental protocol to that in Fig. 2. NS, normal solution. Note large increase in holding current in response to ACh (arrow). Panel *C* shows difference currents obtained by digitally subtracting traces in panel *B* from the corresponding traces in panel *A*. Panel *D* shows current–voltage relationship of early (○) and late (●) difference currents (as defined in Fig. 2). Note voltage-dependent activation of $I_{ACh}$ at potentials in the range of −100 to −50 mV. All recordings were performed in the presence of nifedipine ($10^{-6}$ M).

100 ms voltage steps from a holding potential of −65 mV to potentials ranging from −110 to +40 mV. Test potentials were applied every 2 s. The differences between the currents evoked at each test potential in the absence and presence of ACh were calculated and averaged over the initial pulse interval (~ 10 ms) in order to exclude effects of ACh on outward currents that might be mediated via entry or release of $Ca^{2+}$. The difference current ($I_{ACh}$) was plotted as a function of test potential (Fig. 2*C*).

The current–voltage relationship for $I_{ACh}$ might be obscured by effects of ACh on other voltage-dependent currents. To eliminate contamination by inward calcium current, cells were exposed to $10^{-6}$ M-nifedipine, which has been shown to block $Ca^{2+}$ current in these cells (Vogalis & Sanders, 1989*b*). Elimination of voltage-dependent outward current was more difficult. When caesium-filled pipettes were used (140 mM $[Cs^+]_i$), ACh failed to increase holding current, suggesting that $Cs^+$ blocked the ACh-dependent conductance. Inclusion of 40 mM-tetraethylammonium chloride (TEA-Cl) in the perfusion solution blocked most of the voltage-dependent outward current, but also blocked the ACh-dependent current in the three cells tested.

Figure 2*A* shows currents evoked upon stepping the membrane potential to hyperpolarized and depolarized potentials. Figure 2*B* shows the currents evoked by the same test potentials in the presence of ACh ($10^{-4}$ M). Note the change in holding current in Fig. 2*B*. The differences between the current responses in Fig. 2*A* and *B* (difference currents) are shown in Fig. 2*C*. Figure 2*D* is a plot of the difference current early in the responses (10 ms; ○, $I_{ACh}$) and late in the responses (80 ms; ●) at test potentials ranging from −110 to +40 mV. The curves drawn through the data points are non-linear regression lines fitted using the least-squares method. The two curves are linear and are superimposed at potentials negative to −40 mV but diverge at more positive potentials suggesting that ACh may enhance a component of time-dependent outward current. $I_{ACh}$ reversed in the region of −20 mV. The late difference current reversed at a slightly more negative potentials reflecting activation of outward current later in the pulse.

The *I–V* relationship of $I_{ACh}$ was linear in the range of −110 to −40 mV in ten of twelve cells studied. In the two remaining cells the *I–V* relationship appeared U-shaped in this voltage range (Fig. 3) suggesting that the $I_{ACh}$ may demonstrate some voltage dependence. The reversal potential of $I_{ACh}$ ($E_{ACh}$) averaged $-14{\cdot}5 \pm 2{\cdot}4$ mV ($n = 12$ cells). In nine cells not treated with nifedipine ($10^{-6}$ M), $E_{ACh}$ averaged $-28 \pm 5$ mV.

The reversal potential, $E_{ACh}$, was unaffected by changes in the chloride equilibrium potential ($E_{Cl}$). When $E_{Cl}$ was increased to −86 mV by substitution of potassium gluconate with potassium chloride in the standard pipette solution, $E_{ACh}$ averaged $-20{\cdot}2 \pm 6$ mV ($n = 4$). When $E_{Cl}$ was reduced to −26 mV, $E_{ACh}$ averaged $-15 \pm 4$ mV ($n = 4$ cells). Therefore $Cl^-$ does not significantly contribute to the generation of $I_{ACh}$.

The reversal potential, $E_{ACh}$, lies between the equilibrium potentials of $Na^+$ ($E_{Na}$) and $K^+$ ($E_K$). This values suggests that the $I_{ACh}$ is due to a simultaneous increase in the membrane permeability to both ions. The permeability ratio of $K^+$ to $Na^+$ ($P_K/P_{Na}$) was estimated from the following equation (Hodgkin & Katz, 1949; Goldman, 1943):

$$P_K/P_{Na} = ([Na^+]_o - [Na^+]_i \exp(FE_{ACh}/RT))/([K^+]_i \exp(FE_{ACh}/RT) - [K^+]_o),$$

where $[Na^+]_o$ and $[Na^+]_i$ are the extracellular and intracellular concentrations of $Na^+$ respectively, and $[K^+]_o$ and $[K^+]_i$ are the extracellular and intracellular concentrations of $K^+$ respectively. Assuming that only $Na^+$ and $K^+$ contribute to the ACh-activated conductance, $P_K/P_{Na}$ equalled 1·7.

Elevation of $[K^+]_o$ to 50 mM by equimolar substitution of NaCl with KCl in the

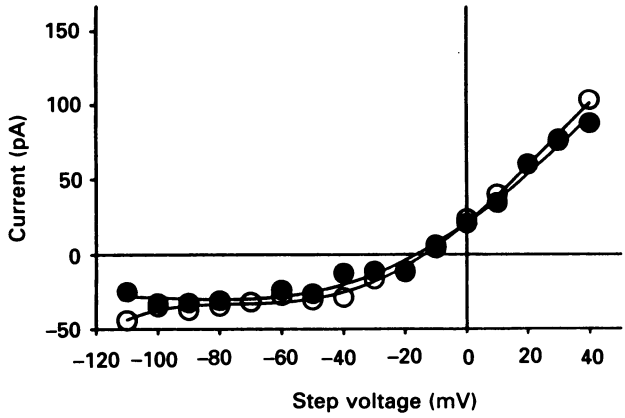


Fig. 4. Effect of external potassium $[K^+]_o$ on the reversal potential of the acetylcholine-activated current ($I_{ACh}$). ○ represent the current–voltage ($I$–$V$) relationship of $I_{ACh}$ in normal perfusion buffer, $[K^+]_o = 5{\cdot}8$ mM. ● represent the $I$–$V$ relationship of $I_{ACh}$ in bathing solution containing 50 mM $[K^+]_o$ (substituted for $Na^+$). The $I$–$V$ relationships are superimposed indicating that the permeability of channels activated by ACh is similar for $K^+$ and $Na^+$. Data were obtained in the presence of nifedipine ($10^{-6}$ M).

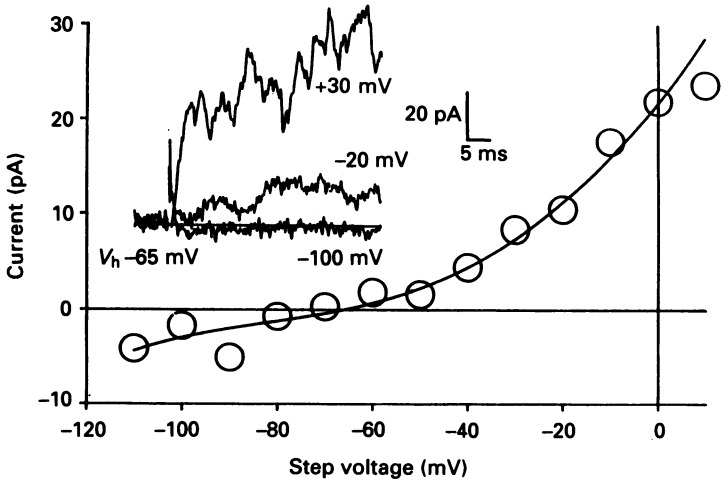


Fig. 5. Effect of $Na^+$ replacement on the acetylcholine (ACh)-activated current ($I_{ACh}$). $[Na^+]_o$ was reduced to 65 mM by substitution with $Tris^+$. This procedure shifted $E_{Na}$ by 20 mV (to +37 mV) and produced a negative shift in the reversal potential of $I_{ACh}$. Inset shows examples of difference currents in reduced external $Na^+$ which were obtained as described in Fig. 2.

perfusing solution, which reduced $[Na^+]_o$ to 85·8 mM, shifted $E_{ACh}$ slightly positively to a mean of $-9 \pm 2{\cdot}8$ mV ($n = 4$ cells) (see Fig: 4). This value of $E_{ACh}$ gave a calculated $P_K/P_{Na}$ of 1·7.

The effects of $Na^+$ replacement on the reversal potential of $I_{ACh}$ was studied in three experiments. Extracellular $Na^+$ was reduced to 65 mM by replacement of half the external $Na^+$ with $Tris^+$. Test potentials ranging from $-110$ to $+40$ mV were

applied before and during exposure to ACh ($10^{-4}$ M). As described above, current responses were subtracted to obtain the difference current. Reduction in external $Na^+$ caused a negative shift in reversal potential of $I_{ACh}$ to an average of $-62 \pm 5$ mV (Fig. 5), and caused the $P_K/P_{Na}$ ratio to change to 8·8.

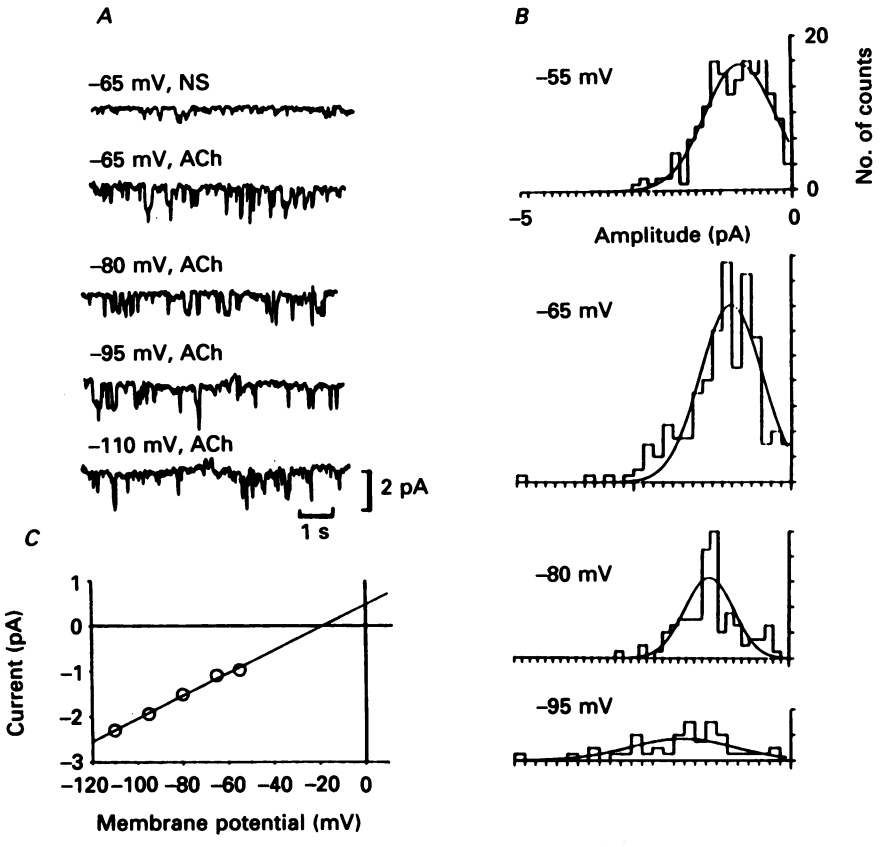


Fig. 6. Acetylcholine (ACh)-activated channels. In some ACh appeared to activate only a few channels, and these could be observed under whole-cell recording conditions. Panel *A* shows channel activity in control solution (NS) and at several holding potentials in response to ACh ($10^{-5}$ M). The amplitude of the ACh-activated single-channel currents increased as the holding potential was made more negative, but the frequency of openings appeared to decrease. Panel *B* shows amplitude histograms of channel openings at four holding potentials for 22 s recording periods. The number of openings decreased at the more negative potentials. Panel *C* is an *I–V* plot of four ACh-activated channels. Reversal potential of ACh-activated channels ($E_{ACh}$) was estimated by plotting the mean current amplitude *versus* holding potential and extrapolating to the zero-current voltage. In this cell the $E_{ACh}$ was calculated to be $-19$ mV, and the slope conductance was 28 pS.

*ACh-activated channel activity*

Under whole-cell recording conditions in voltage-clamp mode openings of single channels were occasionally discernible during application of ACh ($10^{-5}$ M). In three cells the current trace was sufficiently free of noise to permit some analysis of single-

channel amplitude *versus* membrane potential and of channel open times. An example of one of these experiments is shown in Fig. 6. Acetylcholine ($10^{-5}$ M) activated channels at a holding potential of −65 mV (Fig. 6*A*). The amplitude of single-channel currents decreased at more positive holding potentials and the

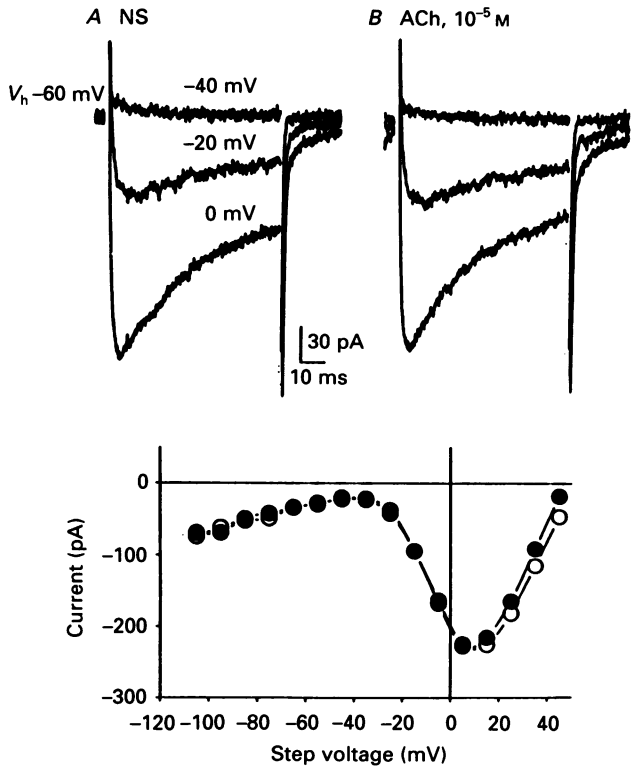


Fig. 7. Effect of acetylcholine (ACh) on inward $Ca^{2+}$ current. Cells were dialysed with pipette solutions containing 140 mM-caesium aspartate to block outward current. Panel *A* shows current responses evoked by stepping the membrane potential from a holding potential ($V_h$) of −60 mV to −40, −20 and 0 mV, in normal perfusion buffer (NS). Panel *B* shows responses to the same protocol in the presence of ACh $10^{-4}$ M. Panel *C* shows current–voltage relationships for the peak inward current elicited in normal solution (○) and in the presence of ACh (●). ACh had little or no effect on the magnitude of the inward current.

frequency of channel openings greatly increased (Fig. 6*A*). The high frequency of openings at potentials positive to −50 mV made it impossible to resolve single-channel currents. Therefore the cells were held at several potentials negative to −50 mV to characterize channel amplitude and slope conductance. Histograms of channel amplitude at four different holding potentials are shown in Fig. 6*B*, each fitted with a curve for a sum of Gaussian distributions using the least-squares method. The histograms indicated that the mean amplitude of the single-channel current increased at more negative potentials while the absolute number of openings

decreased. Figure 6*C* shows a plot of mean single-channel amplitudes (obtained from the histograms in Fig. 6*B*) as a function of membrane potential. In the three cells in which single-channel activity could be resolved, the extrapolated reversal potential averaged $-12 \pm 3$ mV, and the slope conductance averaged $30 \pm 4$ pS.

*Effect of ACh on inward calcium current*

In intact pyloric muscles ACh depolarizes membrane potential and increases the amplitude and duration of slow waves. Neural release of ACh causes excitatory junction potentials which can lead to generation of $Ca^{2+}$ action potentials in some regions of the pylorus. Each of these electrical responses can be associated with an increase in the force of pyloric contractions. Therefore, it could be hypothesized that ACh causes an enhancement in $Ca^{2+}$ influx. Experiments were performed to investigate the possibility that ACh increases the influx of $Ca^{2+}$ through voltage-dependent calcium channels. In these studies outward potassium currents were eliminated by dialysing cells with caesium (140 mM in pipette filling solution). Under these conditions ACh ($10^{-4}$ M) had no effect on the resting conductance at a holding potential of $-60$ mV ($n = 8$), suggesting that $Cs^+$ blocks the ACh-activated channels described above. Cells were stimulated with a series of test potentials ranging from $-105$ to 45 mV before and after ACh. ACh failed to increase the peak amplitude of inward current as shown as in Fig. 7. In fact a slight reduction in peak inward current could usually be observed, which might be attributable to time-dependent run-down of the $Ca^{2+}$ current. The inactivation time constant ($\tau_{in}$) for calcium current (evoked by test potentials from $-60$ to 0 mV) was also unchanged by ACh ($\tau_{in} = 86 \pm 22$ ms in normal solution; $\tau_{in} = 93 \pm 23$ ms in the presence of ACh, $10^{-4}$ M, $n = 8$).

## DISCUSSION

In intact pyloric muscles, activation of cholinergic neurons by transmural nerve stimulation evokes depolarization (excitatory junction potentials; EJPs) which can initiate premature slow waves in the circular muscle near the myenteric border or action potentials in the submucosal region of the circular layer (Vogalis & Sanders, 1989*a*). In the present study we have identified a population of receptor-activated channels that are activated by muscarinic stimulation. These channels may provide an ionic basis for EJPs and may mediate the chronotropic response to muscarinic stimulation in pyloric cells. Activation of these channels increased membrane conductance and generated inward current at physiological resting potentials. The reversal potential for the current evoked by ACh was about $-15$ mV and was sensitive to the $Na^+$ and $K^+$ gradient. These data suggested that the ACh-dependent channels were approximately equally permeable to $K^+$ and $Na^+$. Chloride appeared to play little or no role in the generation of the ACh-dependent current, suggesting that the channels activated by ACh were cation selective. The contribution of $Ca^{2+}$ to the ACh current was not investigated in the present study but Inoue *et al.* (1987) reported that $Ca^{2+}$ and several other divalent cations did not significantly affect the magnitude of the ACh-dependent current. The ACh-dependent current of the motor endplate is also not significantly affected by divalent cations (Adams, Dwyer & Hille, 1980).

Other investigators have found muscarinic activation of non-selective cation channels in other smooth muscles. Inoue *et al.* (1987) have reported that muscarinic stimulation activates a $Na^+$-selective conductance in guinea-pig ileal cells, and Benham *et al.* (1985) have found a non-selective cation current in rabbit jejunal longitudinal cells. Several differences exist between the currents reported previously and the ACh-dependent current observed in the present study. In canine pyloric circular cells the selectivity of the channels appeared to be about equal $K^+$ and $Na^+$ (permeability ratio, $P_K/P_{Na} = 1{\cdot}7$), whereas in ileal cells the selectivity for $Na^+$ appeared to be severalfold greater than for $K^+$ (Inoue *et al.* 1987). The non-selective, ACh-dependent current in jejunal cells demonstrated marked voltage dependence over the range of $-70$ to $-20$ mV (Benham *et al.* 1985), whereas the ACh-dependent current in pyloric cells was less sensitive to potential over this range. Caesium and $TEA^+$ blocked the ACh-dependent inward current in pyloric cells, whereas these ions did not appear to affect the non-selective conductance in ileal cells. The differences between the non-selective conductances may be due to species or differences between circular and longitudinal muscles.

In other gastrointestinal smooth muscles the response to ACh appears to be due to effects on outward conductances. In toad gastric cells ACh suppressed a non-$Ca^{2+}$-dependent conductance, termed an M-current (Sims *et al.* 1985). This caused an increase in membrane resistance and depolarization. Our data showed that the depolarization caused in pyloric cells by ACh was associated with decreased membrane resistance. In canine colonic circular muscle ACh caused a positive shift in the activation of $Ca^{2+}$-activated $K^+$ channels (Cole *et al.* 1989). Thus at a given $Ca^{2+}$ concentration and voltage, the probability of these channels contributing to macroscopic outward currents was reduced by muscarinic stimulation. Since $Ca^{2+}$-dependent outward current appears to contribute to repolarization of slow waves in intact colonic muscles, the action of ACh would be to prolong the duration of slow waves (Sanders & Smith, 1986). In pyloric cells, however, ACh did not reduce outward current. In fact, muscarinic stimulation caused a slight increase in the magnitude of outward current elicited by test potentials positive to $-20$ mV. It is possible that the increased outward current caused by ACh could have been due to an increase in the $Ca^{2+}$-dependent component of this current. Although ACh did not increase the magnitude of $Ca^{2+}$ current, outward current could have been increased by augmenting the release of $Ca^{2+}$ from intracellular stores.

One of the major effects of ACh in gastric muscles is a positive chronotropic action. In canine corpus (Morgan & Szurszewski, 1980), antrum (Publicover & Sanders, 1986) and pylorus (Vogalis & Sanders, 1989*a*), cholinergic agonists increase the frequency of slow waves, and this action is associated with a membrane depolarization. Elevated external $K^+$ in concentrations sufficient to mimic this depolarization also increases slow-wave frequency (Bauer & Sanders, 1985). It is possible therefore that the chronotropic effects of ACh may result from the depolarization caused by activation of the non-selective cation channels characterized in the present study.

Acetylcholine-dependent channels had a mean single-channel conductance of about 30 pS, which is similar to the conductance of channels in the guinea-pig ileum (Inoue *et al.* 1987). In pyloric cells in which single-channel openings could be

observed, the non-selective channels appeared to be voltage-dependent because fewer openings were observed at more negative potentials. This phenomenon may underlie the reduction in the ACh current at more negative potentials in the macroscopic currents recorded from some cells. Positive to −30 mV, the ACh current appeared to rectify outwardly. However this effect may have been due to an indirect activation of $Ca^{2+}$-activated $K^+$ conductance caused by the release of $Ca^{2+}$ from intracellular stores in response to ACh (Somlyo, Bond, Somlyo & Scarpa, 1985). Unfortunately, we could not investigate the ACh current in isolation of the outward current because the standard means to eliminate outward current, replacement of intracellular $K^+$ with $Cs^+$ and bath-applied $TEA^+$ (Langton, Burke & Sanders, 1989), also blocked the ACh-dependent current. It is interesting that we have also found that $TEA^+$ blocks cholinergic EJPs in intact canine pyloric circular muscle (F. Vogalis & K. M. Sanders, unpublished observations).

The amplitude and kinetics of voltage-dependent calcium current was not affected by ACh. The slight reduction in inward current observed in some cells would appear to be contrary to the excitatory role of ACh, but may have been due to $Ca^{2+}$-dependent inactivation of $Ca^{2+}$ current caused by the intracellular release of $Ca^{2+}$ in response to ACh. Previous studies have provided evidence for $Ca^{2+}$ inactivation in canine colonic smooth muscle (Langton *et al.* 1989).

In summary, the activation of a non-selective cation current in response to muscarinic stimulation can explain cholinergically mediated excitatory junction potentials and possibly the chronotropic response in pyloric smooth muscle. Whether this response is mediated directly by an interaction of the receptor with the channel or via production of second messengers is in need of further investigation.

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