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PARTICIPATION OF Ca^{2+}-ACTIVATED K^+ CHANNELS IN ELECTRICAL ACTIVITY OF CANINE GASTRIC SMOOTH MUSCLE

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SUMMARY

1. The hypothesis that Ca^{2+}-activated K^+ channels participate in the repolarization of electrical slow waves was tested in isolated cells and intact muscles of the canine gastric antrum.

2. Freshly dispersed cells from the gastric antrum liberally express large conductance channels that were characterized as Ca^{2+}-activated K^+ channels by several criteria.

3. Mean slope conductance of these channels in symmetrical 140 mM-KCl solutions was 265 ± 25 pS and reversal potential was $1{\cdot}3 \pm 3{\cdot}3$ mV. The reversal potential was shifted when K^+ was partially replaced with Na^+ in a manner consistent with the Nernst equation for the K^+ gradient.

4. Open probability was studied in excised patches in solutions containing 10^{-7}–10^{-6} M-Ca^{2+} with holding potentials ranging from -100 to $+100$ mV. Resulting activation curves were fitted by Boltzmann functions.

5. Increasing $[Ca^{2+}]$ from 10^{-7} to 10^{-6} M shifted the half-maximal activation from $+99$ to 0 mV. These data suggest that Ca^{2+}-activated K^+ channels may be activated in the voltage range and $[Ca^{2+}]_i$ occurring during the plateau phase of the slow wave.

6. In intact muscles loaded with the photolabile Ca^{2+} chelator, nitr-5, photoactivated release of Ca^{2+} during the slow wave cycle produced changes consistent with activation of Ca^{2+}-dependent outward currents.

7. The data are consistent with the idea that Ca^{2+} build-up during electrical slow waves shifts the activation voltage of Ca^{2+}-activated K^+ channels into the range of the plateau potential. Activation of these channels yields outward current and repolarization.

8. Since the force of contractions depends on slow wave amplitude and duration, regulation of these channels may be important in controlling gastric motility.

INTRODUCTION

Gastric contractions are timed and modulated by the occurrence of electrical slow waves (Szurszewski, 1975; Morgan & Szurszewski, 1980). These events are characterized by rapid depolarization from a resting potential of about -70 mV to

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a peak of −30 to −20 mV, partial repolarization, and then a sustained plateau potential that persists at a level of about −35 mV for several seconds before repolarization (see reviews by Szurszewski, 1987; Sanders & Publicover, 1989). The upstroke and plateau phases are associated with muscle contraction (Morgan & Szurszewski, 1980; Morgan, Muir & Szurszewski, 1981), suggesting the entry of Ca^{2+} and an increase in cytoplasmic Ca^{2+} concentration.

The mechanism of slow waves is unknown, but pharmacological and ion replacement experiments (see Tomita, 1981; Sanders & Publicover, 1989) have suggested the involvement of ion channels similar to those in other smooth muscle cells (Benham, Bolton, Lang & Takewaki, 1986; Droogmans & Callewaert, 1986; Singer & Walsh, 1987; Cole & Sanders, 1989*a*; Langton, Burke & Sanders, 1989). Reduction of extracellular Ca^{2+} causes a reduction in upstroke velocity and plateau amplitude (Sanders & Publicover, 1989), and inorganic and organic Ca^{2+} channel blocking drugs also reduce slow waves (El-Sharkawy, Morgan & Szurszewski, 1978; Fujii, Inoue, Yamanaka & Yoshitomi, 1985). Outward potassium currents appear to be involved in repolarization because the duration and amplitude of slow waves are increased by tetraethlyammonium (TEA) ions (Szurszewski, 1978).

Recent studies on isolated gastrointestinal smooth muscle cells have shown that a large portion of the macroscopic outward current generated by these cells is Ca^{2+}-dependent (Walsh & Singer, 1987; Cole & Sanders, 1989*a*). This current is probably carried by Ca^{2+}-activated K^+ channels which are liberally expressed in these cells (Benham *et al.* 1986; Carl & Sanders, 1989). We have characterized the voltage and Ca^{2+} dependence of these channels in canine colonic myocytes and found that at resting levels of membrane potential and intracellular Ca^{2+} there is minimal probability of channel opening (Carl & Sanders, 1989). But during slow wave activity, membrane potential depolarizes and cytoplasmic Ca^{2+} increases as evidenced by contractile activity (Morgan & Szurszewski, 1980; Bauer & Sanders, 1985). Therefore, it is possible that during slow waves the voltage dependence of Ca^{2+}-activated K^+ channels may shift negatively into the voltage range of the plateau potential and participate in slow wave repolarization. This hypothesis is generally supported by the finding that slow wave duration is greatly enhanced by tetraethylammonium ions (Szurszewski, 1978). Unfortunately testing the involvement of Ca^{2+}-activated K^+ channels in slow waves is difficult because: (i) isolated smooth muscle cells do not generate slow waves; (ii) intact gastric muscles are difficult to voltage clamp; (iii) there are no specific blockers of Ca^{2+}-activated K^+ channels that function properly in the physiological ionic gradients necessary to support slow wave activity, e.g. TEA is not a specific blocker of Ca^{2+}-activated K^+ channels (Beech & Bolton, 1989; Cole & Sanders, 1989*a*) and blockade by charybdotoxin may be reduced by physiological concentrations of internal potassium (Anderson, MacKinnon, Smith & Miller, 1988; Carl, Garcia, Kenyon & Sanders, 1990).

In the present study we have characterized the voltage and Ca^{2+} dependence of Ca^{2+}-activated K^+ channels in isolated myocytes from canine antral circular muscle. We have attempted to relate the activity of these channels to the electrical activity of intact antral muscles by loading tissues with 'caged' Ca^{2+} (nitr-5/AM) and photo-releasing Ca^{2+} into cells at various times through the slow wave cycle (Tsien &

Zucker, 1986). We reasoned that release of Ca^{2+} might activate Ca^{2+}-activated K^+ channels, and with intracellular electrical recording techniques we could determine whether resulting voltage changes are consistent with the hypothesis that these channels have a role in the repolarization of slow waves.

METHODS

Preparation of isolated gastric cells and muscle strips

Dogs of either sex were anaesthetized with sodium pentobarbitone (30 mg/kg). Through a midline incision in the abdomen, the entire stomach was removed. Muscle sheets were dissected from the antral region. For patch-clamp experiments, cells were enzymatically dispersed as previously described (Langton *et al.* 1989). For tissue experiments, thin 4 × 20 mm strips were cut parallel to the long axis of the circular fibres.

Patch-clamp experiments

Recordings of single-channel currents were made from 'on-cell' and excised patches using standard patch-clamp techniques (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) as we have applied them previously (Carl & Sanders, 1989). In several experiments voltage commands were applied via pClamp (Axon Instruments) in the form of ramped potentials. This allowed more efficient measurements of slope conductance and channel activation than conventional step depolarizations. Reversal potential and slope conductance were determined for each current response. Activation curves were calculated by averaging current responses to fifteen potential ramps and dividing each data point of the averaged current by the single-channel amplitude at that holding potential after leakage current correction. The rate of change of the applied ramp potentials was sufficiently slow so that the activation curves were not distorted by the time constants of activation or deactivation. This analysis provides a continuous recording of Np_o over the entire voltage range. To obtain values for the steepness of the voltage-dependent activation and half-maximal activation voltage, activation curves were fitted with Boltzmann functions of the form:

$$Np_o = n/\{1+\exp[-K(V-V_{\frac{1}{2}})]\}, \quad (1)$$

where N is the number of channels in the patch, n is the maximal Np_o level, K^{-1} is the steepness of the voltage-dependent activation (change in potential necessary to cause an e-fold increase in activation), and $V_{\frac{1}{2}}$ is the voltage at which there is half-maximal activation.

Calculation of cell membrane area

Fields of cells were photographed under phase-contrast microscopy and images were printed on Kodabrome paper. Individual cells were cut out of the prints and weighed. Calibration was achieved by also photographing a 10 μm graticule at the same magnification used to photograph cells. The image of the graticule was also printed on photographic paper, and an area representing 100 μm^2 was cut out and weighed. Cross-sectional cell area was determined by comparing weights of cells with the 100 μm^2 standard, and cell surface area was estimated by multiplying cross-sectional area by 3·14 (pi). By this method we calculated that gastric cells have an average geometrical area of approximately 4100 ± 1100 μm^2 ($n = 54$).

Voltage recordings from intact gastric muscles

Cells of intact muscle strips were loaded with the membrane-permeable form of a 'caged' calcium compound, nitr-5 (Calbiochem, San Diego). The acetoxymethyl ester (AM) form of nitr-5 was dissolved in dimethylsulphoxide (DMSO) containing 20% (by weight) Pluronic F127 (BASF Inc., Wyandotte, MI, USA). Tissues were loaded in Krebs–bicarbonate solution containing 5–15 μM-nitr-5/AM (with DMSO diluted at least 500:1) for 2–3 h. Strips were then pinned to the Sylgard floor of a Plexiglas recording chamber and perfused with normal Krebs–bicarbonate solution. Experiments were conducted after the appearance of spontaneous slow wave activity which generally occurred within 30 min. All procedures were performed with dim illumination using non-quartz optics to reduce the exposure of the tissue to ultraviolet light.

Electrical activity was recorded with intracellular microelectrodes using conventional techniques and electronics (Publicover & Sanders, 1986). Membrane potential was digitized and recorded on a VCR-instrumentation recorder (Medical Systems). Photo-activated release of Ca^{2+} was triggered by illumination at 300–400 nm using a 200 W mercury arc lamp with a band-pass filter. Timing was controlled by an electronic shutter and light was guided to the surface of the tissue via a fibre optic tube (Dolan–Jenner). Pulses of light (1–4 s duration) were used to activate photolysis. Waveforms were analysed with the aid of microcomputer acquisition (Labmaster) and analysis software (Axon Instruments).

Solutions and drugs

In patch-clamp experiments all recordings were made at 22–24 °C. The standard pipette filling solution contained (in mM): K^+, 140; Ca^{2+}, 1 or less; Cl^-, 142; glucose, 10; and HEPES, 10 (pH 7·4). In most experiments the bath solution contained (in mM): K^+, 140; Ca^{2+}, 0·0154–1·1; Cl^-, 140; glucose, 10; EGTA, 1; and HEPES, 10 (pH 7·4). In some experiments KCl in the bath was replaced partially or completely with NaCl. The pH of these solutions was adjusted with KOH or NaOH where appropriate. The concentration of Ca^{2+} in bath and pipette solutions was buffered by EGTA to obtain activities between 10^{-4} and 10^{-9} M as described previously (Carl & Sanders, 1989).

In on-cell experiments the free Ca^{2+} was adjusted to be 10^{-6} M in both the bath solution and the pipette solution. In all cell-attached patches the standard pipette solution was used and membrane potential was neutralized by bathing the cells in standard bath solution containing 140 mM-K^+.

In tissue experiments, the Krebs–bicarbonate buffer contained (in mM): Na^+, 137·4; K^+, 5·9; Ca^{2+}, 2·5; Mg^{2+}, 1·2; Cl^-, 134; HCO_3^- 15·5; H_2PO_4, 1·2; and dextrose, 11·5.

Data are expressed as averages ± standard deviations. Paired or unpaired *t* tests are used where appropriate to determine differences between data sets.

RESULTS

Cell-attached patches

The dominant channel in cell-attached patches was a large conductance channel which opened more frequently as membrane potential was made more positive. Figure 1 shows current responses to a voltage ramp from +150 to 0 mV. Channel openings were not observed unless patches were depolarized to levels positive to +30 mV. Above 30 mV several channels were activated. Positive to +60 mV the slope conductance decreased and often became negative at potentials positive to +100 mV (see Fig. 1). Average chord conductance was 221 ± 52 pS at +50 mV and 182 ± 53 pS at +100 mV ($n = 12$).

Excised patches

Further experiments were performed on excised patches in either the inside-out or outside-out configuration (Hamill *et al.* 1981). The conductance of channels was studied by measuring the amplitudes of unitary currents at holding potentials from −100 to +100 mV. Data were fitted by least squares and the average slope conductance was calculated to be 264 ± 25 pS ($n = 9$) in symmetrical 140 mM-KCl solutions. In this K^+ gradient the average reversal potential was 1·3 ± 3·3 mV ($n = 9$). Between −50 and +50 mV the slope conductance was voltage independent, while outside this range slope conductance decreased. A permeability value, P_K, of $5{\cdot}1 \pm 0{\cdot}5 \times 10^{-13}$ cm/s was calculated from eqn (2),

$$I_K = P_K EF^2[K^+]/(RT). \quad (2)$$

Equimolar replacement of K^+ with Na^+ in the bath solution caused rectification and a shift in the reversal potential predicted by the Nernst relationship for K^+

(Table 1). The current–voltage relationship for these channels was well described by the Goldman equation (Goldman, 1943).

Ensemble-averaged currents

Activation and deactivation kinetics of the large conductance K^+ channels were studied in excised patches by stepping potential from a holding potential of −50 mV

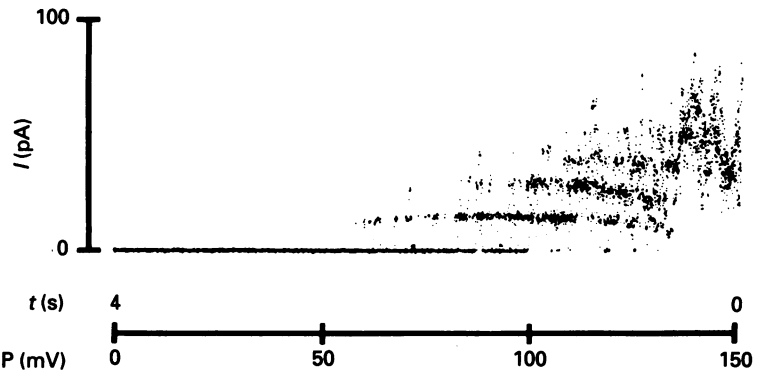

Fig. 1. Current responses to voltage ramp protocol in which holding potential (HP) was changed from +150 to 0 mV over a 4 s interval. Membrane patch was in the on-cell configuration and membrane potential was nulled by bathing the cell in 140 mM-K^+ solution. Multiple channels were observed when potentials exceeded +80 mV, and no channel openings were observed negative to +50 mV.

to test potentials ranging from +40 to +120 mV. The potential was stepped to each test potential fifteen times and the current responses were averaged (Fig. 2). For determination of activation time constants a null trace (single test pulse to −100 mV without channel openings) was subtracted from each record to remove leak conductance and the capacitive transients. Deactivation and activation could be fitted by single exponentials. Deactivation was a faster process ($\tau = 7{\cdot}1 \pm 3{\cdot}9$ ms, range 2·2–10·6, $n = 5$) than activation ($\tau = 15{\cdot}5 \pm 8{\cdot}6$ ms, range 6·3–27·8, $n = 6$). The channels showed no inactivation except at very positive potentials (see Fig. 2).

Calcium dependence

The large conductance K^+ channels were found to be dependent upon intracellular Ca^{2+} concentration. When the free Ca^{2+} in the bath solution was increased from 10^{-7} to 10^{-6} M the channel activity of inside-out patches increased dramatically.

The Ca^{2+} dependence of these channels was studied in detail in the range of 10^{-7}–10^{-8} M using voltage ramp protocols. Figure 4*A* shows the current response obtained from a patch bathed in symmetrical 140 mM-KCl and 0·5 μM-Ca^{2+} in which the potential was ramped from +100 to −100 mV. Patches were slowly hyperpolarized (0·05 mV/ms) from positive holding potentials, taking advantage of the fast deactivation kinetics of the channels to avoid distortion of the data by the deactivation time constant. The patch in Fig. 4*A* contained at least seven channels,

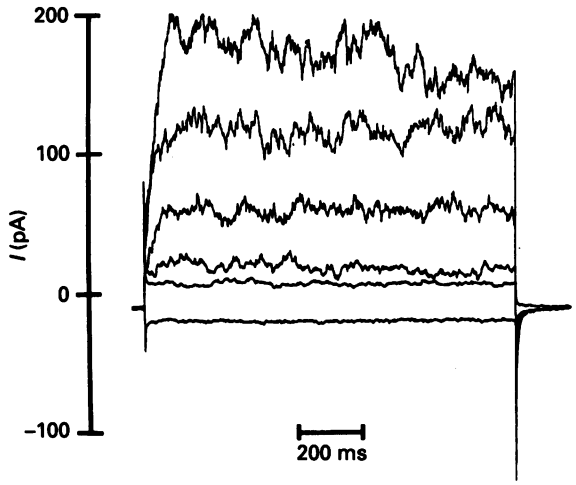

Fig. 2. Plot of ensemble-averaged currents. Excised patches were held at −50 mV. Membrane potential was stepped to −100, +40, +60, +80, +100 and +120 mV and held for 1 s. Current responses for fifteen steps to each potential were averaged. The ensemble currents showed no inactivation, except at very positive potentials. Ca^{2+} in bathing solution was 0·25 μM.

TABLE 1. Effects of substituting sodium ions for potassium ions in the bath on the slope conductance of the Ca^{2+}-activated K^+ channel of dog colon. Slope conductance measured at 0 mV. The theoretical conductance was calculated from the Goldman–Hodgkin–Katz equation (Goldman, 1943)

			Slope conductance (0 mV)		Reversal potential	
Pipette K^+ (mM)	Bath K^+ (mM)	Bath Na^+ (mM)	Measured (S.D.) (pS)	Theoretical (pS)	Measured (S.D.) (mV)	Theoretical (mV)
140	140	0	265 (25)	—	1·3 (3·3)	0
140	70	70	180 (7)	144	23·2 (3·3)	18
140	5·9	134·1	118 (7)	100	61·0 (10·0)	81

as determined by counting the discrete current levels. In patches which were analysed at Ca^{2+} levels high enough to fully activate all channels present, we found an average of 14 ± 8 channels/patch ($n = 5$). Open probability was calculated from the current responses to several voltage ramps at each Ca^{2+} concentration. Figure 4*B* shows a three-dimensional plot of Np_o as a function of voltage and Ca^{2+} concentration. Table 2 summarizes the data from these experiments. A change in Ca^{2+} concentration from 10^{-7} to 10^{-6} M at the inner surface of the patch resulted in approximately a 100 mV negative shift in the voltage for half-maximal activation. The steepness of the activation (K^{-1}) was independent of Ca^{2+} concentration (Table 2).

At 0 mV the activation could be described by a Hill function with a coefficient of $3·4 \pm 0·2$ ($n = 5$), suggesting that three to four Ca^{2+} ions are involved in channel activation. Between 0 and 75 mV there was essentially no voltage dependence on the Hill coefficient (e.g. $3·4 \pm 0·5$ at +50 mV ($n = 3$) and $3·6 \pm 0·5$ at +75 mV ($n = 2$)). Figure 5 shows the open probability of K^+ channels as a function of Ca^{2+}

concentration at a holding potential of −40 mV, which is approximately the level of membrane potential during the plateau phase of antral slow waves (Sanders & Publicover, 1989). These data suggest that increasing intracellular Ca^{2+} in the range of 10^{-7}–10^{-6} M would significantly increase the open probability of Ca^{2+}-activated K^+ channels.

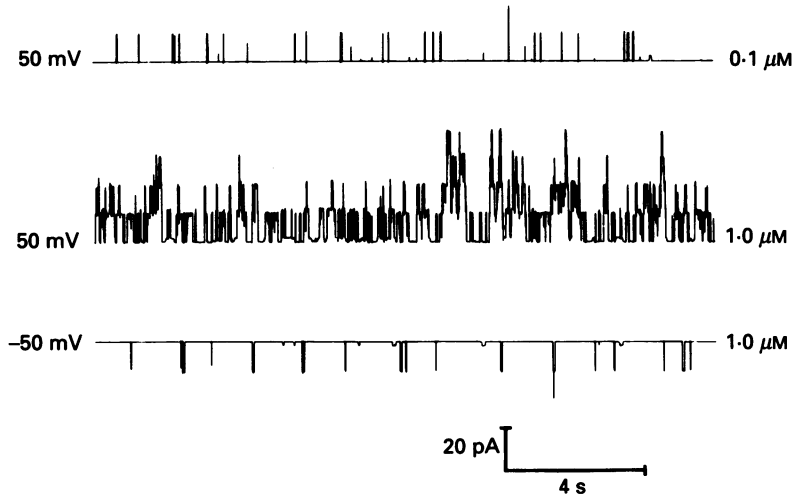

Fig. 3. Effects of Ca^{2+} and voltage on K^+ channel activity. Data were taken from an excised patch in 140 mM/140 mM-KCl gradient. First trace shows channel activity at a holding potential of +50 mV in 0·1 μM-Ca^{2+}. Openings of channels are infrequent and open times are brief. Second trace shows channel activity from the same patch of membrane at the same holding potential with 1 μM-Ca^{2+} in the bath. Note increase in open probability and an increase in the open time. Third trace shows channel activity from the same patch at a holding potential of −50 mV in 1·0 μM. The open probability is about the same as in trace one, suggesting a 100 mV shift in holding potential is about equivalent to a 10-fold increase in Ca^{2+} concentration. There is also a smaller conductance channel apparent in this figure that was observed in 5% of patches. The slope conductance of this channel averaged 60 pS, and its open probability increased with depolarization.

Experiments were also performed to determine the activation of Ca^{2+}-activated K^+ channels in on-cell conditions. Cells were bathed in a solution containing 140 mM-KCl and 10^{-6} M-Ca^{2+}. In fourteen cell-attached patches voltage was ramped from +150 to 0 mV and activation curves were calculated as described above. Half-maximal activation was estimated to be $V_{\frac{1}{2}} = +115 \pm 16$ mV. This value may be underestimated because full activation occurred at +150 mV in only a few of these experiments. These data suggest that intracellular free Ca^{2+} concentration is 10^{-7} M or less under resting conditions. However, the activation of Ca^{2+}-activated K^+ channels might be regulated by intracellular second messengers and therefore may not accurately reflect the cytoplasmic Ca^{2+} concentration.

Effects of K^+ channel blocking drugs

The effects of TEA were tested on Ca^{2+}-activated K^+ channels in excised inside-out and outside-out patches. Addition of TEA (up to 100 mM) to the bath solution with

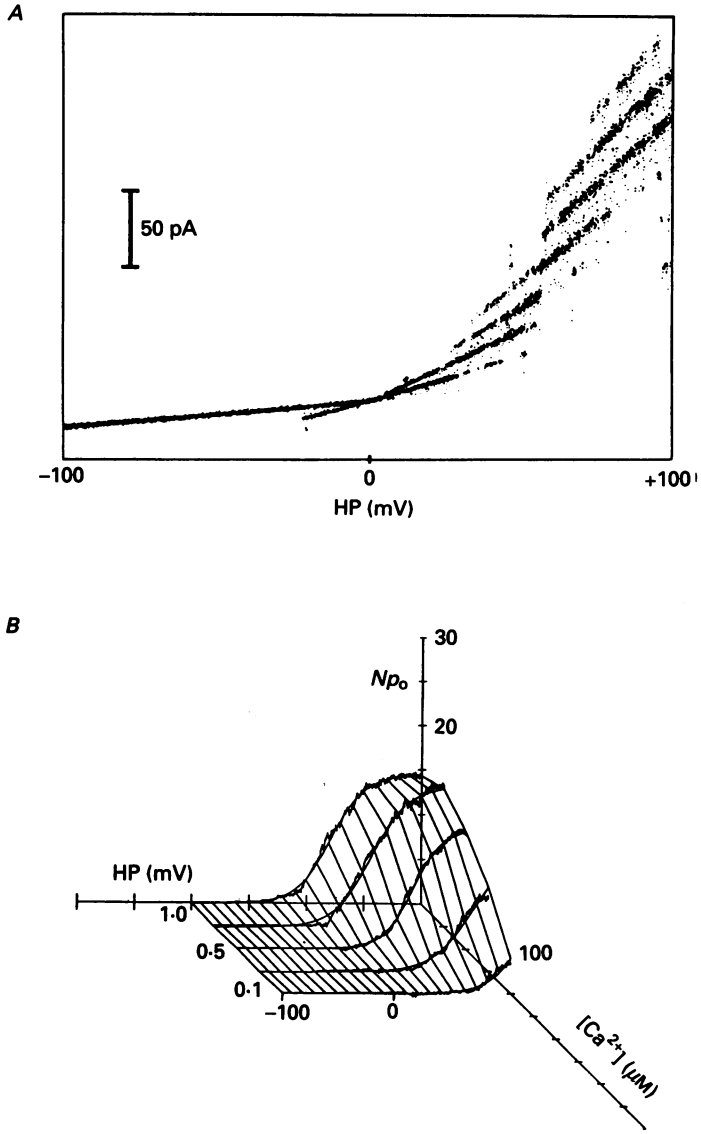

Fig. 4. Current responses of excised patch in symmetrical 140 mM/140 mM-K^+ gradient to voltage ramped from +100 to −100 mV. Single-channel amplitudes are linear over the entire voltage range and reversal potential was observed at 0 mV. This patch contained at least seven channels. Bath Ca^{2+} concentration was 0·5 μM. Averages of fifteen traces like that in *A* were corrected for leakage and driving force to obtain a continuous plot of Np_o as a function of membrane potential. These data describe the voltage dependence of activation at a single Ca^{2+} concentration. *B* shows the voltage and Ca^{2+} dependence of channels from a single patch. Data like those in *A* were collected while bathing the patch in five concentrations of Ca^{2+} ranging from 0·1 to 1·0 μM. Note negative shift in activation voltage with increasing Ca^{2+} concentration.

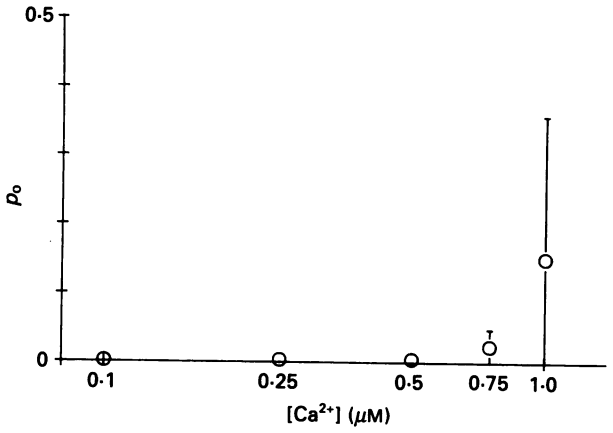

Fig. 5. Ca^{2+} dependence of open probability at −40 mV. Data were averaged from five patches. In intact muscles, the plateau potential persists for several seconds at approximately −40 mV. At resting Ca^{2+} concentrations, there is a very low open probability of these channels (see Fig. 4), but if Ca^{2+} levels rise above 0·5 μM during the plateau potential, there is a significant increase in the open probability of these channels.

TABLE 2. Effect of Ca^{2+} on steepness of voltage-dependent activation (voltage needed (mV) for an e-fold change in open probability) and half-maximal voltage of Ca^{2+}-activated K^+ channels. Data from five patches

[Ca^{2+}] (μM)	K^{-1} (mv)	$V_{\frac{1}{2}}$ (mV)
0·1	16·7±4·6	99±18 (n = 4)
0·25	17·9±4·1	80±28 (n = 4)
0·5	15·6±2·8	54±14 (n = 5)
0·75	14·8±3·2	25±18 (n = 5)
1·0	15·1±4·1	0±24 (n = 4)

inside-out patches had no effect on channel activity or conductance (Fig. 6*A*). TEA was very effective when applied to the extracellular side of the membrane in outside-out patches. TEA (10^{-7}–10^{-2} M) reduced single-channel amplitude in a dose-dependent (Fig. 6*B*) and voltage-independent manner. The half-maximal concentration for inhibition was at $1{\cdot}8 \pm 0{\cdot}3 \times 10^{-4}$ M (n = 5).

Activation of Ca^{2+}-activated K^+ channels by photo-activated release of calcium

The data above suggest that at resting Ca^{2+} levels the probability of Ca^{2+}-activated K^+ channel opening is low. Slow wave depolarization causes an increase in cytoplasmic Ca^{2+} as evidenced by contraction. The increase in Ca^{2+} would cause a negative shift in the voltage dependence of Ca^{2+}-dependent outward current. If the shift is large enough to cause an increase in the activation of Ca^{2+}-activated K^+ channels at physiological voltages (plateau potential), then the increase in outward current could result in repolarization. We studied the participation of Ca^{2+}-activated K^+ channels in slow waves of spontaneously active intact muscles using nitr-5, a 'caged' Ca^{2+} complex.

After loading of tissues with nitr-5, cells were impaled to obtain control recordings of slow wave activity. Then at desired times during the slow wave cycle, the tissues

were illuminated for 1–4 s with ultraviolet light. When tissues were illuminated during the upstroke depolarization, slow waves repolarized prematurely (Fig. 7*A*). Slow-wave duration was reduced from an average of $7{\cdot}2 \pm 1{\cdot}1$ to $3{\cdot}6 \pm 0{\cdot}9$ s ($n = 7$). The decrease in slow wave duration was primarily due to a large decrease in the time

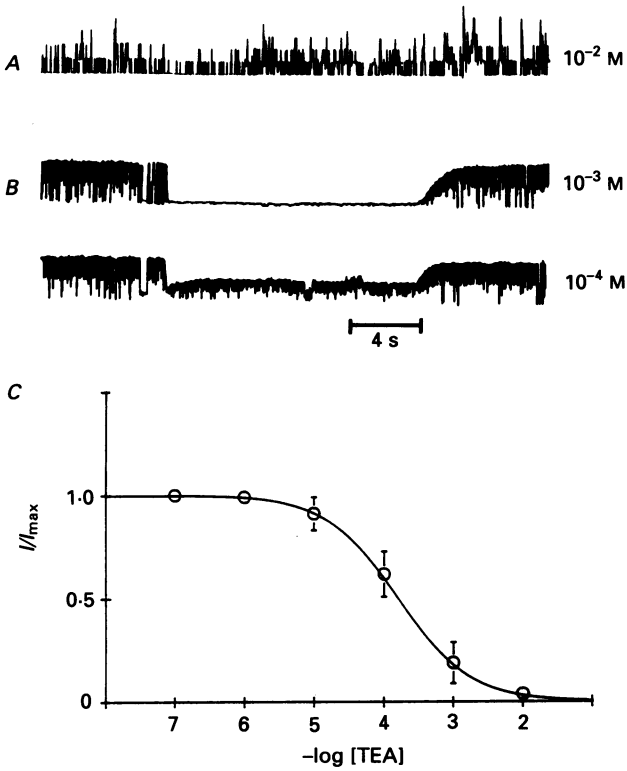

Fig. 6. Blockade of Ca^{2+}-activated K^+ channel by TEA. *A*, TEA applied to inside surface of excised membrane (inside-out patch) in concentrations up to 10^{-2} M had no effect on channel amplitude or open probability. *B*, application of TEA to outside surface of membrane (outside-out patch) caused dose-dependent reduction in single-channel amplitude. *C* shows normalized single-channel current amplitude (I/I_{max}) as a function of TEA concentration. Data were averaged from five patches. Half-maximal concentration for inhibition was $1{\cdot}8 \times 10^{-4}$ M.

course of the plateau phase. Figure 7*B* illustrates an experiment in which photoactivation was triggered just prior to a slow wave upstroke, and in this case the plateau phase was completely abolished.

Illumination at the mid-point of the plateau phase caused abrupt repolarization (Fig. 8*A* and *B*). In some experiments in which the concentration of nitr-5/AM and the loading times were reduced, photolysis during the plateau resulted in a transient repolarization followed by a return of membrane potential to the plateau level. Illumination near the end of the plateau phase caused after-hyperpolarization averaging $1{\cdot}6 \pm 0{\cdot}7$ mV ($n = 9$; Fig. 8*B*).

Illumination between slow waves produced a transient hyperpolarization and delayed the occurrence of the next spontaneous event (Fig. 8*C* and *D*). The

amplitude of the maximal hyperpolarization below resting membrane potential was $1{\cdot}7 \pm 0{\cdot}2$ mV ($n = 10$). Release of Ca^{2+} during the inter-slow-wave interval also affected slow wave frequency. The delay produced by photo-activation between slow wave events varied from preparation to preparation. This may have resulted from

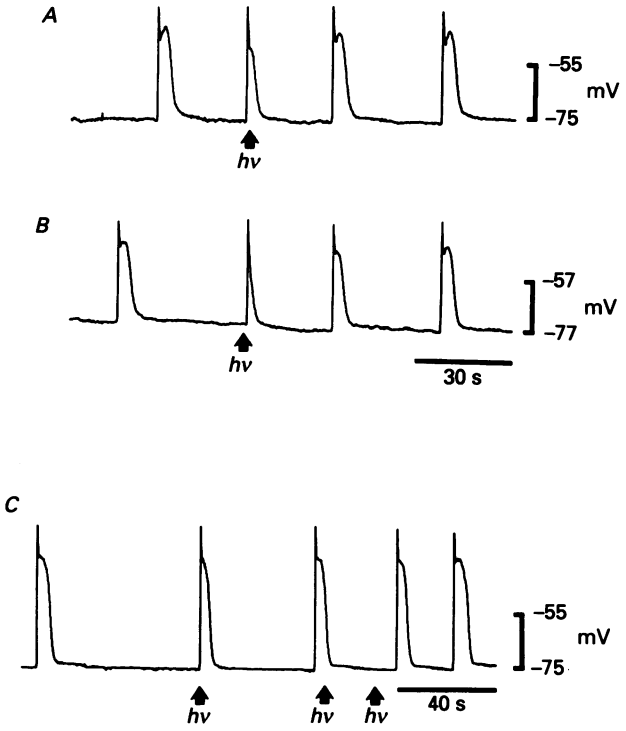

Fig. 7. Photo-activated release of Ca^{2+} near upstroke phase of slow wave. During recording of spontaneous slow waves tissues were illuminated (*hv*) at approximately the same time as the upstroke event (indicated by arrows). This caused premature (*A*) repolarization of the slow wave. If the light was applied just prior to the upstroke, the plateau phase could be abolished (*B*). *C* shows illumination of a muscle not loaded with nitr-5. This caused a small decrease in the plateau potential when the stimulus was applied near the upstroke (first arrow), but illumination late in the plateau (second arrow) or between slow waves (third arrow) failed to elicit the responses caused in nitr-5-loaded muscles. These results were unaffected by the sequence of the applied stimuli.

variations in the degree of nitr-5 loading. In one experiment, for instance, photolysis caused a 5 min hiatus in spontaneous slow wave activity (Fig. 8*D*). In all experiments the normal pattern of slow waves returned following the prolonged intervals.

Control experiments were also performed on muscles exposed to Pluronic and DMSO but not loaded with nitr-5. Illumination with the same intensity and wavelength of light used in the nitr-5 experiments produced a small decrease in slow wave duration (averaging $19 \pm 12\%$; $n = 13$) when the light was applied during the upstroke phase of the slow wave. This effect was smaller than in muscles loaded with

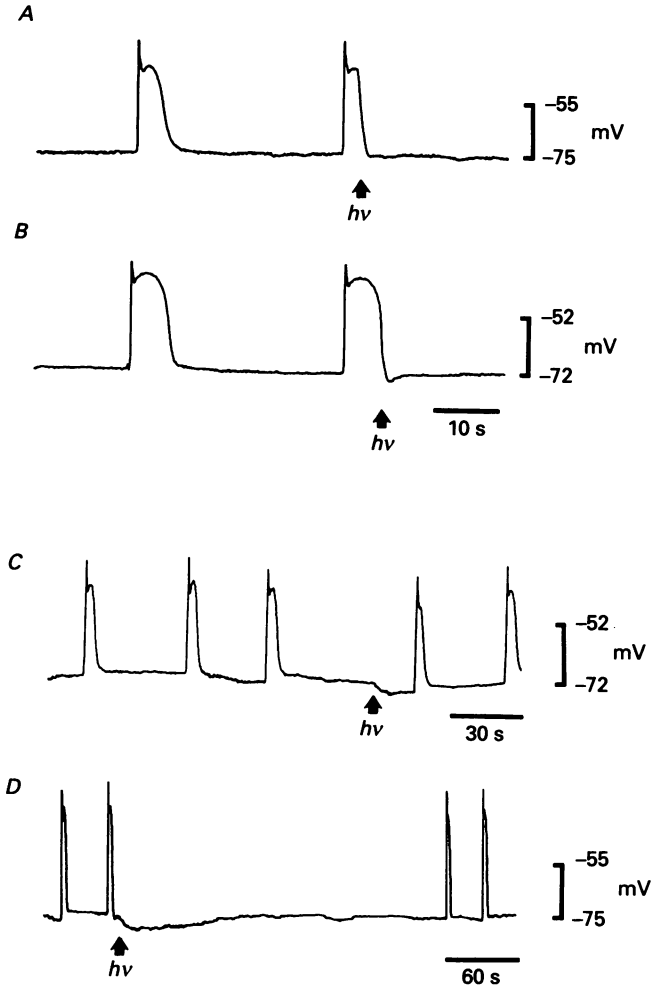

Fig. 8. Photo-activated release of Ca^{2+} during the slow wave cycle. *A* shows two spontaneous slow waves. During the second event, the muscle was irradiated at the mid-point of the plateau. This caused immediate repolarization. If illumination was delayed until the period of repolarization, a pronounced after-hyperpolarization was elicited (*B*). Photo-activated release of Ca^{2+} during the inter-slow-wave interval caused hyperpolarization and delayed the occurrence of the next slow wave. *C* shows illumination late in the diastolic period. This causes a pronounced hyperpolarization and delayed the next slow wave. *D* shows another example of this phenomenon. Illumination early in the diastolic period delayed the next slow wave for several minutes. In *C* and *D* the normal spontaneous frequency was restored shortly after the delayed event.

nitr-5 (average $50 \pm 11\%$ reduction in slow wave duration; $n = 10$; $P < 0{\cdot}002$). Illumination during the plateau potential, repolarization, and the period between slow waves had no effect. Representative control experiments are shown in Fig. 7*C*.

Previously we have reported that gastric slow waves are followed by a lengthy refractory period (Publicover & Sanders, 1986); often an interval of several seconds is needed between slow waves for the plateau phase to recover completely.

Experiments were also performed to test the effect of photolysis of nitr-5 on the refractory period. A series of slow waves was evoked by direct electrical stimulation (single pulses, 150 ms in duration). With a sufficient inter-slow-wave interval between evoked slow waves, full recovery of the plateau was observed (Fig. 9*A*). If,

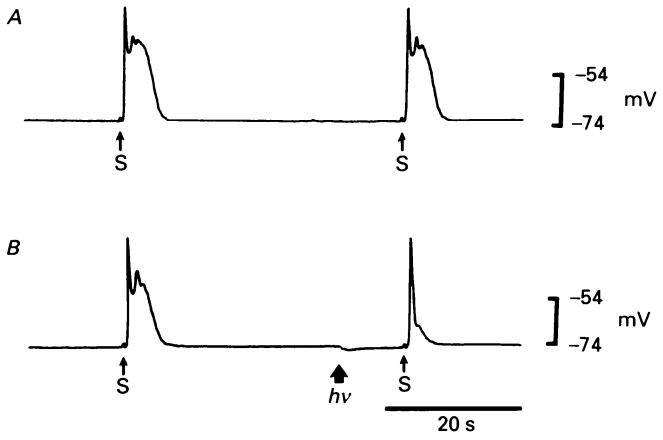

Fig. 9. Lengthening of refractory period by photo-activated release of Ca^{2+} during inter-slow-wave interval. Normally several seconds are required between slow waves for full recovery of the slow wave in antral muscles. In *A* two slow waves were stimulated (S) with a sufficient inter-slow-wave interval to allow full recovery of the plateau phase. In *B* two slow waves were stimulated in the same cell with the same inter-slow-wave interval. But in this case, Ca^{2+} was released at the time indicated by the arrow. The second slow wave was significantly reduced in amplitude and duration.

with the same inter-slow-wave interval, muscles were irradiated between slow waves, the amplitude and duration of the next evoked slow wave was reduced, suggesting a lengthening of the slow wave refractory period (Fig. 9*B*). These data suggest the hypothesis that Ca^{2+} remaining in cells after slow wave repolarization may contribute to the mechanism of the refractory period.

DISCUSSION

This study characterizes Ca^{2+}-activated K^+ channels in antral myocytes. These channels are liberally expressed in the membranes of these cells; we measured an average of fourteen channels per patch in our experiments. This corresponds to about 19000 channels per cell based on our calculations of cell area ($4100 \pm 1100\ \mu m^2$; see Methods) and assuming a patch area of $3\ \mu m^2$.

In situ the resting membrane potentials of canine gastric antral circular muscle cells average between -73 mV in cells near the myenteric border and -64 mV in cells near the submucosal surface (Bauer, Reed & Sanders, 1985). Our data would predict that the open probability of Ca^{2+}-activated K^+ channels is low under resting conditions. Nevertheless, because of the large conductance and number of these channels, a contribution to resting conductance by these channels cannot be entirely excluded.

On the basis of our estimate of channel number and the Boltzmann fits for open probability one can estimate the current provided by Ca^{2+}-activated K^+ channels under resting conditions and during activation (i.e. during slow-wave activity). Based on measurements of intracellular Ca^{2+} concentration in other smooth muscles, one can assume it is low during the diastolic period between slow waves, probably in the 100 nM range (Williams & Fay, 1986; Sato, Ozaki & Karaki, 1988). At a resting potential of −70 mV, the open probability of Ca^{2+}-activated K^+ channels would be about 4×10^{-5}, and this would produce 0·4 pA of outward current (corrected for driving force). During slow waves membrane potential depolarizes to about −30 mV during the plateau phase. This change in potential alone would yield an increase in open probability to 4×10^{-4} and an increase in outward current to about 26 pA. The slow wave plateau appears to be the result of a balance between the sustained inward current and the outward currents that are elicited in the range of −40 to −30 mV (Cole & Sanders, 1989*a*; Langton *et al.* 1989). The sustained inward current is carried by calcium ions, and can provide a significant increase in cell Ca^{2+} content (Langton *et al.* 1989). During slow waves, Ca^{2+} activity must rise, as evidenced by the development of force (Morgan & Szurszewski, 1980; Sanders & Publicover, 1989). If $[Ca^{2+}]_i$ increases into the range of 500–1000 nM during slow waves, the open probability of Ca^{2+}-activated K^+ channels would increase to between 5×10^{-3} and $1{\cdot}1 \times 10^{-1}$. This increase in open probability would result in an increase in outward current to between 270 and 7100 pA. Since gastrointestinal smooth muscle cells have input resistances in excess of 0·5 GΩ (Bolton, Lang, Takewaki & Benham, 1985; Yamaguchi, Honeyman & Fay, 1988; Langton *et al.* 1989), it is likely that currents of this magnitude could easily cause repolarization. Clearly the actual levels of $[Ca^{2+}]_i$ and the time course of the changes in $[Ca^{2+}]_i$ must be measured to fully test this hypothesis.

Two findings support the hypothesis that Ca^{2+}-activated K^+ channels contribute to the repolarization phase of slow waves: (i) as discussed in the previous paragraph, increases in $[Ca^{2+}]_i$ that occur during slow waves would increase the open probability of these channels; (ii) illumination of intact muscles loaded with nitr-5/AM results in significant reduction in the amplitude and duration of slow waves. We suggest that the nitr-5 effect is due to a release of Ca^{2+} in antral cells which results in an increased activation of Ca^{2+}-activated K^+ channels. The resulting outward current may be responsible for premature slow wave repolarization, development of after-hyperpolarizations, and inter-slow-wave hyperpolarizations depending upon the timing of photolysis.

Nitr-5 is one of a family of photolabile Ca^{2+} chelators that differ in Ca^{2+} affinities and release time constants (Gurney, Tsien & Lester, 1987). The dynamics of these chelators have previously been studied by comparing their effects on the Ca^{2+}-activated K^+ current in rat sympathetic neurons. Nitr-5 releases Ca^{2+} upon illumination with ultraviolet light with a time constant of less than 0·3 ms. Its affinity to Ca^{2+} decreases by a factor of 40 after absorption of ultraviolet light (300–400 nm). At an ionic strength of 0·1–0·15 M its dissociation constant increases to about 6 μM after photolysis.

In addition to activation of Ca^{2+}-activated K^+ channels, photolytic release of Ca^{2+} might also cause inactivation of Ca^{2+} channels (Tillotson, 1979). In colonic cells we

have found that inward Ca^{2+} current elicited by depolarization occurs in two phases: (i) an initial fast transient phase which inactivates within 10–20 ms, and (ii) a small sustained current phase which can persist for many seconds (Langton *et al.* 1989). It is the latter current that appears to be involved in maintenance of the plateau potential (Langton *et al.* 1989), and it is conceivable that inactivation of this current could reduce slow-wave amplitude and duration. Two findings argue against the Ca^{2+} inactivation mechanism for the nitr-5 effects: (i) Photolysis timed to occur near the end of slow waves caused repolarization and the development of after-hyperpolarization. (ii) Illumination between slow waves caused hyperpolarization and delayed the occurrence of the next slow wave. These data are consistent with activation of an outward potassium current, but inconsistent with inactivation of voltage-dependent Ca^{2+} currents.

Irradiation with light in the range of 280–450 nm has been reported to produce relaxation of aortic muscles from rabbit (Matsunaga & Furchgott, 1989), cat and rat and coronary arteries of dog (Ehrreich & Furchgott, 1968). Other smooth muscles have also demonstrated photosensitivity, but to a lesser degree than aortic muscle. With aorta the effects elicited by irradiation were independent of endothelial cells but were potentiated by the presence of $NaNO_2$. It has been suggested that photo-irradiation causes production of nitric oxide, which may mediate the relaxation effect (Matsunaga & Furchgott, 1989). In our studies, control irradiation with 300–400 nm light also elicited some reduction in the amplitude of slow waves (see Fig. 7*C*), but these effects were significantly less than those produced by irradiation of muscles loaded with nitr-5. This suggests that generation of other active by-products by irradiation cannot fully explain the effects attributed to photo-activated release of Ca^{2+} from nitr-5.

We have previously characterized the voltage and Ca^{2+} dependence of Ca^{2+}-activated K^+ channels in colonic myocytes (Carl & Sanders, 1989). The channels observed in gastric myocytes are very similar to the colonic channels in terms of their voltage and Ca^{2+} dependence. In both cell types the level of expression is high, exceeding ten channels per patch. Other gastrointestinal smooth muscles have also been shown to express these channels. For example Benham *et al.* (1986) have characterized similar channels in rabbit jejunal cells and Ohya, Kitamura & Kuriyama (1987) have found Ca^{2+}-dependent currents in rabbit ileal cells. These tissues also generate electrical slow waves, and these events have been shown to be coupled to contractions in small intestinal muscles (Sanders, 1983). It is possible that a contribution from Ca^{2+}-dependent K^+ channels to slow-wave repolarization is a common feature of rhythmic, gastrointestinal smooth muscles. Recently we have found that cholinergic stimulation of colonic myocytes causes a significant positive shift in the voltage-dependent activation of Ca^{2+}-activated K^+ channels (Cole, Carl & Sanders, 1989; Cole & Sanders, 1989*b*). This effect could explain the prolongation of slow waves caused by cholinergic stimulation in colonic (see Sanders & Smith, 1989) and gastric (Szurszewski, 1975) muscles. It appears, although considerably more study is needed, that regulation of Ca^{2+}-activated K^+ channels may be an important and common mechanism for controlling gastrointestinal motility.

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