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A STUDY OF PACE-MAKER ACTIVITY IN INTESTINAL SMOOTH MUSCLE

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SUMMARY

1. Electrical activity of longitudinal muscle from cat intestine was recorded in the double sucrose gap.

2. Approximately 20 % of the preparations demonstrated slow, spontaneous fluctuations of membrane voltage, slow waves. This activity, although quite uniform in a given preparation, showed considerable inter-preparation variation with respect to amplitude, frequency and wave form.

3. Application of steady hyperpolarizing current decreased slow-wave frequency and increased slow-wave amplitude while depolarizing currents increased frequency and decreased amplitude.

4. Some preparations with no spontaneous slow-wave activity developed slow waves when the membrane was hyperpolarized into a given range which, depending on the preparation, varied in size from 10 to 40 mV. Step or ramp depolarization of the membrane from hyperpolarized levels triggered slow waves in some preparations.

5. When the membrane potential of a slow-wave generating preparation was clamped at the resting potential, spontaneous inward-directed current transients were observed.

6. No changes in membrane conductance were observed during the course of a slow wave.

7. The slow-wave pattern was simulated for individual preparations by applying the membrane current measured under voltage clamp to the passive membrane resistance and capacitance measured independently under current clamp.

8. In addition to the defined slow-wave activity, voltage-dependent oscillations in membrane potential were sometimes observed.

9. Application of 10^{-5} M ouabain irreversibly blocked slow waves and

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produced a membrane depolarization equal to or slightly greater than the slow wave crest. Repolarization of the membrane to the resting potential, or hyperpolarization, failed to restore slow-wave activity.

10. Removal of external potassium produced a reversible sequence of events almost identical to those following ouabain application.

11. Replacement of 50 % of the external sodium chloride with sucrose produced no changes in slow-wave activity with respect to rates of rise or fall, maximum amplitude or frequency. Sucrose replacement of all external sodium chloride eliminated slow waves after 5 min; however, activity could be restored by a slight hyperpolarization. Longer exposures to the modified bath abolished activity.

12. Following a conditioning exposure to potassium-free Krebs solution, readmission of potassium at normal concentration produced a mean hyperpolarization of 20·5 mV and in spontaneous preparations an arrest of activity.

13. Pump current in sodium-loaded, non-spontaneously active preparations was measured by voltage clamp and was observed to be voltage-dependent.

14. The results of this study indicate that an electrogenic pump is present in longitudinal muscle of cat duodenum, and that oscillations in the level of pump current produce slow waves.

INTRODUCTION

The longitudinal muscle of mammalian small intestine gives rise to spontaneous, rhythmic depolarizations which have a duration of seconds and a relatively uniform amplitude within a single preparation. In different preparations amplitudes can range from 5 to 15 mV. These voltage changes have been termed slow waves and serve to pace muscular contraction in the intact intestine by lowering the membrane potential to spike threshold in both the longitudinal and circular muscle layers (Prosser & Bortoff, 1968). Previous studies have demonstrated that slow-wave activity may be disrupted by a variety of factors: among them low temperature (Job, 1969), anoxia (Daniel, Honour & Bogoch, 1960), metabolic inhibitors (Bortoff, 1961; Daniel, 1965; Job, 1969), ouabain (Daniel, 1965; Kobayashi, Prosser & Nagai, 1967; Liu, Prosser & Job, 1969) and low external sodium (Daniel, 1965; Kobayashi *et al.* 1967; Bolton, 1971; Liu *et al.* 1969). These findings indicate a close coupling between slow-wave generation and metabolism which suggests the possibility that the active transport of sodium is involved in slow-wave production (Daniel, 1965; Job, 1969). Job (1969) has reported a maximal rate of extrusion of labelled sodium early in the repolarization phase of the slow wave cycle. Although there

is an extensive literature on electrogenic ion transport in excitable membranes (Cross, Keynes & Rybova, 1965; Kerkut & Thomas, 1965; Adrian & Slayman, 1966; Thomas, 1972; DeWeer & Geduldig, 1973; Casteels, Droogmans & Hendricks, 1971), we are unaware of other examples where the transport rate may be spontaneously and rhythmically modulated.

Other investigators have favoured the idea that membrane conductance changes underlie slow-wave generation. Bolton (1971) has studied the effects of carbachol on guinea-pig ileum and has demonstrated the existence of dose-dependent oscillations in membrane potential. These oscillations are similar in gross appearance to spontaneous slow waves observed in cat and rabbit in the absence of drugs. At low concentrations of carbachol, oscillations develop only when membrane potential is decreased below a threshold value. A pronounced conductance increase occurs during the upstroke of an oscillation.

Tomita & Watanabe (1973) proposed for slow oscillations in ovarian and uterine smooth muscle that the depolarizing phase is brought about by a potassium conductance decrease and that the repolarization to base line is mediated by a return of the potassium conductance to normal. These investigators suggested that this cycle may be coupled to metabolic activity by an oscillating calcium transport system. That is, when the transport rate is low, intracellular calcium will be at a relatively high level, mediating a high potassium conductance. Increasing the transport rate of calcium lowers internal calcium concentration and consequently potassium conductance.

The present study was undertaken to explore the mechanism of slow wave generation in longitudinal muscle of cat duodenum using a preparation compatible with the double-sucrose gap. Electrical characteristics of the slow wave process and the effects of various ions on the generating mechanism are explored and the implications of these effects with regard to the nature of the primary oscillatory process are considered.

Parts of this communication have appeared in a preliminary report (Weems, Connor & Prosser, 1973).

METHODS

Preparation

Segments of duodenum were removed from adult cats anaesthetized with α-chloralose (65 mg/kg body wt.) and were held for 1 hr before use at 37° C in oxygenated Krebs solution. For dissection, a glass rod was inserted into the intestinal lumen and watchmaker forceps were used to obtain strips of superficial longitudinal muscle 100–150 μm wide and 2–3 cm in length. A number of preparations were stained with methylene blue (0·03%) following electrical recording to check for the presence of neurones or neural processes. No neural tissue was ever found.

Equipment

Electrical measurements were made in a double sucrose-gap chamber which employs a combination of features described elsewhere (Julian, Moore & Goldman, 1962; Berger & Barr, 1969; Kumamoto & Horn, 1970). The central or test node was formed by the flowing boundaries between the sucrose cuffs and the central stream of Krebs solution. Test node width could be varied over a range of 70–600 μm by suitable adjustment of flow rates. Rubber membranes with a central perforation were used to separate the sucrose cuffs from the end pool solutions.

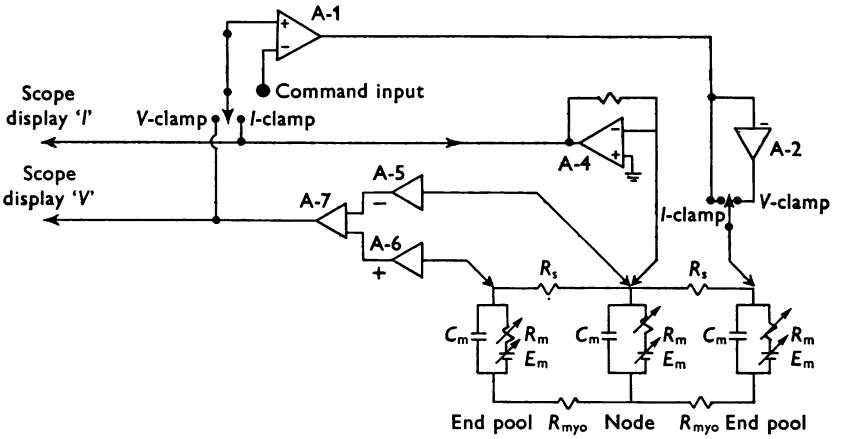

Fig. 1. Circuit diagram for current and voltage clamping. The lumped circuit approximation for a smooth muscle strip in a double sucrose-gap chamber is in the lower right corner of the diagram. R_{myo} is resistance through the myoplasm; R_s is resistance through the sucrose, C_m, R_m, and E_m are membrane capacitance, resistance and e.m.f.

The general arrangement for voltage and current clamping is shown in Fig. 1. The clamping circuits were similar to those described by Connor & Stevens (1971) except that an external voltage reference electrode was employed. All three pools of the chamber were connected to silver–silver chloride electrodes through Krebs-agar bridges.

Solutions

The Krebs solution had the following composition (mM): NaCl 120, KCl 5·9, $CaCl_2$ 2·5, $NaHCO_3$ 15, NAH_2PO_4 1·2, $MgCl_2$ 1·2, glucose 11, and was equilibrated with 95% O_2 and 5% CO_2. Isotonic KCl had a composition of 150 mM-KCl. Sodium replacement was by equivalent concentration of sucrose, lithium or Tris (Tris hydroxymethylaminomethane chloride). Ouabain (10^{-5} M) was added to some solutions as indicated.

Experimental considerations

Resting potential

Measurements of resting potential obtained with the double sucrose-gap technique are influenced by at least three factors: (1) the ratio of $R_s/(R_{myo}+R_m+R_s)$; (2) the amount of sucrose-gap hyperpolarization (Julian *et al.* 1962); and (3) the value of

V_m in the voltage pool following application of isotonic KCl or K_2SO_4. Since the observed steady voltage measured in the chamber may not accurately reflect the true resting potential and may vary in the degree of uncertainty between preparations, we have not tried to express resting potential in absolute units. Instead, we have referred all measurements to the steady membrane voltage in quiescent preparations or else to the 'trough' voltage where slow waves are present. A correspondence between this sucrose-gap resting voltage and absolute membrane potential may be estimated from the spike threshold. If one assumes that the absolute value for spike threshold is invariant among preparations and also invariant whether sucrose-gap or micro-electrode recording is employed, then a comparison of our value of spike threshold relative to the micro-electrode-determined value of -38 to -42 mV (Liu *et al.* 1969) indicates that our reference voltage, depending upon the preparation, generally lay between -40 and -70 mV absolute.

Voltage homogeneity

For making certain of our measurements it is imperative not to have appreciable non-uniformities in the membrane voltages of muscle cells within the test node. The method of choice for demonstrating non-uniformity of potential has been to measure the membrane voltage of a large population of cells within an experimental test node with a microelectrode as Giebish & Weidmann (1971) have done with cardiac muscle in a single sucrose-gap arrangement. In order to minimize shunt pathways and to achieve a high degree of test node stability, our chamber is sealed and precludes this measurement. We have therefore relied upon several indirect criteria for demonstrating spatial uniformity of voltage.

(A). *Core conduction considerations.* In intestinal smooth muscle, individual muscle cells are electrically coupled through nexuses (Barr, Berger & Dewey, 1968) giving to a muscle bundle certain of the electrical properties of a single long cell. Decremental spread of voltage due to injected current has been measured in smooth muscle by a number of investigators (Abe & Tomita, 1968; Kobayashi, Nagai & Prosser, 1966; Tomita, 1970). In analysis of these measurements the space constant, λ, the distance between injection site and the measuring point at which the voltage change has decayed exponentially to the factor $1/e$, is commonly calculated. This quantity is strictly defined only for single cells with tubular geometry, but has been used, for better or worse, with regularity in describing voltage spread in multicellular tissue such as smooth and cardiac muscle. The point has been repeatedly made (cf. Johnson & Lieberman, 1971) that if spatial uniformity is to occur within the test node even under the best of conditions the width of the test node must be much less than a space constant.

Potential spread away from a large surface electrode in intestinal longitudinal muscle has been measured by Kobayashi *et al.* (1967). Along the muscle bundle axis the $1/e$ value of decrement occurs at recording-injection site separations of from 1 to 1·5 mm. This method is not completely equivalent to the uniform current density injection of a sucrose gap but should give a low estimate of λ due to the fact that there is tangential dispersion of current. Our test node width, 100–120 μm, is thus on the order of 1/10 of space constant estimates on our tissue. Furthermore, the double sucrose-gap approaches an open-circuit termination to a segment of nerve or muscle. That is, instead of the length of muscle in the central node being part of an effectively infinite core conductor, longitudinal current flow is stopped at the end of the node nearer the voltage pool. For this configuration it can be shown (Cole, 1968) that the profile of membrane voltage versus distance should approach a $V_0[\cosh(x-L)/\lambda]/\cosh(L/\lambda)$ rather than the steeper exponential one.

The foregoing considerations are applicable for steady-state voltage changes with

membrane resistance at its resting value. Activation of the time- and voltage-dependent conductance changes of the action potential cycle drastically reduce total membrane resistance, hence the space constant. The large transmembrane currents which flow as a result of these conductance changes must also flow through the rather restricted volume of the extracellular space. For all but the most superficial layer of cells the extracellular space introduces a sizeable resistance in series with the cell membranes which, during periods when transmembrane current density is high, causes the apparent voltage across the membrane to be different from what it

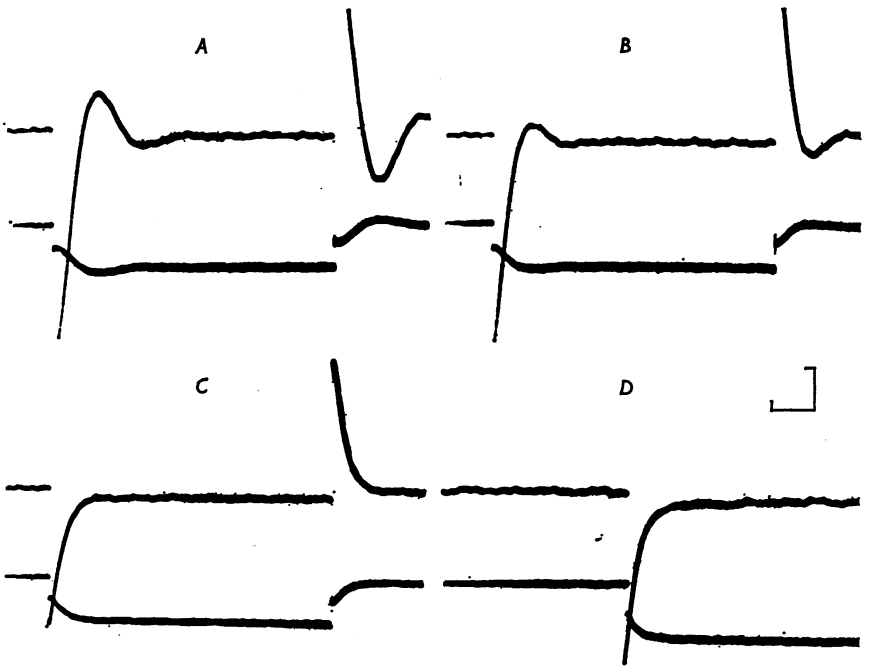

Fig. 2. Elimination of oscillations in the voltage-clamp pattern by reduction of test node width. Node widths are as follows: *A*, 600 μm, *B*, 275 μm, *C*, 200 μm, *D*, 100 μm. Calibration 20 msec, 50 nA, 5 mV.

actually is. These complications and their possible implications to experimental data have been recognized, if not surmounted, by previous investigators (cf. Anderson, 1969) and have been subject to analysis (Johnson & Lieberman, 1971; Kootsey & Johnson, 1972). The measurements reported here should not be affected by these considerations for the following reasons: (1) there was no detectable change in membrane resistance from the resting value during slow-wave activity, (2) the spontaneously occurring voltage changes are slow, (3) when voltage control (clamp) was utilized, it was to maintain membrane potential at a steady level or to change it at a very low rate (5 mV/sec), and not to measure time courses of large currents soon after the imposition of a step change, (4) estimates of external series resistance based upon measurement of the initial voltage jump following a step of applied current give a value of approximately $10^4\,\Omega$. This value is low enough that the measured ion currents, in our experiments less than 20 nA, should not cause the actual transmembrane voltage to be appreciably different from that measured.

(B) *Analysis of the capacitive transients.* If one is indeed able to impose a given voltage step across all of the cell membrane in the test node, the total charge flow during the capacitive transient should provide an estimate of membrane area. This estimate should be equal to or greater than an estimate made by geometrical means. By determining the area under the capacitive transient in Fig. 2*d* it was calculated that $1{\cdot}14 \times 10^{-9}$ C of charge were required to hyperpolarize the membrane by 5·4 mV in a 100 μm node. If the membrane is assumed to have a specific capacitance of 1×10^{-6} F cm^{-2}, then an estimate on the lower bound of membrane area that is being charged within the node is $1{\cdot}75 \times 10^{-1}$ cm^2. When membrane area is calculated from the total volume of tissue within the test node, assuming that 35% of the volume is extracellular space (Burnstock, 1970) and that the average cell diameter is 5 μm, a value of $1{\cdot}57 \times 10^{-2}$ cm^2 for total membrane area was obtained. The value for membrane area computed from geometric consideration is much smaller than that 'measured' electrically. There are numerous possible reasons for this. The volume of the extracellular space may be less than the 35% used and, more importantly, we know of no realistic way of accounting for the increase in membrane area known to result from the irregularity of the cell surface (Burnstock, 1970; Goodford, 1970). This irregularity results primarily from the presence of vesicles in the membrane which in taenia coli increase the over-all surface area of the cells by more than 70% (Goodford, 1970). In any case, the fact that much more membrane is apparently being charged than these calculations of surface area indicate, suggests that little of the membrane area within the node is isolated from the control circuitry.

(C) *Node width manipulations.* When the test node width was greater than 200 μm, oscillations in the current pattern could be observed following a hyperpolarizing step (Fig. 2). Node widths smaller than 200 μm resulted in a capacitive transient of the standard wave form with the long duration typical of smooth muscle (Anderson, 1969). We view these oscillations as arising because the electrical delay across the test node is sufficient to give a 180° phase shift at the ringing frequency (cf. Kootsey & Johnson, 1972). In the cases where ringing is present, there is demonstrable, transient, spatial inhomogeneity. The converse statement cannot, unfortunately, be made with any certainty; however, we suggest that the minimum oscillatory length sets a maximum upper bound on a useful test node width.

RESULTS

Electrical characteristics

General observations

Preparations were routinely tested for electrical continuity by applying hyperpolarizing and depolarizing current pulses to the muscle strip 5 min after establishment of the test node. Membrane voltage responses were exponential (Fig. 3) with time constants of 50–150 msec. A linear current–voltage (I–V) relationship was observed (Fig. 4). Spontaneous electrical activity was not observed during this initial period, but approximately 15% of the preparations demonstrated some delayed rectification at depolarizations greater than 20 mV. Following this test, preparations were allowed to equilibrate in Krebs solution with no sucrose flow for 1 hr, after which a different I–V relationship was observed (Fig. 4). Membrane slope resistance near resting potential increased and delayed rectification was

pronounced for depolarizing current steps. Depolarizing current pulses produced self-supporting spikes that had thresholds of 2–10 mV above resting potential. The range of observed time constants increased to 200–400 msec and the total resistance varied from 0·5 to 1·3 MΩ. Typical voltage responses to depolarizing and hyperpolarizing current pulses after the 1 hr equilibration in Krebs solution are shown in Fig. 5.

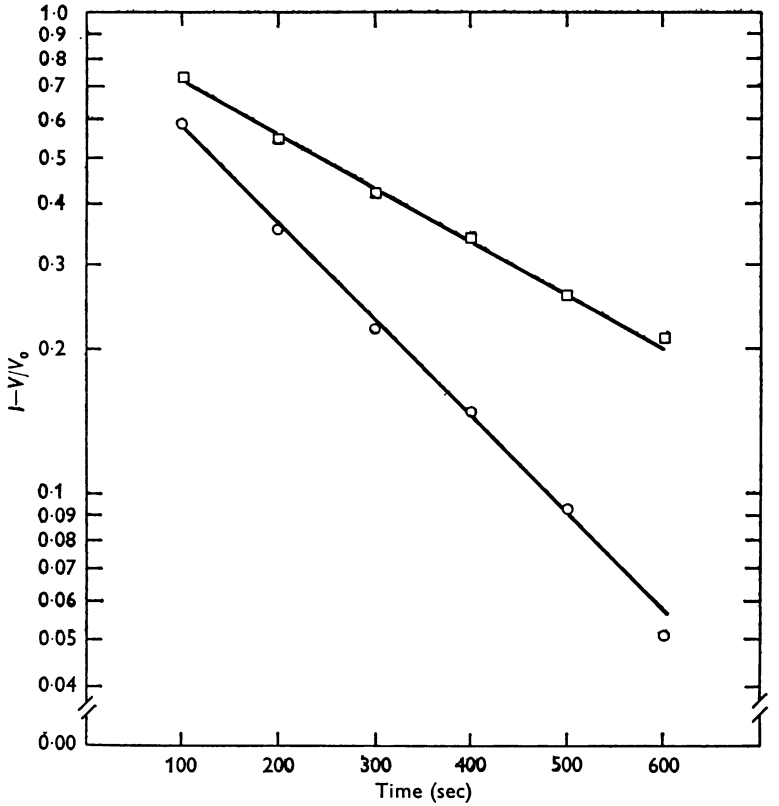

Fig. 3. Semi-logarithmic plot showing the time course of membrane voltage response to a long hyperpolarizing pulse. ○, membrane response 5 min after establishment of the test node in freshly dissected muscle. □, membrane response 5 min after establishment of the test node following a 1 hr soak in Krebs solution.

Approximately 20% of the preparations demonstrated spontaneous slow waves in the sucrose gap after the 1 hr equilibration period. Typical records are shown in Fig. 6. Slow-wave activity, although quite uniform in a given preparation, showed wide interpreparation variation. Frequencies, for example, ranged from 5 to 18 per minute, amplitude from 2 to 12 mV and duration from 2 to 7 sec. Slow-wave depolarization reached

spike threshold in only a few of the preparations tested while in the remaining preparations the slow-wave peak was usually no more than 5 mV below spike threshold. This finding is in contrast to the situation in the intact gut where spikes are usually seen superimposed on the peak of the slow wave.

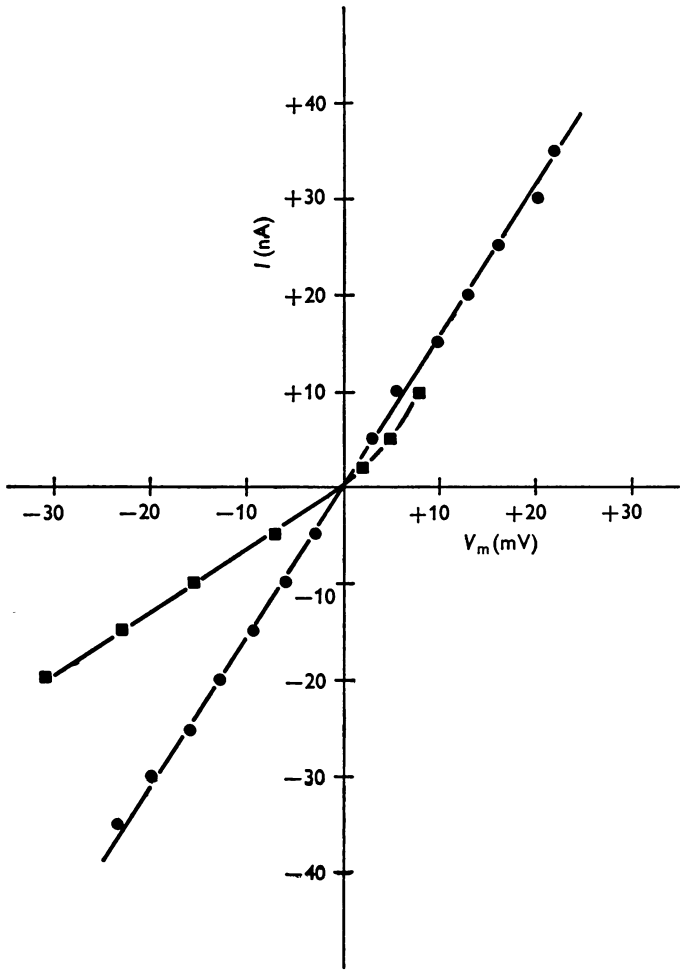

Fig. 4. Current–voltage (I–V) relation of longitudinal smooth muscle from cat duodenum. ●, 5 min after establishment of the test node in freshly dissected muscle. ■, 5 min after establishment of the test node following a 1 hr soak in Krebs solution.

Slow-wave dependence on membrane potential

Application of steady hyperpolarizing currents decreased slow-wave frequency and increased slow-wave amplitude while depolarizing currents

increased frequency and decreased amplitude. Frequency and amplitude as functions of membrane potential for a single preparation are presented in Fig. 7. Spontaneous slow waves were abolished by depolarizing the membrane to approximately the spike threshold or by hyperpolarizing the membrane by more than 30 mV, thus giving a 'window' for activity of roughly 40 mV. Several preparations, which had no spontaneous slow-wave activity, developed slow waves when the membrane potential was

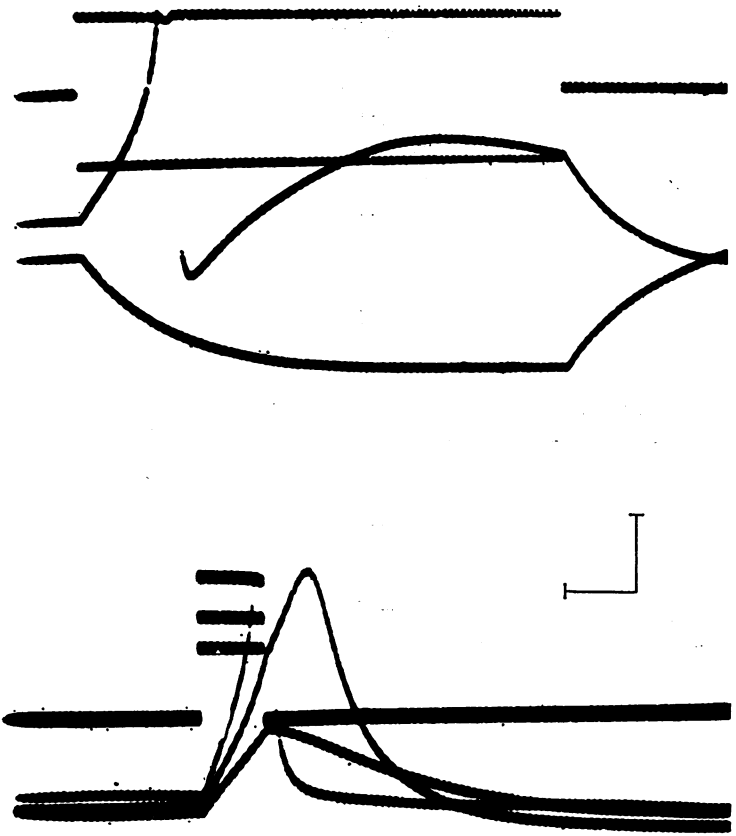

Fig. 5. Top: responses of membrane potential to a hyperpolarizing and to a depolarizing current pulse. Bottom: effects of varying the intensities of three depolarizing current pulses. Action potential peak is off scale in both cases. Calibration: 200 msec, 10 nA, 5 mV.

hyperpolarized into a given range which, depending on the preparation, varied in size from 10 to 40 mV. Within this voltage range, slow-wave frequency and amplitude demonstrated the same relationship to membrane potential as previously described. Some preparations, showing a simple pattern of slow-wave activity, developed upon hyperpolarization

a complex notched pattern indicating possible multiple sites of slow-wave production.

Step or ramp depolarization (as slow as 2 mV sec^{-1}) of the membrane from hyperpolarized levels sometimes triggered slow waves in quiescent preparations and often disrupted the slow-wave rhythm in spontaneous preparations by triggering 'extra' slow waves. The efficacy of electrical stimulation of slow waves was very poor however.

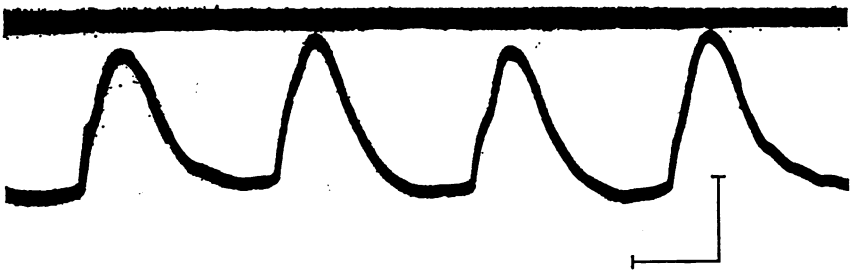

Fig. 6. Spontaneous slow-wave activity. Calibration: 2 sec, 5 mV.

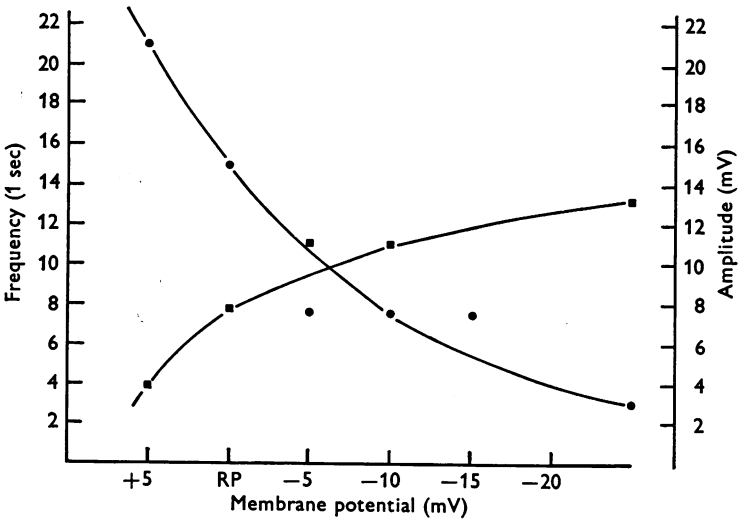

Fig. 7. Slow-wave frequency (dots) and slow-wave amplitude (squares) plotted against membrane potential (abscissa). Resting potential is defined as that voltage at the bottom (trough) of the slow wave. Negative values indicate hyperpolarization and positive values depolarization.

Membrane current behaviour under voltage clamp

When the membrane voltage of a spontaneous preparation was clamped at the resting potential (arbitrarily defined as the bottom of the oscillating wave form), spontaneous, inward-directed current transients were

observed (Fig. 8). The current transients were of the same frequency as the voltage wave form but the rise time of the current was faster, and the duration slightly shorter. The level of membrane voltage had exactly the same effect upon the oscillatory current wave form as it did for the voltage wave form. That is, clamping the membrane to potentials more negative than the resting potential produced a decrease in the frequency of the current transients and increased their amplitude. Clamping to potentials more positive than the resting potential increased current frequency and decreased current amplitude. Spontaneous current transients were abolished when the holding potential was equal to or more positive than spike threshold or when it was more negative than 30 mV below the resting potential. We were unable to detect a reversal potential for the current transients.

Fig. 8. Spontaneous, inwardly directed current transients (upward deflexion) observed during voltage clamp. Following the slow wave (lower trace) the membrane potential was clamped at the resting potential (arrow) and current transients appeared (upper trace) with the same frequency as slow waves. Calibration: 2·5 sec, 5 mV, 10 nA.

The observation of spontaneous inward-directed current transients under voltage clamp indicates that slow waves need not result from conductance changes, at least those that are triggered by changes in membrane potential. Rather, the observation suggests that slow waves could result from either conductance changes regulated by factors other than voltage or by the cyclic activity of an electrogenic active transport system or pump. To test for the occurrence of conductance changes, rectangular, hyperpolarizing current pulses were applied at various times during the course of the slow wave and the magnitude of the voltage responses observed. Measurements of this type are difficult to make because the long membrane time constant of these preparations made

impossible, in most cases, the attainment of a steady-state voltage during any phase of the slow wave. This type of measure therefore relied heavily upon those few preparations which displayed very long periods of depolarization. Conductance measurements were also made under voltage clamp by observing the leak current change in response to step changes in holding potential applied during the current transient. No conductance changes were observed using either test procedure.

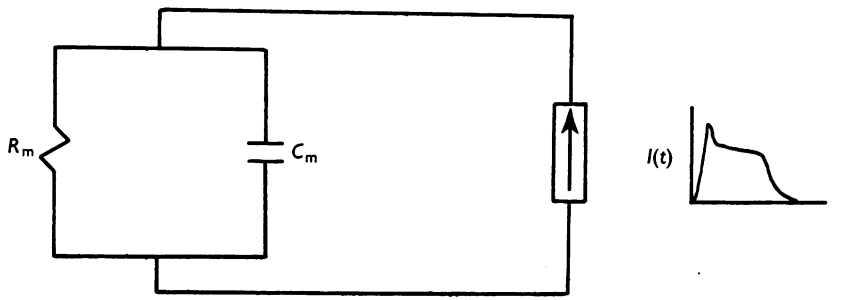

Fig. 9. Model of the slow wave mechanism. $I(t)$ is the time-varying current measured under voltage clamp (cf. Fig. 8). R_m and C_m are the passive membrane resistance and capacitance and can be measured for a single preparation under current clamp. If R_m, C_m, and $I(t)$ are obtained from a single preparation, the computed voltage response of this network to $I(t)$ should predict the slow wave time course for that preparation.

If one is to discard the notion of conductance variations as underlying slow-wave generation and suppose that a cycling electrogenic pump produces the activity, then the observed voltage changes must be due to a time-varying current flowing through a nearly constant membrane resistance and capacitance combination. As a test of this model, we reconstructed a slow wave, for a single preparation, from membrane current measured under voltage clamp, and passive membrane resistance and capacitance measured independently under current clamp (Fig. 9). The convolution integral was used to compute the voltage wave form. The computed voltage response of this network to $I(t)$ and its relation to the measured wave form are shown in Fig. 10. Agreement between measured and reconstituted slow waves is excellent. It could have been made slightly better or worse by taking advantage of the small variability in slow waves and picking another voltage record for comparison.

Voltage-dependent oscillations

In addition to the slow-wave activity discussed above, an unstable membrane potential, which often triggered spikes, was sometimes observed. This activity manifested itself only when the membrane voltage

was within 1–4 mV of spike threshold (Fig. 11). This electrical activity, from all but one of twenty-five preparations, had a pattern that was distinctly different from that of the slow wave and could always be

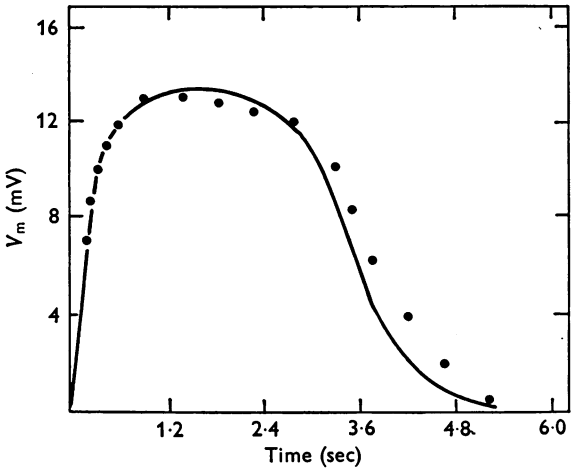

Fig. 10. Comparison, for a single preparation, of a slow wave computed from the model in Fig. 9 (continuous curve) and a measured slow wave (dots).

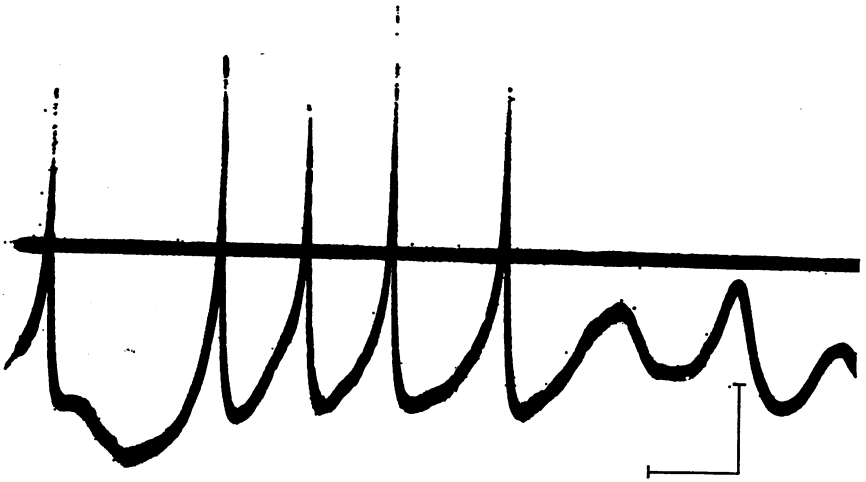

Fig. 11. Spontaneous voltage-dependent oscillations of membrane potential. Action potentials frequently occur on the crest of the waves. This activity may be prepotential activity (see text). Calibration: 2 sec, 5 mV.

distinguished from slow waves in the following manner. (1) Spontaneous current transients were not observed under voltage clamp, (2) the activity was abolished by hyperpolarizations of 3–5 mV whereas slow waves were

not abolished (Figs. 12 and 13), and (3) the oscillations could often be induced by depolarization to near threshold. These data suggest that the oscillations do not result from the same mechanism responsible for slow-wave generation, but probably arise from a voltage- and time-dependent

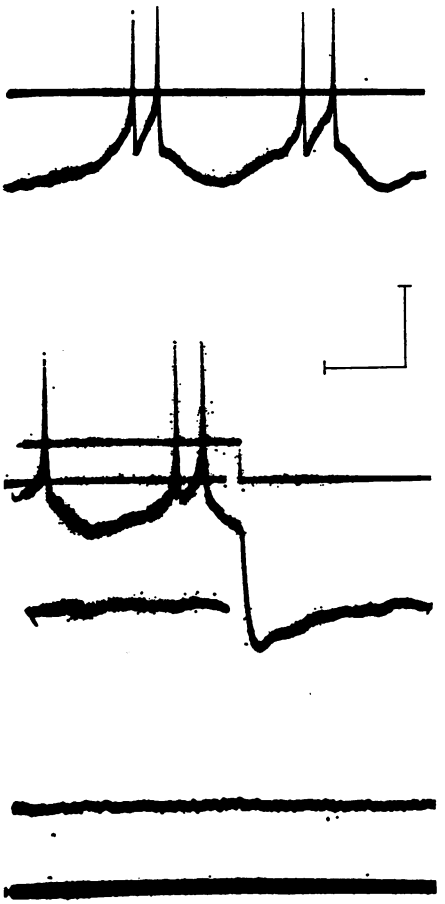

Fig. 12. Top: spontaneous prepotential activity giving rise to spikes. Middle: abolishment of prepotential activity by a 10 mV hyperpolarization. Bottom: absence of spontaneous inwardly directed current transients during voltage clamp. Upper traces, current; lower, voltage. Calibration: 4 sec, 20 nA, 10 mV.

ion conductance. This electrical activity is most likely identical to prepotential activity which has been described by other investigators as occurring at the top of slow waves and usually giving rise to spikes (Bortoff, 1961; Gonella, 1965; Liu *et al.* 1969). This conclusion is further supported by the fact that several of our preparations gave rise to slow

waves with a small-amplitude component (1–3 mV) at the top of the wave. This small-amplitude component was absent when the membrane was hyperpolarized by more than 5 mV. Since this second type of activity is oscillatory and can resemble slow waves, we feel that it is possible, under certain conditions, to regard it mistakenly as being slow-wave activity.

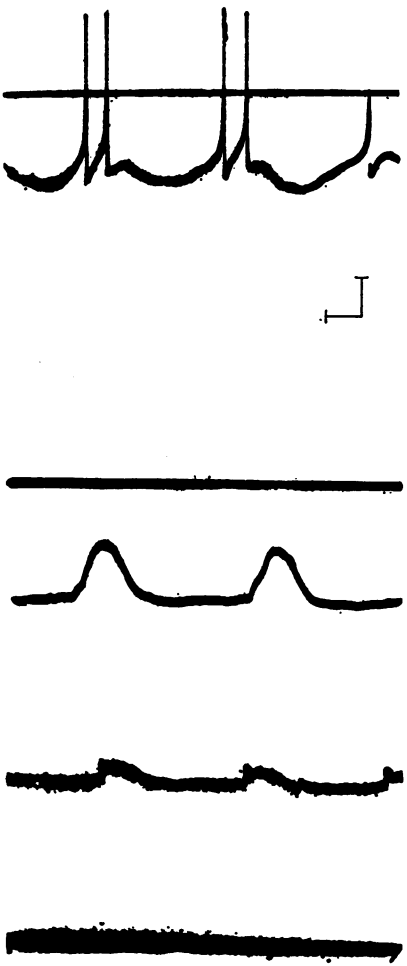

Fig. 13. Top: spontaneous slow waves giving rise to spikes. Middle: spike-free slow waves during a 10 mV hyperpolarization. Bottom: spontaneous inwardly directed current transients during voltage clamp. Calibration: 2 sec, 10 mV (top), 5 mV (middle and bottom traces), 10 nA.

Multiple pace-maker sites

Evidence was obtained that slow waves may not be produced along the entire isolated strip of muscle prior to its introduction into the chamber,

but may be restricted to localized regions. Spontaneous slow waves of reverse polarity could be recorded from some preparations before isotonic potassium chloride was added to the voltage pool. This activity was associated with the membrane in the voltage pool and was always abolished upon addition of potassium chloride. No preparation was observed to have slow-wave activity in both the test node and in the voltage pool. Occasionally slow waves of normal polarity were observed within 1 or 2 min of the start of sucrose flow, but disappeared as sucrose replacement of extracellular ions in the sucrose pool became more complete. Imposed depolarization did not restore activity. This observation suggests that a membrane region adjacent to the membrane area finally covered by the test node was producing slow waves and was part of the node prior to its complete isolation by sucrose.

Recordings from some preparations demonstrated a complex pattern of slow-wave activity that had a variable time-interval between individual slow waves that were sometimes of different amplitude. Individual slow waves often had notches or humps which also varied with time as to their position or occurrence on a slow wave. The occurrence of these notches on the rising phase usually followed a decreasing interval between slow waves and seemed to appear as a result of overlap of these waves. These data suggest that slow waves can be independently produced by two or more populations of cells within the node.

Inhibitors of active transport

Ouabain

Oubain eliminates slow waves when applied to whole intestine (Daniel, 1965; Liu *et al.* 1969). Application of 10^{-5} M ouabain to our preparation abolished slow waves within 1 min of application and produced a membrane depolarization that was equal to or no more than 4 mV greater than the slow wave crest (Fig. 14). Repolarization to the resting potential or hyperpolarization failed to restore slow-wave activity. The inward current transients observed under voltage clamp were also abolished following exposure to ouabain.

Application of ouabain to viable preparations with no spontaneous slow-wave activity always produced a depolarization of 4–8 mV. Such a finding is generally taken as evidence that an electrogenic pump is functioning at a steady level (Cross *et al.* 1965; Thomas, 1972). No change in membrane resistance, as determined by the size of the voltage response to applied current pulse, was detected following ouabain application. Ouabain effects were not reversible.

Low external potassium

The effects of removing external potassium were reversible but were otherwise almost identical to the effects of ouabain (Fig. 15). The membrane depolarized and slow waves were abolished. Repolarization to resting potential did not restore slow-wave activity. During a long hyperpolarization of 30 mV or more a single slow wave was sometimes seen once every

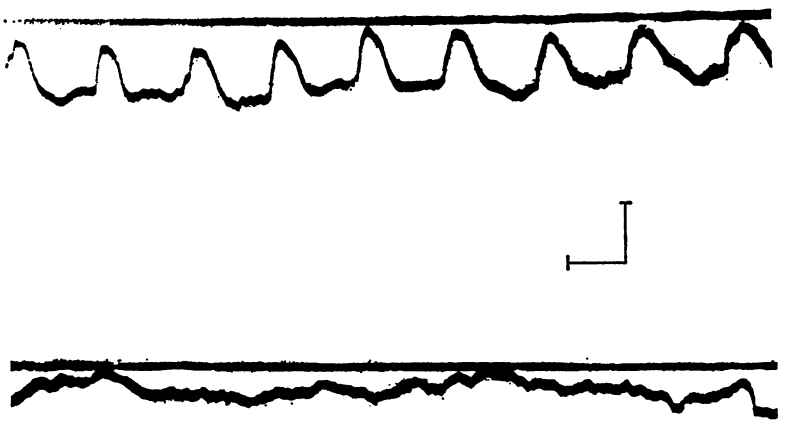

Fig. 14. Action of ouabain (10^{-5} M) on slow-wave activity and resting membrane potential. Top: control showing spontaneous slow waves. Bottom: recording begins 45 sec after ouabain application. Calibration: 5 sec, 5 mV. Upper traces, current, have been left in the records in Figs. 1, 2, 4 and 5 to provide a reference level between frames.

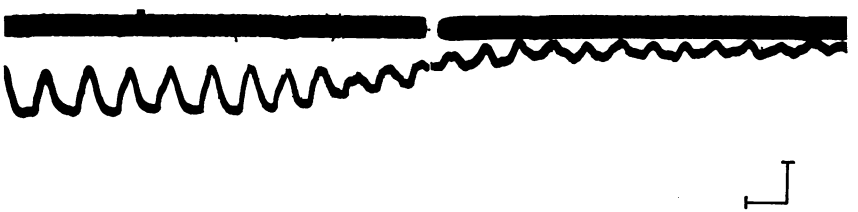

Fig. 15. Effect of potassium-free Krebs on slow waves and membrane potential. The preparation was exposed to potassium-free solution at the beginning of the trace. Calibration: 5 sec, 1 mV.

1 or 2 min. Preparations with no spontaneous activity also became depolarized (4–8 mV) when exposed to potassium-free solution.

Ouabain application and exposure to potassium-free solution did not abolish oscillatory prepotential activity in preparations where it was present. On the contrary, in some non-spontaneous preparations, de-

polarization produced by ouabain application or potassium removal brought the membrane voltage into the appropriate range to elicit pre-potentials and spikes.

Fig. 16. Recording of spontaneous slow-wave activity following a 10 min exposure to Krebs solution containing 50% (68 mM) sodium chloride (sucrose replacement). The pattern of small-amplitude slow waves interspersed between normal-appearing slow waves is typical after this treatment. Calibration: 2 sec, 5 mV.

Depletion of internal sodium

Replacement of 50% of the external sodium chloride with sucrose produced no changes in slow-wave activity either with respect to rates of rise or fall, maximum amplitude, or frequency. Some individual slow waves in an ongoing train, however, decreased in amplitude and this resulted in a pattern of normal-amplitude slow waves interspersed with low-amplitude slow waves (Fig. 16). The effect was reversible upon return to normal Krebs solution.

Replacement of all sodium chloride in the bathing medium with sucrose, leaving only the sodium (15 mM) in the buffer, left activity during the first 5 min very much as it was following a 50% sodium chloride reduction. After approximately 5 min in the 15 mM sodium solution, slow-wave activity vanished. However, the normal pattern of slow waves could be restored by hyperpolarizing the nodal membrane by a small amount, generally around 5 mV. This activity often continued for 10–15 min. Exposure to the modified bathing solution beyond this time led to the generation of extremely long-duration slow waves (10–20 sec) which had a normal rate of rise but a slowed rate of return to the resting level (Fig. 17, top). During the next 15 min, this pattern of long-duration slow waves degenerated and membrane voltage approached a steady base line approximately equal to the value of the slow-wave peak (Fig. 17, middle). Hyperpolarization or depolarization by applied current to any potential within

40 mV of the resting level failed to restore slow-wave activity. The elapsed time between exposure to the low sodium chloride bath and complete abolition of slow waves was 20–30 min in the six preparations tested. This time compares favourably with the 30 min reported by Casteels, Droogmans & Hendricks (1973) required to deplete internal sodium in taenia coli.

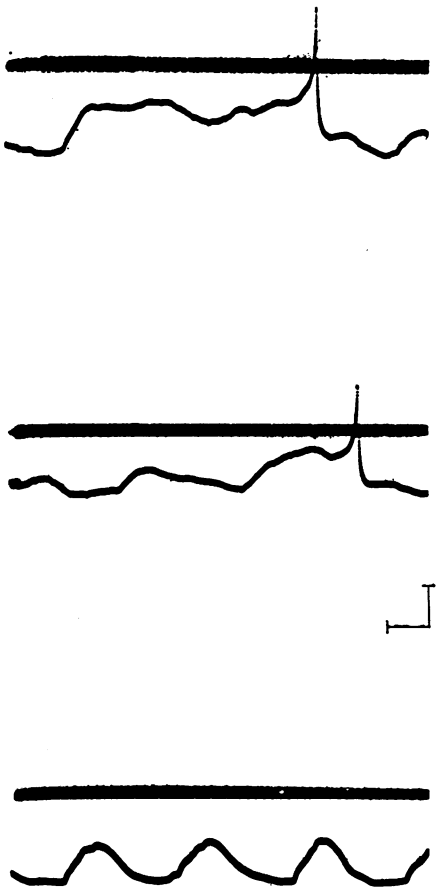

Fig. 17. Electrical activity during exposure to Krebs containing 15 mM sodium (sucrose replacement). Top: long duration slow wave, after 15 min in 15 mM sodium Krebs, with pre-potentials and a spike. Middle: activity after 30 min. Slow waves appear to be absent and the membrane is 5 mV depolarized. Prepotentials and spikes are still present. Bottom: spontaneous slow-wave activity after return to normal Krebs. Calibration: 2 sec, 5 mV.

Activity in sodium-depleted preparations could be restored by returning normal sodium concentration to the bath. The recovery process required approximately 30 min for the preparations tested, during which time a

pattern of long low-amplitude waves first developed. This pattern then underwent a gradual transition, duration decreasing and frequency increasing, to the normal slow-wave pattern. There was no pronounced voltage shift immediately upon restoration of normal sodium.

Replacement of external sodium by lithium led to essentially the same sequence of events as sucrose replacement except that the pattern of alternating amplitudes did not develop and spontaneous activity was maintained without external alteration of the membrane potential. Slow-wave amplitude uniformly decreased with time in the lithium saline and cyclic activity disappeared after exposure for about 45 min. Duration increased prior to the loss of activity and the steady potential was approximately equal to the slow-wave crest.

Substitution of Tris for sodium resulted in the reversible abolition of both spontaneous slow waves and of driven spikes within a few minutes.

Effects of high active transport rates

The preceding series of results indicates that agents and manipulations of the cell environment which commonly inhibit electrogenic transport interrupt slow-wave generation and reduce resting potential. When electrogenic transport is augmented, the rhythmic generation of slow waves is interrupted and resting potential is increased. Taylor, Patton & Daniel (1970); Casteels *et al.* (1971, 1973) and Bolton (1973) have demonstrated that the rate of electrogenic pumping can be increased in other smooth-muscle preparations by loading the cells with sodium, accomplished by exposing the preparation to potassium-free Krebs solution for a length of time. This operation slows the active extrusion of sodium and allows the passive influx to increase internal sodium concentration. A depolarization accompanies exposure of the preparation to potassium-free medium (Fig. 18*A*). Upon restoration of external potassium, increased internal sodium concentration increases the pump rate over normal levels.

In quiescent preparations (no slow waves) we have uniformly observed that sodium loading produces a membrane hyperpolarization. In six preparations following exposure to potassium-free Krebs solution for 30 min, 5·9 mM potassium was readmitted to the external bath and a mean hyperpolarization of 20·5 mV resulted. An example of the time course of the resulting hyperpolarization is shown in Fig. 18*B*. This hyperpolarization was readily abolished by ouabain or return to potassium-free solution.

Voltage clamp was used to measure the amount of pump current in sodium-loaded, non-spontaneously active preparations. This technique has been successfully employed by Thomas (1969) to investigate the activity of an electrogenic pump in snail neurones and by Kostyuk, Krishal & Pidoplichko (1973) to investigate the voltage dependency of this pump

current. Membrane voltage was clamped at the resting level following a 30 min exposure to potassium-free Krebs solution. Following restoration of normal potassium to the external medium a large outward (hyper-

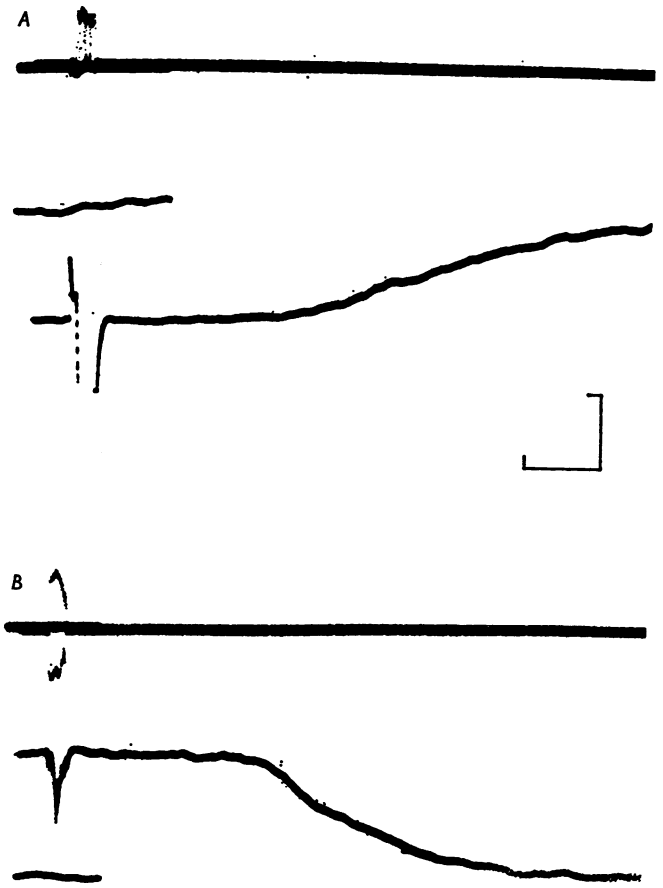

Fig. 18. This preparation was soaked in potassium-free Krebs solution for 30 min and returned to normal Krebs for 2 min. *A*: membrane depolarization resulting from exposure to potassium-free Krebs (beginning of trace). *B:* hyperpolarization on readmitting potassium following the 30 min soak period in potassium-free Krebs. The vertical deflexion in the upper traces of *A* and *B* marks the time at which solutions were changed. Final voltage levels are indicated by the partial sweeps of *A* and *B*. Calibration: 5 sec, 10 mV.

polarizing) current flow developed (see Fig. 19*A*). This current was ouabain-sensitive. We conclude, in agreement with others, that the extra current measured under voltage clamp is a measure of the current produced by an electrogenic pump.

The following test was run in order to ascertain whether the magnitude of the pump current is potential-dependent as is pump current in some snail neurons (Kostyuk *et al.* 1973). The preparation was first bathed for 30 min in potassium-free Krebs and then normal Krebs was readmitted during measurement of current while the voltage was clamped at various

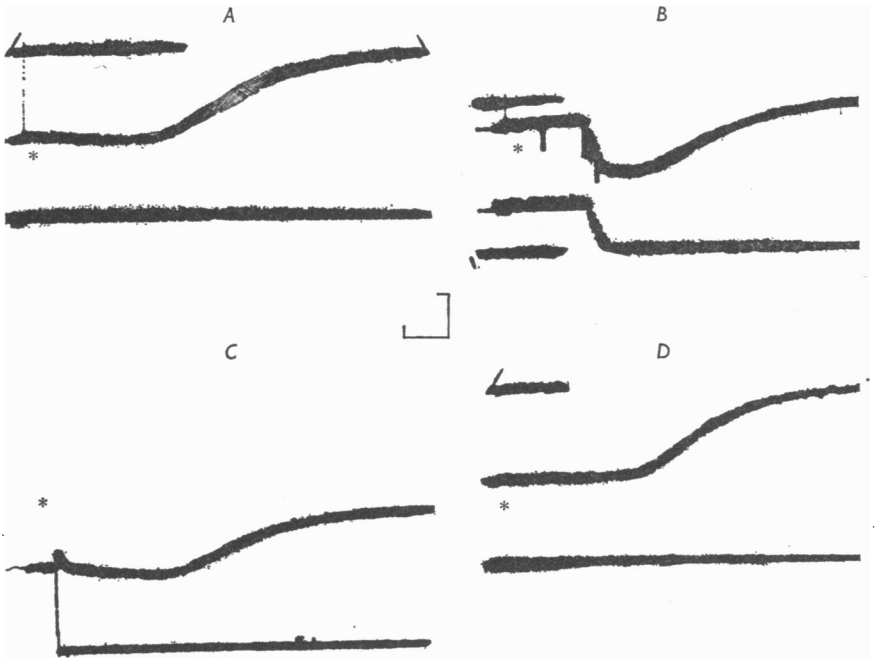

Fig. 19. Measurements of pump current (upper traces) by voltage clamping at different membrane potentials; resting level (*A* and *D*), 10 mV hyperpolarized (*B*) and 20 mV hyperpolarized (*C*). Before making this set of records the preparation had been soaked for 30 min in potassium-free Krebs. Potassium was readmitted at the beginning of each trace (*) and was removed for 1 min at the end of each trace before repeating the measurement at a different membrane potential. In *A*, *B*, and *D* a partial second sweep is displayed in the left half of the oscilloscope trace to show steady-state levels. Calibration: 5 sec, 20 nA, 10 mV.

levels. In Fig. 19*A* the voltage was held at the normal resting level; in Fig. 19*B*, 10 mV more negative; 19*C*, 20 mV more negative and in 19*D* again at the resting level. Between each run, which lasted approximately 2 min, the preparation was exposed to potassium-free Krebs for 1 min. The results show that with increasing hyperpolarization the pump current declines.

Sodium loading of slow-wave-generating preparations led to a temporary arrest of spontaneous activity during the period of hyperpolarization.

There was no significant difference between spontaneous and quiescent preparations in the magnitude of voltage shift which resulted on restoration of external potassium. Depolarization by applied current during the period of high transport rate did not restore slow-wave activity. Approximately 20 min after potassium restoration, small-amplitude slow waves appeared at control frequency and increased in amplitude until the control level was reached.

We conclude that all of our viable preparations had a resting potential with an electrogenic component, but only a small percentage of these preparations had cyclic pump activity.

DISCUSSION

We have been led to reject the idea that changes in membrane conductance play a significant part in generating slow waves for the following reasons: (1) we have been unable to detect conductance changes during the course of the slow wave using either current or voltage clamp techniques, (2) the slow-wave amplitude and time course can be simulated by applying the membrane current measured under voltage clamp to the passive membrane resistance and capacitance measured independently under current clamp, and (3) the nature of alterations in slow-wave activity produced by inhibitors of active transport and by ionic substitutions (sodium and potassium) are not consistent with a mechanism involving ion-specific conductance changes.

We conclude that slow waves in cat duodenum result from the rhythmic modulation of current from an electrogenic ion transport system, most probably the sodium–potassium pump. The action of such a mechanism is as follows (see Fig. 20). Between the periodic slow depolarization, the electrogenic transport system is operating at a basal level. At a given time the electrogenic transport rate drops, either because the total transport rate decreases or the coupling ratio of inward to outward flux approaches unity. This decrease in net outward flux leaves a now unbalanced inward leakage flux which depolarizes the membrane and creates the rising phase of the slow wave. Under voltage clamp such a decrease in outward current would appear as an inward current. Following the undershooting decrease in transport rate, the pump level settles to a quasi-stable value from which it builds back to the basal level. Correspondingly, the membrane voltage, which lags the current because of the membrane capacitance, reaches a peak depolarization and then declines.

Our observations and the resulting conclusions differ from those in previous studies of slow-wave activity in rabbit (Mills & Taylor, 1971) and guinea-pig ileal muscle (Bolton, 1971). Figures published by Mills &

Taylor (1971) showing the electrotonic potential responses to applied current pulses during the slow wave, suggest that the time constant of their preparation was also too large to permit conductance measurements to be made by current application during the rising or falling phases. They did observe, however, an increased conductance at the peak of the slow

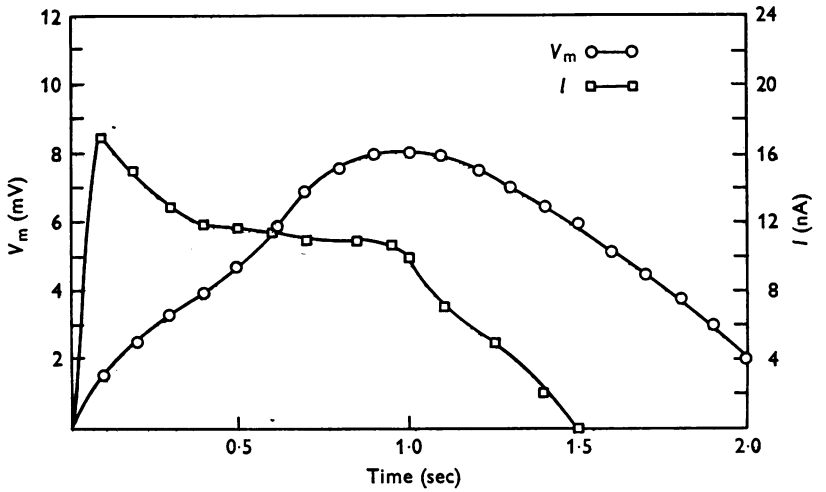

Fig. 20. Simultaneous plot of membrane current and the slow-wave voltage from a single preparation. Inward membrane current is plotted as positive to aid in comparing the two quantities.

wave. It is possible that rabbit ileal muscle is different from that of cat duodenum with respect to the slow-wave mechanism, but we consider this unlikely since the rest of their data agrees with our observations and those of other investigators (Liu *et al.* 1969). An explanation of the Mills & Taylor measurement may be that the voltage at the slow-wave peak lay in the delayed rectification region; thus, the movement of the membrane potential into this range by the slow-wave mechanism might induce an increase in membrane conductance but this would be a result of slow-wave activity and not a cause.

The slow oscillatory behaviour described for guinea-pig ileum and jejunum appears to arise from a different mechanism from the one we have proposed for slow waves. Kuriyama, Osa & Toida (1967*a*, *b*) have described two types of slow potential changes in guinea-pig jejunum, slow waves and prepotentials. Tetrodotoxin and atropine abolished these slow waves while having no effect on the prepotential. Bolton (1973) reported that 'slow waves' could only be observed from guinea-pig ileum in the presence of carbachol or acetylcholine, and that they were abolished by small hyperpolarizations, atropine and hyoscine. Sizeable conductance

changes, up to 17% of resting conductance, were measured during the rising phase of the depolarization with maximum conductance occurring at the wave-form crest.

Rabbit and cat slow waves, however, are insensitive to tetrodotoxin and atropine (Liu *et al.* 1969; Mills & Taylor, 1971) and we find that slow waves are only abolished by hyperpolarization greater than 30 mV. Since guinea-pig 'slow waves' respond differently from those of rabbit or cat to various treatments, have a different wave form, and appear to be associated with conductance changes, we suggest that they may result from a voltage-dependent conductance mechanism that is similar to the mechanism underlying the second type of oscillatory activity that we have observed and called oscillatory pre-potential activity (see Figs. 11 and 12). Our data indicate that even quiescent preparations have a resting potential partially determined by an electrogenic pump, much as in taenia coli (Casteels *et al.* 1971). The slow-wave amplitude is well within the range of this electrogenic component and it appears that both slow waves and the steady electrogenic component in quiescent preparations result from the same pumping mechanism since both systems respond similarly to ouabain and potassium removal, produce a measurable pump current, and are stimulated by increases in the internal sodium concentration. The only apparent difference between the electrogenic systems of these two types of preparation is that in those demonstrating slow-wave activity the electrogenic pump oscillates to produce slow waves while in the quiescent preparations the pump rate remains constant and produces the observed steady electrogenic component of the resting potential.

In attempting to ascertain why the pump mechanism should oscillate, it is important to emphasize that the initial event in our scheme of the slow-wave cycle is a sudden drop in the electrogenic transport rate of an ion pump. The most direct way to account for a sudden decrease in transport is to assume either that a depletion of some necessary substrate has occurred or that there is a build-up of an inhibitory product. In order to obtain oscillations, in either case, one must assume the existence of thresholds for activation and inactivation of the transport mechanism, or alternatively, a transport lag between the sites of manufacture and depletion of substrate necessary for transport. One possible example of a depletion mechanism that could account for the qualitative features of slow-wave activity, is the following: if the basal rate of outward sodium transport exceeds the passive influx of sodium, the internal sodium concentration will decrease. When the internal concentration reaches a critical value the transport system slows down and the membrane depolarizes. Sodium continues to leak in and the internal sodium concentration then increases to a second critical level, which reactivates the transport system.

Electrogenic transport then returns to the basal rate and the membrane voltage returns to the potential observed at the bottom of the slow wave.

Although this scheme predicts many of the observed responses (e.g. the relation of slow-wave frequency and amplitude to membrane potential), it is probably not responsible for the oscillatory behaviour since slow-wave frequency is insensitive to external sodium over the short run, and slow-wave generation continues after long exposure to low sodium which should deplete a large fraction of the internal store. Activity stops only when most of the internal sodium would reasonably be expected to be gone (Casteels *et al.* 1973). Were the pump oscillations determined by internal sodium, one would expect a rather quick cessation of activity or at least a marked frequency change as the internal level of sodium was being depleted. A similar mechanism involving external potassium could be considered but several preliminary studies have not given supporting evidence.

Our data from sodium-loaded cells indicate that whatever the mechanism is for modulating the electrogenic transport rate, it can be overridden. That is, slow-wave activity is transiently interrupted after loading with sodium in a potassium-free medium. During the quiescent period there is a steady electrogenic flux which persists for minutes at a rate which, at its maximum, is several times that of the periodically varying currents. It is thus possible that the internal sodium must be reduced below a critical level before the transport system will oscillate.

The finding that pumping can occur at a steady, high rate eliminates from consideration a simple scheme which would postulate periodic exhaustion or depletion of a cellular energy source such as ATP (Job, 1969, 1971) unless the increased internal sodium concentration induces the cell to provide an ATP supply sufficient to support an increased pumping rate or unless it alters the thresholds of activation and inactivation.

Since slow waves appear to result from cyclic decreases in pump current from a given basal level, an increase in slow-wave amplitude must be accompanied either by a greater decrease in pump current from the basal level, or by an increase in the basal current if pump current drops to zero during a slow-wave crest. Membrane hyperpolarization increases slow-wave amplitude, but it is also observed to decrease the pump current in sodium-loaded, electrically quiescent preparations. This is consistent with the report that pump current from sodium-loaded snail neurones is also decreased by hyperpolarization (Kostyuk *et al.* 1973). These results are in agreement with the idea that slow-wave amplitude is regulated by the degree to which the transport system is inactivated during a slow wave.

The finding that the slow-wave mechanism can function rhythmically in the absence of any voltage change indicates that the 600–1000 cells within the node must be using something other than the electrotonic spread of voltage as a direct or a primary mechanism to synchronize their respective generators. We have been unable to identify this primary mechanism which in all probability accounts for both coupling and setting the rate of oscillation. Whatever the nature of this coupling mechanism may be, it is not completely insensitive to voltage since we were occasionally able to induce a single slow wave in quiescent preparations by the appropriate current or voltage stimulus and the rate of spontaneous oscillation is influenced by steady applied current (Fig. 7). Also, multiple pacemakers appeared in some preparations upon hyperpolarization. This might result if the transport mechanisms of a population of cells within the node falls into asynchrony or if a population of quiescent cells having a slightly different natural frequency becomes activated due to the applied voltage. The idea that voltage changes in a population of cells may be entrained by a factor other than voltage does not have great intuitive appeal. However, there is some precedent for it in the literature. Studies by Bass, Code & Lambert (1961) and Kobayashi *et al.* (1966) of electrical activity in the intact gut have shown that slow waves are synchronous to the limits of measurement around the intestinal circumference, but that the spread of activity in the longitudinal direction is slow enough to allow easy detection of phase lag over a distance of half circumference. The low velocity of longitudinal spread is expected from the large capacitance of the tissue (see Experimental considerations section) and is similar to the velocity of spread in taenia (Bülbring, Burnstock & Holman, 1958). The synchrony around the circumference is therefore unexpected and could possibly be due to the coupling mechanism observed in our isolated muscle preparation.

Slow-wave activity was not recorded from the majority of preparations tested. In the cases where it was recorded, it was restricted to localized regions along the small muscle strand preparations. This stands in contrast to intact intestine where slow waves can be recorded from all regions of longitudinal muscle (Prosser & Bortoff, 1968). The reason for this is not clear although we would make the following speculation. It appears certain that the dissection and mounting procedures initially damage the muscle since passive and active membrane properties are altered during 1 hr equilibration period in Krebs. These procedures may permanently damage the slow-wave mechanism of given preparations or regions of a preparation so that these regions are incapable of spontaneous slow-wave activity. If this lack of activity does result from damage to the preparation, the nature of the damage must be very specific since those preparations

not having slow-wave activity have functioning, but non-oscillating electrogenic pumps and do not differ from preparations demonstrating slow waves with respect to passive membrane properties or spike generation. Previous studies indicate that this inactive condition may result from the gross removal of the longitudinal muscle from the intestine and not from the micro-dissection required to obtain our small preparations since slow-wave activity has also been reported to be localized in large isolated sheets of longitudinal muscle (Kobayashi *et al.* 1966). It is possible that not all regions of longitudinal muscle in intact intestine actively produce slow waves, but that slow potential changes are observed at some regions due to electrotonic spread from a pacemaker site. If this is the case, then some of the electrical coupling from one longitudinal muscle region to another may be by way of the circular muscle. These two layers are known to be physically interconnected by muscle strands which run between the two muscle layers (Bortoff, 1965; Kobayashi *et al.* 1966). Thus, the apparent localization of slow-wave activity in isolated longitudinal muscle may result from the loss of electrotonic coupling via these connecting muscle strands.

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