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Relation between oxidative metabolism and slow rhythmic potentials in mammalian intestinal muscle

(smooth muscle/cell metabolism)

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ABSTRACT **Intact muscle layers separated from the small intestine of the cat were mounted in a specially designed chamber to measure electrical slow waves and NADH fluorescence simultaneously. Cooling the muscle to 17° eliminated slow waves and simultaneously increased the level of fluorescence. Likewise, superfusing the muscle with a N_2-bubbled glucose-free Krebs solution decreased the amplitude of slow waves and concomitantly increased fluorescence emission. In both cases, return to normal conditions reversed the effects on both slow waves and fluorescence. When signals were averaged over 30–70 slow waves, a pattern emerged with the fluorescence oscillations in phase with the electric oscillations. The NADH:NAD^+ ratio reached a maximum at the most depolarized point of the slow waves and a minimum at the most polarized point between slow waves. This indicates maximum ATP utilization during the repolarization process. The correlation between redox oscillations and electrical slow waves is consistent with previous evidence that slow wave generation is associated with cell metabolism.**

The small intestine of most mammals shows rhythmic slow potentials which correspond in frequency to segmental contractions, 6–18/min. These waves originate in the longitudinal muscle layer and spread electrotonically to the circular muscle where they may initiate spikes which trigger contractions. Evidence has been presented (1–3) that the slow waves may result from oscillations in an electrogenic sodium pump. The slow waves are abolished by agents which block Na–K pumps, e.g., ouabain, K-free medium; and when these agents depolarize, the waves are not restored by repolarization. In addition, a Na^+ pump contributes to resting potential, and slow wave amplitude is maximal at the potassium concentration of maximal pump contribution (4). Under voltage clamp, current pulses can be recorded with period and ionic properties like the voltage waves (1). Furthermore, tracer sodium is extruded during the repolarizing phase (2) and resistance changes are not detected during the slow waves (1). Slow waves are sensitive to metabolic factors; they are diminished or eliminated by some metabolic inhibitors, anoxia, or cooling (5). Likewise, there is a slow wave-frequency gradient decreasing from duodenum to terminal ileum which is accompanied by a proportionate decline in CO_2 production (6). It is postulated that a rhythmic pacemaker may be located in the metabolic paths which make ATP available to the pump or in a feedback pathway between ion pump and metabolic cycle. This report presents evidence that the slow waves in intestinal smooth muscle are correlated with oscillations in NADH fluorescence and suggests that these oscillations are related energetically to rhythmic oscillations in electrogenic ion transport.

METHODS

Segments of cat jejunum were removed under chloralose anesthesia (65 mg/kg) and were immediately placed in Krebs solution of the following composition (mM): NaCl, 118.5; KCl, 4.7; $MgCl_2$, 1.2; $NaHCO_3$, 23.8; KH_2PO_4, 1.2; $CaCl_2$, 2.5, and glucose, 5.5. The solution was bubbled with O_2–CO_2 (95%–5%) which maintained a pH of 7.35 ± 0.05. D-600 (methoxyverapamil) was obtained from Knoll Pharmaceutical, West Germany. For testing the effects of anoxia, the solution was bubbled with N_2–CO_2 (95%–5%).

(*a*) Measurement of NADH Fluorescence. Pieces of the muscularis approximately 3 × 1.5 cm were separated from the intestinal mucosa and were mounted in a chamber (Fig. 1A) such that the longitudinal muscle layer faced the exciting light beam coming from above the chamber. The muscle was stretched slightly from "rest" length and pieces were allowed to equilibrate at 37° for at least 1 hr before recording.

The muscle chamber (Fig. 1) consists of a black plexiglass base in which the muscle is pinned on Sylgard (Dow Corning). The plexiglass top is fastened to the base with six screws and a seal is maintained with a rubber "O" ring. At the centers of both the top and the base are aligned quartz windows 5 mm × 5 mm. The base has inlet and outlet holes at opposite ends for flow of solutions over the muscle.

Light from a high-intensity xenon lamp (Ushio Electric Inc.) is focused onto a grating-type monochromator (Jarrel-Ash). The output of the monochromator is focused via a lens and planar mirror onto the muscle through the quartz window in the top of the chamber. The exciting wavelength was that which gave maximal fluorescence in a test solution (0.1 mg/ml) of NADH (364 nm). An RCA-931 photomultiplier (PM) tube was mounted in a shielded box below the chamber. A 1 M $NaNO_2$ filter and a Corning 3-72 filter are placed between the chamber and the PM tube. In some experiments, a bandpass filter with transmission peak at 470 nm (70-01-2 Baird Atomic) is placed on the emission side of the chamber. The output of the PM is fed to a variable gain preamplifier which is connected to one channel of an oscilloscope.

External electrodes are mounted through holes in the top of the chamber next to the quartz window; one electrode (E_1) is angled so that it contacts the muscle in the region of illumination. The configuration of the recording apparatus is dictated by the particular experiment. For recording baseline shifts of the fluorescent signal while altering oxygen concentration and temperature, the outputs of the oscilloscope for both voltage and fluorescence are connected to a polygraph (Grass Instruments, Inc.). To average the phasic changes in the fluorescent signal during individual slow waves, the oscilloscope output is fed to a computer of average transients (CAT, Technical Measurements Corp.) which is manually triggered at a given phase of the slow waves.

(*b*) Assay of Muscle ATP. ATP content was measured in pieces of longitudinal muscle removed during rising and falling phases of the slow waves. Assay was by the luciferase method (7); a fluorometer (Aminco Inc.) with a modified input circuit was used. Samples of muscle were removed by a tonsil biopsy

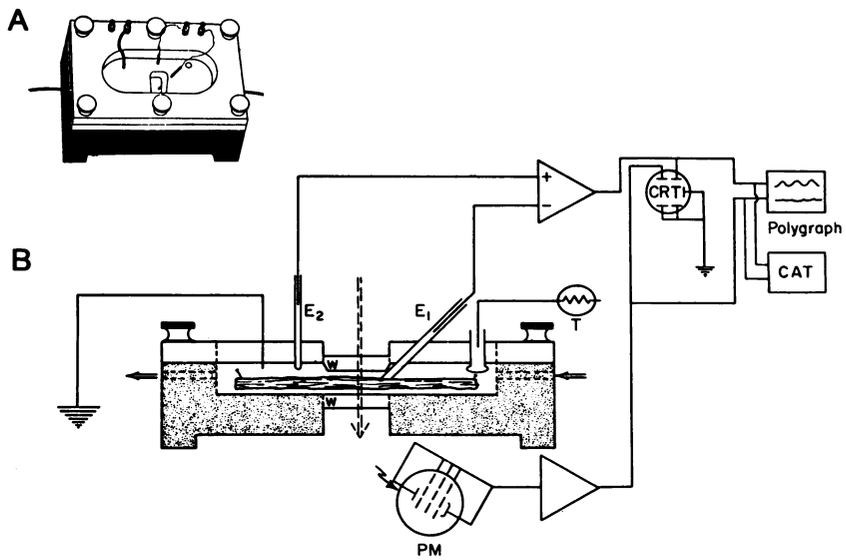

FIG. 1. Apparatus for measuring fluorescence and electrical activity simultaneously. (A) Overview of the chamber which measures 7.5 × 5 × 2.5 cm. (B) Cross-section of chamber with schematic drawing of recording equipment. E_1 and E_2 are the recording and indifferent pressure electrodes, respectively; E_1 is placed on the muscle at the edge of the quartz windows (W). An inlet and an outlet hole (arrows) at either end of the chamber provide for constant flow of solutions. Other symbols: broken arrow, excitation beam; T, thermistor; CAT, computer of average transients; PM, photomultiplier tube; CRT, cathode ray tube. See *text* for further details.

instrument during continuous monitoring of slow waves by a pressure electrode 1–2 mm from the point of biopsy. The biopsy tool was chilled in liquid nitrogen before use and the tissue samples were plunged into liquid nitrogen within 1 sec after removal. Frozen samples were pulverized at −80° in a mortar containing a few drops of perchloroacetic acid; after thawing, the sample was neutralized with NaOH. The precipitated protein was removed by centrifugation and the chilled supernatant was assayed. Luciferase (Sigma) was added and the emitted light was recorded on a polygraph or read at 10 sec intervals on a meter. A standard curve for ATP (maximum emitted light versus ATP concentration) was constructed before each experimental series. Since the muscle samples (5–15 mg each) could not be weighed before freezing, ATP content was expressed per total inorganic phosphate or protein. In other samples the equivalent wet weight was measured for P_i or protein so that ATP could be calculated on a weight basis.

RESULTS

The electrical recordings made with external electrodes showed typical slow waves with amplitude of approximately 1.0 mV and frequency of 10–15/min (Fig. 2). In preparations in which spiking and contraction occurred, these activities were stopped by addition of D-600 (10 μM) or 60 mM sucrose. During periods of normal slow-wave activity, there were no noticeable systematic changes in the light signal which could be correlated with a specific phase of a single slow wave; noise from the photomultiplier and background fluorescence exceeded possible changes which may have occurred during one slow wave.

Slow waves have been shown to be dependent on oxygen, on metabolizable substrates and to be sensitive to temperature (2, 5). As a means of evaluating the optical system, ascertaining whether the fluorescent signal could be correlated with cell metabolism, and relating slow wave activity to metabolism, we subjected muscles to hypoxic glucose-free Krebs solution and to cold (Fig. 2). Hypoxia plus substrate deprivation produced results similar to those obtained with cooling. After perfusion with N_2-bubbled glucose-free Krebs solution for 20 min, amplitude of slow waves was reduced to less than 22% of control level and this was accompanied by a gradual increase of 1.7% in light intensity (Fig. 2A). After returning the muscle to O_2-bubbled glucose-containing Krebs solution, slow waves gradually increased in amplitude and the light signal intensity decreased concomitantly. When precooled (15°), Krebs solution flowed through the chamber slow waves were eliminated as the temperature reached 17°. From 37° to 18° the frequency decreased with a Q_{10} (relative change in rate for each 10° change in temperature) of 2.1. This decrease in electrical activity corresponded to an increase of 2% in intensity of the total fluorescent signal, indicating more NADH (Fig. 2B). On rewarming to above 20°, the slow-wave activity returned and the intensity of the light signal decreased. Thus slow-wave elimination by either cold or anoxia was accompanied by an increase in levels of the reduced form, NADH; return to normal activity was accompanied by a decrease in NADH. In both cases the average change in the light emission represented 2% of the total fluorescent signal.

Because no changes in fluorescent emission could be discerned in phase with single slow electrical waves, the signals were averaged during many slow waves on a computer of average transients. Fig. 3 gives examples of signals averaged over 30–70 sweeps; each sweep was triggered manually at the same point on the rising phase of a slow wave in a pair. The averaged transients showed a phase-locked change in the light intensity at the same frequency as the electrical slow waves. In other words, averaging uncovered a correlation between voltage and the ratio of NADH/NAD. In nine series of measurements on six preparations with the same fiber orientation, the maximum fluorescent signal (corresponding to maximum NADH concentration) occurred in the repolarizing phase of the slow waves in eight of nine experiments. The fluorescent signal was minimum during the final phase of the trough or early in the depolarization phase of the slow wave in all nine experiments. When the light signal was averaged over pairs of consecutive slow waves, the phase relationship was the same for both slow waves.

As controls for nonspecific changes, such as movement artifact or changes in light scattering, the wavelength of the exci-

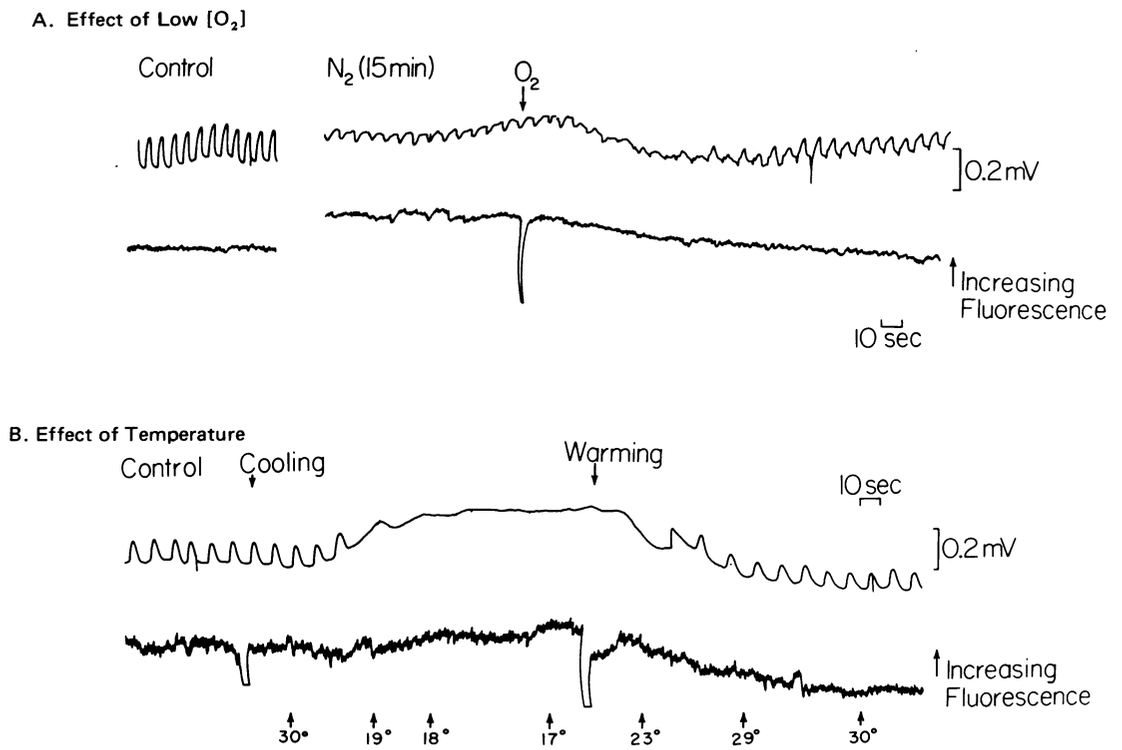

FIG. 2. Effects of anoxia and temperature on electrical slow waves and NADH fluorescence. (A) Superfusing the muscle with N_2-bubbled glucose-free Krebs for 15 min increases the fluorescence and decreases the slow wave amplitude. Return of normal Krebs (O_2) reverses these effects. (B) Quickly cooling the muscle to 17° by superfusing with cold Krebs rapidly eliminates electrical oscillations and simultaneously increases NADH fluorescence; this was reversed by rewarming the muscle.

tation source was changed from the optimal excitation wavelength to 410 nm. Under these conditions, fluorescence changes in phase with the slow waves were eliminated. Also when the computer of average transients was randomly triggered, no correlation was observed and no consistent summed pattern emerged for either the voltage or the light signal. It is concluded that the ratio of NADH/NAD is maximal (reduced form in highest concentration) in the repolarizing and early trough phases of a slow wave.

The ATP content of longitudinal muscle samples removed at different phases of the slow wave and immediately frozen in liquid nitrogen ranged from 5 to 50 nmol per sample (approximately 10 mg). Total phosphorus and protein averaged 1.45 mg/g of wet weight and 108.5 mg/g of wet weight, respectively. The average of 12 ATP determinations on paired samples removed during the depolarizing phase of the slow wave was 0.915 μmol of ATP per g of wet weight (8.43 nmol of ATP per mg of protein); and during the repolarizing phase

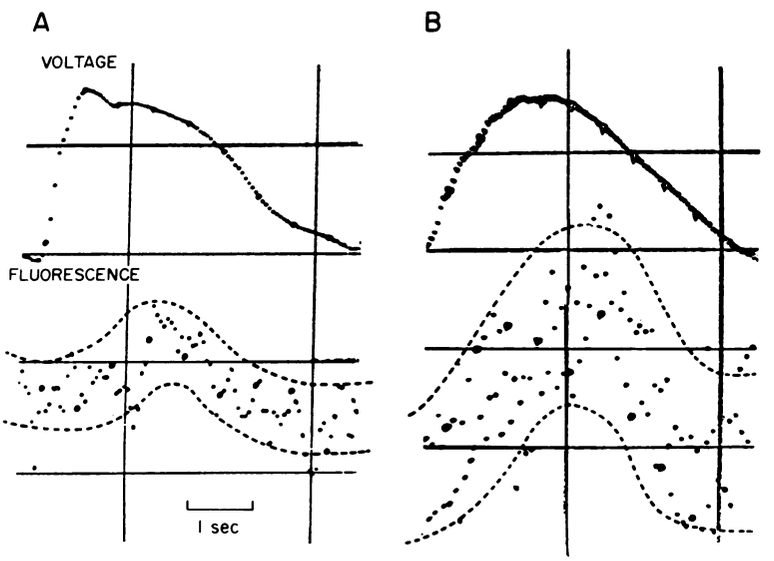

FIG. 3. Summed voltage and fluorescence signals from two preparations using a computer of average transients. Dashed lines above and below fluorescence signal are approximate limits drawn to outline changes in intensity. Because each trace is the average of numerous events the scales are relative. (A) Averages of 30 slow waves. (B) Averages of 70 slow waves.

was 0.740 μmol of ATP per g of wet weight (0.683 μmol ATP per mg of protein). Based upon 5×10^8 cells per g of tissue, the ATP content was 1.83 fmol per cell on depolarization and 1.48 fmol per cell on repolarization. Statistical analysis (*t* test) revealed no significant differences between the depolarizing and repolarizing phase ($P > 0.1$).

DISCUSSION

These observations demonstrate a correlation between redox state and the slow electrical rhythm of cat intestinal muscle. This correlation in and of itself is not sufficient to establish that a cycling transport system directly underlies generation of the slow waves. For example, the cycling level of NADH could reflect a demand on the transport system brought about by periodic membrane conductance changes which themselves generate the slow waves. The evidence summarized earlier favors the cycling transport system.

Both hypoxia and cooling reduce or eliminate the slow waves as they increase the level of NADH fluorescence. Hypoxia has little effect on frequency while cooling causes a reduction in frequency. The observed phase relation between NADH fluorescence and voltage indicates a maximum of the NADH/NAD ratio at the peak of slow waves and a minimum at the trough. Thus, maximum ATP utilization and NADH oxidation occur during the repolarizing phase of the slow wave when sodium ions are being extruded. Conversely, during or immediately prior to the depolarizing phase, ATP utilization and NADH oxidation are at a minimum when the outward transport of sodium ions is low (2). This suggests that a sodium pump is actively extruding sodium ions during repolarization and that the pump is consuming less energy during depolarization. There presumably is some phase lag between the metabolic and electrical events, but the time resolution of the apparatus is inadequate to establish any precise phase differences. Lack of precise phasing between different preparations may have been due to different amounts of asynchrony between muscle fibers under the recording electrode and the majority of those in the window region. However, the general phase relation is probably accurate since there is electrical synchrony over large areas of tissue when the circular fibers are attached to longitudinal and since in one preparation rotating the muscle fiber orientation 90° changed the phase relation of the voltage and fluorescence an equivalent amount.

While the effects of metabolic depletion on slow wave amplitude are well documented, it is difficult consistently to induce alterations in slow wave frequency by any other means than changing temperature. Job (5) found a 50% reduction of slow wave amplitude after 20 min of hypoxia with little effect on frequency. Several metabolic inhibitors such as cyanide and antimycin A reduced amplitude at low concentrations but reduced frequency only in very high concentrations. Temperature, on the other hand, decreases the rates of rise and fall of the slow waves with a Q_{10} (relative change in rate for each 10° change in temperature) of 2.5 (2), and decreases the frequency but has only a small effect on amplitude. This situation is similar to that reported by Betz and Chance (8) for the oscillations of reduced pyridine nucleotides in yeast cells where the amplitude of the oscillations is reduced by metabolic inhibitors but the frequency is not affected. Frequency, on the other hand, is affected by temperature with a Q_{10} of 2.2 to 2.4 above 20°. While the present results indicate a link between ATP utilization and slow oscillations of membrane potential, the frequency of the slow waves is not likely determined by a simple redox oscillation but probably by some more complex interaction of the metabolic pathways with membrane ion pump activity.

Failure to detect a change in NADH fluorescence or tissue ATP concentration during single slow waves without averaging may be due to compartmentalization and to masking of effects by high levels of metabolism. To estimate the magnitude of changes in cellular ATP concentration during slow wave activity, we calculated the amount of ATP consumed per slow wave by assuming an on-off ion pump oscillation. This means that during repolarization to produce maximal membrane polarization the pump is *on* and during depolarization the pump is *off*. The maximum change of cellular ATP concentration is the difference between pump ATP consumption in the two states. Assumptions by analogy with other Na–K pumps (9) are: one ATP hydrolyzed per net Na^+ transferred, pump ratio 3 Na^+/2 K^+. Geometry of smooth muscle from prior measurements (10) gives: double cone 200 μm long, 5 μm maximum diameter, volume 1.3 pl, surface area 1.5×10^{-5} cm^2, extracellular space 35% of volume, number of cells per cm^3, 5×10^8. Electrical properties measured previously by microelectrodes and double sucrose gap are: slow wave amplitude 10 mV, current under voltage clamp 10 nA, node volume in sucrose gap 2.6 μl (1), input impedance 70 MΩ, membrane resistance 1 kohm cm^2 (10). Cellular concentration of ATP is approximately 1 μM/g (ref. 11, see above). We start with the magnitude of the slow wave current transient under voltage clamp in the double sucrose gap of 10 nA for 2.5 sec, and then calculate:

$$2.5 \times 10^{-8} \text{ A sec} = 6 \times 10^{18} \text{ charges/coulomb}$$
$$= 1.5 \times 10^{11} \text{ } Na^+ \text{ transferred/slow wave per node}$$
$$= 1.5 \times 10^{11} \text{ molecules of ATP hydrolyzed/slow wave per node}$$

Dividing by node volume:

$$\frac{1.5 \times 10^{11} \text{ ATP molecules/slow wave per node}}{2.6 \times 10^{-6} \text{ } cm^3\text{/node}}$$
$$= 5.8 \times 10^{16} \text{ ATP/}cm^3 \text{ of tissue}$$

Assuming 35% extracellular space:

$$= 8.8 \times 10^{16} \text{ ATP molecules/}cm^3 \text{ of cells}$$
$$= 1.46 \times 10^{-7} \text{ mol of ATP/}cm^3 \text{ cells}$$

or for the volume of one cell:

$$= 1.9 \times 10^{-16} \text{ mol of ATP/cell per slow wave}$$

which represents about 10% of cellular ATP content. The change in cellular ATP was also calculated on the basis of other assumptions and measurements. Based on a measured intracellular voltage of 10 mV for a slow wave in a cell with an input impedance of 70 MΩ, the current is 0.14 nA/cell and 3.6 fmol of ATP per cell/slow wave. This calculation is questionable because of uncertainty as to the true impedance across which the voltage drop occurs. Another type of calculation was based on the assumptions of 300 pump sites per μm^2 of membrane (12) and pump rate of transfer of 100 times/sec. This calculation gave 0.18 fmol of ATP per cell/slow wave. Thus, the ATP values given by these two methods are of the same order as that calculated from the current measurements under voltage clamp which is approximately 10% of total cellular ATP. This represents the maximum change in cellular ATP concentration because a change of this magnitude would require that for a 5 sec slow wave period the pump is in the "on" state for 2.5 sec and in the "off" for 2.5 sec and changes instantaneously between the two states. More importantly, the calculations do not take into account replacement and utilization of cellular ATP by ongoing metabolism.

The fact that measurements made by the biopsy method are within statistical variation and that signal averaging was required to detect fluorescence oscillations during slow waves is probably an indication that changes in concentration of ATP (and therefore NAD) were considerably less than the calculated maximum. This is substantiated by the observation that the maximum increase in static NADH fluorescence after 20 min in N_2, glucose-free solution was only 2% of the total fluorescent signal.

Oscillations of metabolic intermediates correlated with oscillations in the redox state as measured by NADH fluorescence have been demonstrated in other systems (13). These fluctuations are reported in intact as well as in cell-free yeast cultures (14), in continuous cultures of *Klebsiella aerogenes* (15) and in rat heart (16). The oscillations occur with a period of 1–100 min and usually are initiated by some perturbation of the system such as anoxic shock or administration of cyanide. In most cases the oscillations are damped after 5–10 cycles. The mechanism for these oscillations is thought to reside in a metabolic feedback system involving intermediates in glycolytic or respiratory reactions.

Similar oscillations, which in addition are associated with membrane ion transport, have been reported in the epithelium of the bladder of *Bufo marinus* (17) and in the plasma membrane of the *poky f* mutant of *Neurospora crassa* (18). In both cases a metabolic perturbation initiates the damped oscillations and transport returns to a steady state after 4–5 cycles. In the *Neurospora* mutant the oscillations are dependent upon partial maintenance of respiration but they are not necessarily associated with changes in ATP concentration. This suggests that the ATP system is critically damped in order to maintain levels of cellular ATP and that oscillations of the same frequency of the electrogenic hydrogen ion pump must be occurring at some other metabolic site.

The fact that voltage oscillations in intestinal smooth muscle have been correlated with changes in the redox state of the tissue suggests some similarity with these other systems. This similarity most likely lies in the basic metabolic mechanisms contributing to the oscillations, although the precise steps are not yet understood. However, there are three striking differences between the intestinal system and microbial systems: (*i*) slow wave oscillations are spontaneous and do not appear to require an external perturbation to be initiated. The perturbation which does lead to the oscillations would be the primary pacemaker and probably would be the most significant determinant of slow wave frequency. (*ii*) Slow waves continue undamped indefinitely; in this respect they are unlike the negative feedback loops proposed for many other oscillating systems which gradually return to an equilibrium state. (*iii*) The period of the intestinal slow waves is much shorter than oscillations in other systems, less than 10 sec as compared with one to several minutes. These differences are probably reflections of the unique characteristics of the primary pacemaker reactions in the longitudinal muscle of the intestine.

These observations demonstrate a correlation between NADH oxidation and slow electrical waves in intestinal muscle. Changes in fluorescence correlate well with amplitude of slow waves and the two events appear to synchronize. However, these data do not delineate whether the oscillations in NADH/NAD ratio are a reflection of rhythmic changes in the hydrolysis of ATP or in formation of ATP. The data do not permit the conclusion that the molecular pacemaker is in a redox oscillation, nor do they preclude it. A more likely hypothesis is that some sort of feedback interaction exists between the ATP utilization by the Na-K pump and the generation of ATP.

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