Wright (*3*) actually reported blockade of neuromuscular transmission with curare, but only at high concentrations (10^{-3} g/ml). The tonic muscles studied here are functionally quite different from the limb muscles previously studied. For example, it would be easy to miss a behavioral effect of transmitter blockade in these muscles, since they control only the posture of the tail and most righting and swimming reflexes would remain essentially unaltered.

The following lines of evidence presented in this study support the identification of ACh as a transmitter to these tonic flexor muscles in crayfish: (i) a depolarizing response to iontophoresis of ACh applied at the synaptic region, (ii) neuromuscular blockade by curare without accompanying alteration of membrane potential or conductance, (iii) enhancement of EJP's by a cholinesterase inhibitor, and (iv) possible ACh desensitization of postsynaptic receptors. One criterion for assigning transmitter function has not been met: ACh has not been shown to be normally present in the motor axon studied, but neither have other putative excitatory transmitters. On present evidence, the crustacea now appear unique in possessing two independent transmitter systems for the excitation of skeletal muscle.

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9 December 1971

Cilia: Activation Coupled to Mechanical Stimulation by Calcium Influx

Abstract. *Ciliated epithelial cells in the oviduct of* Necturus maculosus *were stimulated mechanically by brief dimpling with a microstylus. This treatment produced a transient depolarization of the membrane, and a transient increase in the frequency of ciliary beating. The increase in frequency of ciliary beating was related to the concentration of extracellular calcium ion, decreasing with reduction in calcium. Addition of lanthanum was followed by a decrease in spontaneous ciliary activity and a hyperpolarization of the membrane. In the presence of lanthanum, the transient depolarization in response to mechanical stimulation had a shorter time course, and the concomitant increase in ciliary frequency was greatly reduced. It is concluded that calcium ions enter the cell as a result of mechanical stimulation of the membrane, and that calcium influx leads to an increase in the frequency of ciliary activity.*

There is growing evidence that the periodicity of beating in an active cilium is inherent in the motile apparatus itself or in structures closely associated with it, and that the periodicity occurs without the need of triggering or phasing by extrinsic pacemakers or membrane-borne signals (*1*, *2*). The frequency with which the cycle of ciliary movement occurs, however, can be modulated by exogenous factors such as nervous activity (*3*), membrane potential (*4*), and the chemical environment (*5*). An increase in frequency of beating has also been observed in some ciliated epithelia in response to mechanical stimulation of the cell surface. We now present data indicating how mechanical stimulation of the cell membrane evokes increased ciliary activity.

The funnel-shaped ostium of the oviduct of the salamander *Necturus maculosus* contains large ciliated cells (~ 30 μm in diameter) scattered in groups among nonciliated cells. The salamanders selected were 30 to 33 cm long with well-developed ovaries, and with eggs of at least 4 mm in diameter. A piece of the thin tissue forming the ostium was isolated and wrapped around a 0.1-mm-thick glass plate for mounting in physiological saline under a ×40 water immersion objective. At the edge of the plate the cilia projected from the surface of the epithelium in the plane of focus of the objective; the cilia were

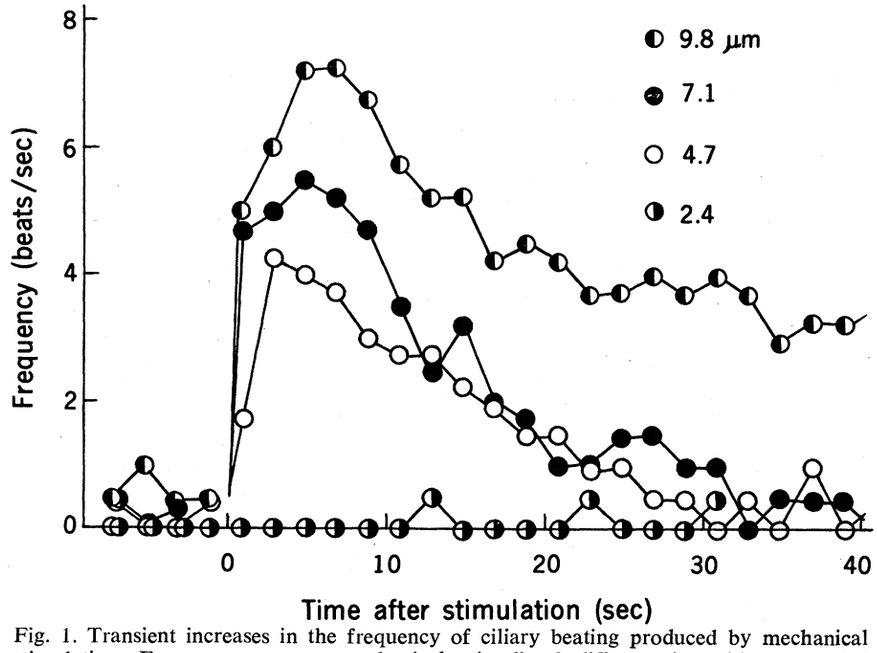

Fig. 1. Transient increases in the frequency of ciliary beating produced by mechanical stimulation. Four responses to mechanical stimuli of different intensities, recorded from the same cell, are superimposed. Spontaneous activity just prior to mechanical stimulation is shown to the left of time zero. Intensity of stimuli is expressed as the displacement, in micrometers, of the tip of the glass stylus. Frequencies are plotted as numbers of beats per second averaged every 2 seconds.

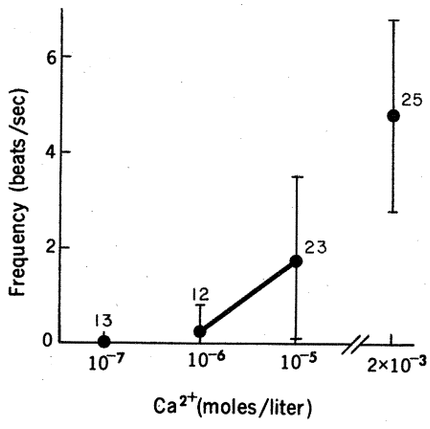

Fig. 2. Maximal frequencies (mean ± S.D.) of responses to mechanical stimulation obtained in the control saline solution ($2 \times 10^{-3}M$ Ca^{2+}), and in solutions of identical composition except that Ca^{2+} concentrations were buffered from 10^{-7} to $10^{-5}M$ with 10 mM EGTA. Stylus displacement, or intensity of mechanical stimulus, was 10 μm. Cells were stimulated after at least 15 minutes of exposure to each test solution. Numbers next to plotted points indicate the number of measurements at each concentration. Free calcium concentrations in calcium, EGTA buffer were calculated by the method of Hagiwara and Nakajima (*16*). Responses to mechanical stimulation disappeared in solutions containing $10^{-7}M$ free calcium or less.

thus observed in profile. Movements of the cilia were monitored photometrically by projecting the microscope image onto a screen bearing an aperture with an equivalent diameter (that is, in the object plane) of 0.5 μm. The photocathode of a photomultiplier tube (EMI 9524B) placed behind the aperture detected the fluctuations in light intensity that were caused by movement of the optical interface that lay between the edge of the ciliary population and the free solution across the aperture. The photocurrent was amplified and displayed with capacity coupling on a strip chart recorder. Each cycle of movement produced one diphasic wave in the pen recording.

The control saline contained 2 mM Ca^{2+}, 109 mM NaCl, 3 mM KCl, and 11 mM glucose. For concentrations of Ca^{2+} from 10^{-8} to $10^{-5}M$, the sodium salt of ethylene glycol bis(β-aminoethyl-ether)-*N*,*N*′-tetraacetic acid (EGTA) (Lamont Laboratories), was added in a concentration of $10^{-2}M$ and $CaCl_2$ was adjusted to give desired concentrations of dissociated Ca^{2+} (*6*). Saline was buffered with 10 mM tris(hydroxymethyl)aminomethane-maleate to *p*H 7.0 ± 0.1. Temperatures ranged from 18° to 21°C, except where noted otherwise.

Quiescent cells were stimulated mechanically with a glass microstylus (tip diameter ~ 5 μm) driven against the cell membrane by a piezoelectric phonocartridge that was energized by pulses from a square-wave generator. Displacement of the stylus was adjusted from 0 to 12.5 μm, while duration of displacement was held constant at 200 msec, except where noted otherwise.

Individual ciliated epithelial cells, or clusters of these cells, found in the mucosal epithelium of the amphibian oviduct show spontaneous fluctuations of beating from 0 to 13 beats per second (*7*). Activation (increase in frequency of beating) occurs when these cells are mechanically stimulated by turbulence of the bath solution or by dimpling of the cell membrane with a microstylus.

The cilia responded to the dimpling of the cell surface (with the microstylus) with a transient increase in frequency of beating (Fig. 1). The peak frequency varied with stylus displacement, reaching a maximum of about 12 beats per second. Excessive stimulation produced a strong and prolonged activation, similar to that produced by puncture of the cell membrane with a microneedle (*2*).

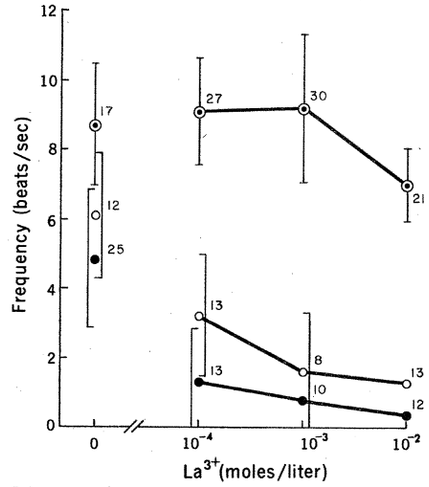

Fig. 3. Effect of La^{3+} on maximal frequencies (mean ± S.D.) of responses elicited by mechanical stimulation (stylus displacement: ●, 10 μm; ○, 12.5 μm) or by puncture of the cells with a microneedle (⊙). Experiments were performed after the cells had been present in the test solutions for 30 minutes. Numbers near plotted points indicate the number of measurements at each concentration of La^{3+}.

The increase in beating frequency produced by mechanical stimulation depends on the concentration of extracellular Ca^{2+} (Fig. 2). In the control saline solution, containing 2 mM Ca^{2+}, the mean rise in beating frequency was 5 beats per second. The frequency dropped with EGTA-buffered Ca^{2+} concentrations of 10^{-5} and 10^{-6} mole/liter. At calcium concentrations below 10^{-7} mole/liter, mechanical stimulation evoked no response even through activity still appeared spontaneously (*8*). Dependence of activation on the external concentration of calcium suggested that activation may involve an influx of Ca^{2+} across the stimulated membrane.

The lanthanum ion is known to block Ca^{2+} influx across certain cell membranes that normally undergo increased calcium conductance during excitation (*9*). The La^{2+} ion was therefore used to determine if it interferes with the activation of cilia in response to mechanical stimulation. Cells were given a constant mechanical stimulus while they were in solutions containing several different concentrations of La^{3+} and a constant concentration of 1 mM Ca^{2+}. Ciliary activation in response to mechanical stimulation was then plotted against the La^{3+} concentration (Fig. 3). As La^{3+} concentration was increased from 0 to 10^{-4} and $10^{-3}M$ (*10*) the activation produced by a constant mechanical stimulus decreased progressively from a mean of 6 beats per second in the absence of La^{3+} to a mean of 1.6 beats per second in $10^{-3}M$ La^{3+}. Spontaneous beating continued at this concentration of La^{3+}, but was somewhat depressed. Although La^{3+} strongly depresses the response to dimpling of the cell surface, it does not reduce the activation produced by membrane puncture at concentrations of La^{3+} between 0 and 1 mM, the range over which La^{3+} has its most pronounced effect on mechanically stimulated activation (Fig. 3, upper curve). This suggests that La^{3+} interferes with Ca^{2+} movement across the stimulated membrane, but does not prevent the stimulating action of calcium which diffuses through a puncture in the membrane (*2*). The lowering of puncture-induced activation in $10^{-2}M$ La^{3+} may be due to an antagonism between La^{3+} and Ca^{2+} at an intracellular site.

The membrane potential was recorded (standard glass capillary electrodes filled with $0.2M$ KCl) to determine if

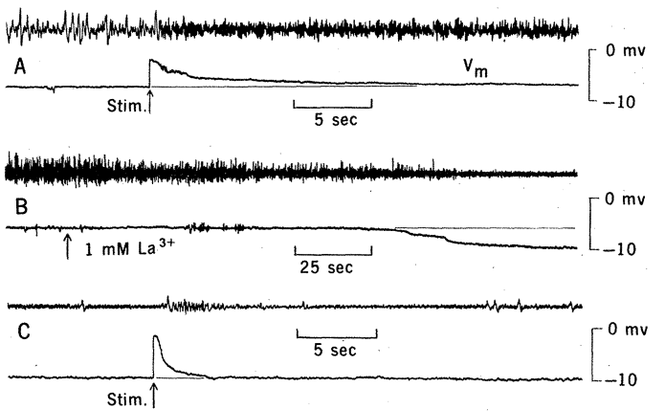

Fig. 4. The relation between membrane potential and ciliary activity. The upper tracing in each part records the photometrically monitored ciliary movement (*2*), and the lower tracing indicates the membrane potential V_m (intracellular potential minus extracellular potential). (A) Response to 10-msec mechanical stimulus in control saline (no La^{3+}). (B) Addition of 1 m*M* La^{3+} produces a gradual reduction in the frequency of spontaneous ciliary activity that is related to an increase in membrane potential. (C) Response to a 10-msec mechanical stimulus while cell is in saline containing 1 m*M* La^{3+}. The peak of the mechanically evoked depolarization reached the same level as in (A), but repolarization was more rapid. Increased resting potential, increased rate of repolarization, and reduced ciliary activity occurred consistently in each of the cells tested. All data in this figure were obtained from one cell with (A), (B), and (C) recorded in that order. Temperature was maintained at 24°C. In order to reduce the activation produced by electrode insertion, 1 m*M* $MgCl_2$ was added to the control solutions and to the solutions containing La^{3+}.

ciliary activity is related to membrane potential. Resting potentials were generally low (< 25 mv) in the control preparations of saline, but consistently increased by as much as 10 mv after addition of 1 m*M* La^{3+} (Fig. 4B). The increased potential difference was generally accompanied by a reduction in the frequency of spontaneous ciliary beating (Fig. 4B).

Mechanical stimulation in the control saline preparation produced a transient depolarization with a rapid upstroke followed by a slow repolarization (Fig. 4A). The depolarization was followed by an increase in ciliary activity with a delay of 1 second or less after the onset of depolarization. In the presence of 1 m*M* La^{3+} the duration of the mechanically evoked depolarization was reduced, as indicated by the accelerated rate of repolarization (Fig. 4C). There was also a concomitant decrease in ciliary activation consistent with the results presented in Fig. 3.

The results show that mechanical stimulation produces a transient increase in frequency of ciliary beating that varies with the intensity of the mechanical stimulus and is dependent on the concentration of extracellular calcium (Figs. 1 and 2). Mechanical stimulation also produces a depolarization of the cell membrane that is consistent with a net inward positive current. That current may be due in part to an influx of Ca^{2+} across the stimulated membrane. In the presence of La^{3+}, known to suppress Ca^{2+} conductance in other membranes (*9*), there is an increased rate of repolarization of the potential transient of the cell membrane (*11*), and a concomitant reduction in ciliary activation (Fig. 4). We propose that mechanical stimulation increases the permeability of the membrane to Ca^{2+} (and perhaps to other ions as well) permitting Ca^{2+} to leak through a stimulated membrane when its extracellular concentration is several orders of magnitude higher than its intracellular concentration. Such an inward Ca^{2+} current in response to mechanical stimulation was demonstrated in *Paramecium* (*12*). There is evidence (*2*) that ciliary activity requires intracellular Ca^{2+} in the living cell. Our results therefore suggest that the intracellular concentration of Ca^{2+} is a limiting factor in the frequency of beating, and that an increase in concentration of intracellular Ca^{2+} produced by a calcium influx through the stimulated membrane permits a higher frequency of beating.

How does an increase in intracellular Ca^{2+} stimulate ciliary activity? The Ca^{2+} ion is not required for reactivation of ciliary beating of extracted models of ciliated cells with adenosine triphosphate (*13*). The Mg^{2+} ion acts as the cofactor for the ciliary adenosine triphosphatase, dynein (*14*). Therefore, it is likely that the stimulatory effect of Ca^{2+} influx is due not to a direct action of Ca^{2+} on the ciliary apparatus, but is due instead to an indirect effect of Ca^{2+}, perhaps on one or more steps in the pathway of energy metabolism (*15*).

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11. The effect of La^{3+} is more apparent on the rate of repolarization than on the initial amplitude of the depolarization. This suggests that the ionic conductance changes that produce the upstroke are less sensitive to La^{3+} than those which maintain the depolarization. Differences between the early and later conductances that produce depolarization also occur in amphibian cardiac muscle. In this muscle tissue the plateau of the action potential appears to be produced in part by an increased calcium conductance that continues after the initial upstroke that is primarily due to a short-lived increase in sodium conductance [R. Niedergerke and R. Orkand, *J. Physiol.* **184**, 291 (1966); S. Hagiwara and S. Nakajima, *J. Gen. Physiol.* **49**, 793 (1966)].
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17. Work was supported by PHS grant NS-8364. This report is dedicated to Professor H. Kinosita on the occasion of his retirement from the University of Tokyo.
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