moved for analysis of DPG and for determination of hemoglobin. The samples were then centrifuged for 10 minutes at 3000 rev/min in a PR-2 centrifuge at room temperature. The plasma was removed, and a hemolyzate was prepared by the addition of equal volumes of distilled, deionized water. After thorough mixing, the hemolyzate was allowed to stand for an additional 10 minutes, and another portion was removed for subsequent determination of DPG content. The lysate was then centrifuged at 3500 rev/min at room temperature for 10 minutes; the clear hemoglobin supernatant was removed from the lower layers that contained stroma. The supernatant was then passed through a capillary tube (0.5 by 15 cm) containing a Pyrex wool plug to remove all residual red cell stroma. The effluent was centrifuged for 15 minutes at 3500 rev/min, and the clear hemoglobin solution was again sampled for its content of both DPG and hemoglobin.

Propranolol was administered to nonsmoking human volunteers in a dosage schedule of 10 mg every 4 hours for a total dose of 40 mg. Blood samples were obtained before the drug was administered, 2 hours after, and 24 hours after the initial dose. The samples were studied for the measurement of P_{50}, the total content of DPG in red cells, and the fraction of bound and unbound DPG in the red cell. The determination of DPG and P_{50} were performed by techniques previously described (*5*).

It was found, in 20 studies in vitro, that 69.7 ± 4.9 percent of DPG in the red cell was present in the stroma-free hemoglobin ("unbound") (Table 1). In the presence of propranolol with a final concentration of $3.3 \times 10^{-5} M$, all of the DPG was recoverable in the stroma-free hemoglobin. Changes in the ratio of "bound" to "unbound" DPG were observable at propranolol concentrations of $1.0 \times 10^{-5} M$. Incubation of red cells with propranolol in the concentrations tested produced no change in the total content of DPG in the red cell. The prior addition of epinephrine in equimolar concentrations completely prevented the "unbinding" effect of propranolol.

The administration of propranolol to the human volunteers produced observable effects within 2 hours of its initial administration (Table 2). By 24 hours after the start of drug administration, virtually all of the DPG in the red cell was unbound and the P_{50} had risen by a mean of 2.5 mm-Hg. A shift to the right of the P_{50} curve was observed in blood from each individual. The P_{50} increases ranged from 1.5 to 4.0 mm-Hg.

Table 2. The effects of propranolol administration to humans on red cell P_{50} and the percentage of "unbound" 2,3-diphosphoglycerate in the red cell. Results are expressed as the mean of the determinations obtained from blood samples of seven nonsmoking adults.

Time (hr)	DPG in the red cell (μmole per gram of hemoglobin)	P_{50} (mm-Hg)	"Unbound" (%)
0	15.8	28.5	76.1
2	15.6	30.2	96.2
24	15.7	31.0	97.5

Propranolol has been reported to be actively incorporated into the human erythrocyte (*6*). Our findings suggest that it produces conformational changes in the red cell membrane, resulting in the release of bound organic phosphate. The presence of adenosine triphosphate that is bound to the red cell membrane has been demonstrated (*7*). It would appear that a certain fraction of DPG in the red cell may also be normally bound to the membrane and does not interact with hemoglobin to alter the hemoglobin affinity for oxygen. If approximately 30 percent of the normal concentration of DPG in the red cell (5.0 μmole per milliliter of red cells) is not normally interacting with hemoglobin, the red cell has a potential reserve of DPG that, when "unbound," could increase the P_{50} by approximately 3.0 mm-Hg (*2*). The observation that epinephrine prevents the effect of propranolol on the red cell membrane strengthens the argument that the red cell is responsive to vasoactive agents (*8*), and suggests that the red cell may contain adrenergic receptors. The efficacy of propranolol in relieving the symptoms of angina pectoris may be mediated, in part, by the ability of the drug to cause more oxygen to be delivered to the myocardium as a consequence of the altered hemoglobin-oxygen affinity. These data represent the first observations, in humans, of a pharmacologic agent that can be used to alter the position of the oxygen-hemoglobin curve for potential therapeutic benefit.

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Acetylcholine: Possible Neuromuscular Transmitter in Crustacea

Abstract. *The tonic flexor muscles of the crayfish abdomen respond with a large depolarizing potential to acetylcholine iontophoresed onto a neuromuscular junction, but not to glutamate. Excitatory junctional potentials are abolished by* d-*tubocurarine and enhanced by a cholinesterase inhibitor. The membrane is depolarized and the junctional potentials are desensitized by excess acetylcholine. Thus acetylcholine is thought to be the neuromuscular transmitter.*

Although the presence of acetylcholine (ACh) and cholinesterase has been reported in crustacean nerves and muscles (*1*), respectively, crustacean neuromuscular junctions have previously not been thought to be cholinergic (*2, 3*). Instead, glutamate has been proposed as the excitatory transmitter at these junctions (*4*). I now present evidence that ACh may be the transmitter in at least one neuromuscular system of the crayfish.

The tonic postural flexor muscles in each hemisegment of the abdomen of the crayfish *Procambarus clarkii* are innervated by five excitatory neurons and one inhibitor (*5*). In most experiments the largest excitatory axon was selectively stimulated with trains of brief pulses delivered through a suction

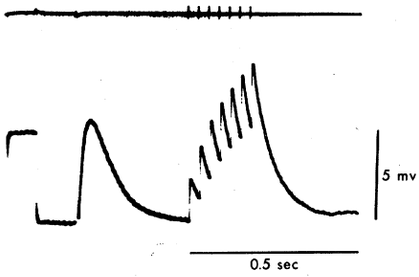

Fig. 1. The lower trace shows an intracellular sequence of a 5-mv calibration signal, an ACh potential, and summated EJP's. The ACh potential was obtained by passing current of 2×10^{-7} amp for 10 msec through a pipette containing 2*M* ACh. The upper trace shows monitored motor nerve spikes.

electrode placed on the nerve, and the axonal responses were monitored with a second electrode. Excitatory junctional potentials (EJP's) were recorded intracellularly from muscle fibers with the use of 3*M* KCl- or K citrate–filled glass micropipettes with impedances of 10 to 30 megohms. A second intracellular electrode was used in some experiments to deliver current pulses for measurement of membrane conductance changes. Acetylcholine was applied iontophoretically to the junction from an extracellular microelectrode containing 2*M* ACh by means of outward current pulses in the range of 1 to 5×10^{-7} amp for periods of from 1 to 30 msec. The junctions were localized by using the recording electrode in an extracellular search for presynaptic spikes followed by large negative responses. The negative responses, which signify a current sink, were maximized by careful electrode manipulation in order to localize the synaptic region. This recording electrode was then advanced to penetrate the muscle fiber, and the iontophoresis electrode was positioned extracellularly for maximum response in the same region. The placement of the iontophoresis electrode was extremely crucial since only a very restricted region of subjunctional membrane responded to ACh. Van Harreveld's physiological solution at *p*H 7.2 was used throughout.

Figure 1 shows a depolarizing ACh potential obtained by the above-described procedure. The effective quantity of ACh delivered (2×10^{-9} coulomb) is within the range used for iontophoretic stimulation of vertebrate motor end plates (*6*). Even in quantities several times greater, glutamate was without effect in other experiments in which the same procedure was used.

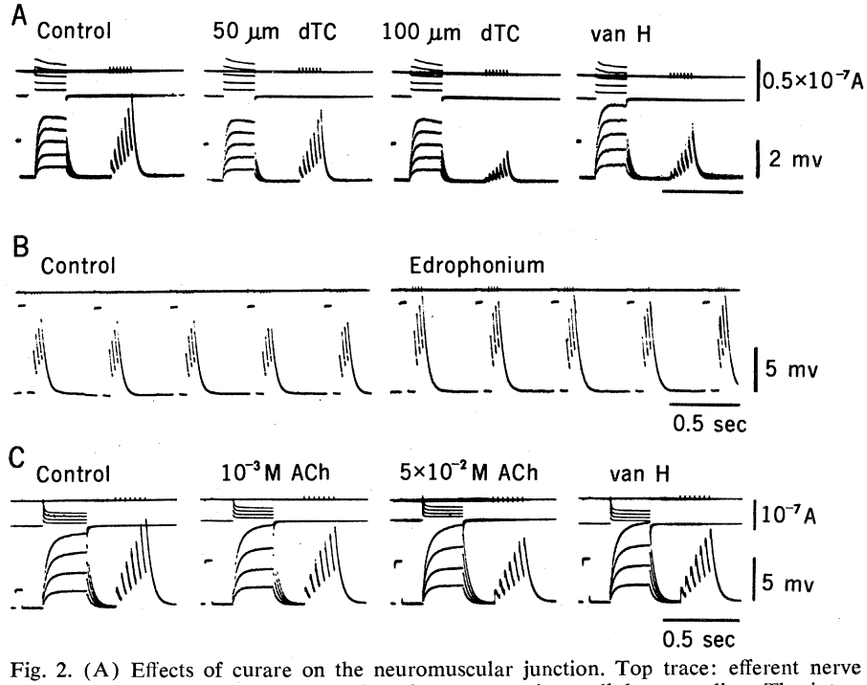

Fig. 2. (A) Effects of curare on the neuromuscular junction. Top trace: efferent nerve impulses; middle trace: current monitor; lower trace: intracellular recording. The intracellular record consists of a calibration signal, depolarizing response to current pulse, and summated EJP's. Each frame consists of five superimposed traces, taken at five different current levels. (B) Two segments of a continuous record taken before and after treatment with a cholinesterase inhibitor, edrophonium chloride. (C) Effects on the neuromuscular junction of bath perfusion with ACh. The same paradigm as in (A) is used at four current levels. Abbreviations: *dTC*, *d*-tubocurarine; *van H*, van Harreveld's physiological solution; *A*, ampere.

To explore further the possibility that the evoked EJP's are cholinergic, I studied the effects of facilitators and inhibitors of cholinergic transmission on the response of muscle fibers to nerve stimulation. *d*-Tubocurarine is commonly believed to act competitively for ACh receptor sites; Fig. 2A shows its blocking effect on evoked EJP's with bath perfusion. This effect is shown at two concentrations of *d*-tubocurarine, 50 μM and 100 μM (where 100 $\mu M = 7 \times 10^{-5}$ g/ml). These concentrations are comparable to those used to inactivate torpedo electroplaques [10^{-4} g/ml (*7*)], to abolish ACh-induced depolarizing potentials in snail cells [5×10^{-5} g/ml (*8*)], and to inactivate both H and D membrane responses to ACh in snail cells [10^{-4} g/ml (*9*)]. The responses to current pulses show that there is no change in membrane conductance when the EJP's are diminished in amplitude. The first three frames were taken at 15-minute intervals, and the fourth, showing the reversibility of the curare effect, 30 minutes after a wash with fresh van Harreveld's solution. There was no change in membrane potential during the experiment.

If ACh is the transmitter at this junction, then a cholinesterase inhibitor might be expected to enhance the EJP's (*10*). Figure 2B shows an augmentation of EJP amplitude produced by edrophonium chloride (Tensilon) within 2 minutes after 200 ml of a 10^{-4} g/ml solution was introduced into the bath. Data from this and other experiments show no conductance or membrane potential changes due to the perfusion.

Finally, bath perfusion with 5×10^{-4} to $5 \times 10^{-2}M$ ACh was used to demonstrate possible desensitization (*11*) of naturally evoked EJP's; the results are illustrated in Fig. 2C. A progressive diminution of EJP amplitude was produced by increasing concentrations of ACh. Although high concentrations of ACh do reversibly depolarize the membrane, the decrease in EJP amplitude was determined experimentally not to be attributable to this depolarization. Although these experiments do not completely rule out possible presynaptic effects, it seems likely that the decrement is at least partially due to postsynaptic receptor desensitization.

Others who have evaluated a possible transmitter role for ACh at crayfish neuromuscular junctions and come to negative conclusions used muscles of the claw, or employed behavioral tests.

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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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

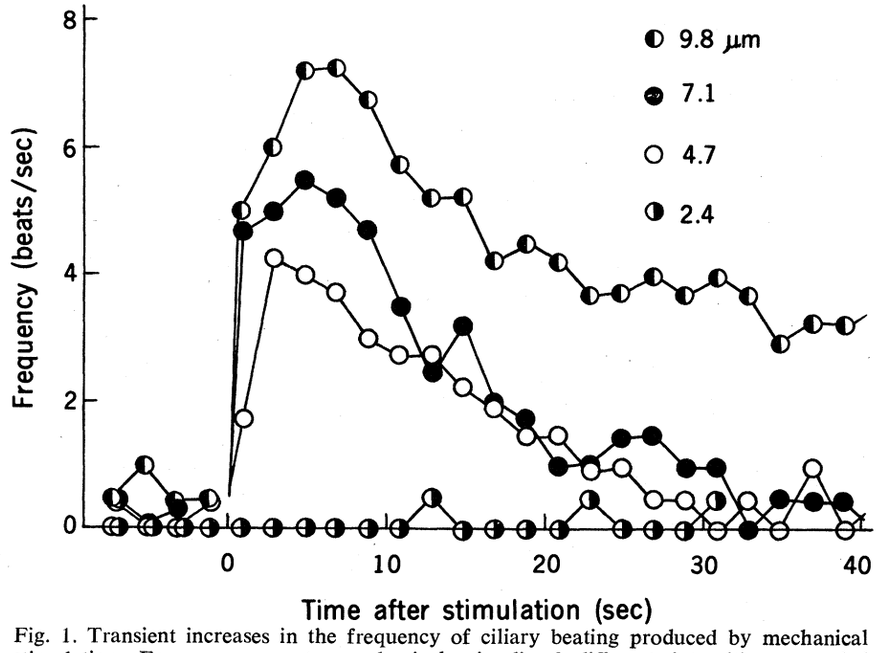

Fig. 1. Transient increases in the frequency of ciliary beating produced by mechanical stimulation. Four responses to mechanical stimuli of different intensities, recorded from the same cell, are superimposed. Spontaneous activity just prior to mechanical stimulation is shown to the left of time zero. 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.