The Distribution of Pre- and Postsynaptic Inhibition at Crustacean Neuromuscular Junctions

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ABSTRACT The relative contribution of pre- and postsynaptic mechanisms to peripheral inhibition has been analyzed in the abdominal slow flexor muscles of crayfish and lobsters. The conductance of the muscle fiber membrane may be increased to five or more times its resting value by repetitive stimulation of the peripheral inhibitory axon, and this effect accounts for all of the attenuation exerted by the inhibitor against excitatory junctional potentials. No "critical interval" has been found at which an inhibitory nerve impulse produces anomalously large reduction of a following depolarizing junctional potential; electrotonic depolarizations and junctional potentials are identically affected under all phase conditions. The presynaptic inhibitory mechanism is, therefore, absent in this system. In the dactyl opener muscle, on the contrary, most of the attenuation of excitatory junctional potentials is achieved presynaptically, though equally large postjunctional conductance changes are also seen (Dudel and Kuffler, 1961). The difference is correlated with a difference in the reflex operation of the two muscles. Reflex inhibition in the abdominal slow flexors is primarily central, whereas in the dactyl opener, inhibition is brought about by an increase in inhibitory nerve discharge frequency without central suppression of the single excitatory axon. The function of peripheral inhibition in the abdominal flexors is presumably to terminate residual depolarization by reducing the long time-constant of the muscle fibers.

INTRODUCTION

Inhibition at crustacean neuromuscular junctions, as in the mammalian spinal cord, may be brought about by two different mechanisms. One, postsynaptic inhibition, imposes a reduction in the response of postsynaptic membrane by increasing conductance of the ion(s) which have equilibrium potentials near the resting membrane potential. The other mechanism affects the presynaptic terminals instead, reducing the number of transmitter quanta released from them by a nerve impulse. Dudel and Kuffler (1961) showed that both mechanisms operate at junctions on the abductor muscle of the crayfish dactylopodite. Though the postsynaptic conductance change due to repetitive activity

of the peripheral inhibitor nerve is substantial, it accounts for only a small part of the reduction observed in concurrently generated excitatory junctional potentials (ejp's) when the inhibitory impulses are timed so as to arrive at a critical interval preceding excitatory ones. That the major part of the inhibitory effect is due to a reduction in presynaptic transmitter release was proved by Dudel and Kuffler, who demonstrated a drop in the quantal content of ejp's evoked during inhibitory nerve stimulation. Presynaptic inhibition is achieved by endings of the same axon that causes the postjunctional conductance change, whereas presynaptic inhibitory systems in the mammalian spinal cord involve interposed interneurons (Eccles, Kostyuk, and Schmidt, 1962). At crustacean junctions presynaptic inhibition occurs through a conductance increase at the excitatory terminals (Dudel, 1965 *b*), which would not necessarily produce lingering depolarization of the type found in mammalian afferent terminals.

The mixture of inhibitory effects exerted by the same efferent axon in the crustacean system raises questions about the integrative significance, distribution, and evolutionary origin of these two ways of achieving inhibition in neural systems. An opportunity to pursue the problem in a comparative way is presented by the fact that different crustacean neuromuscular systems achieve reciprocal reflex inhibition by radically different means. In the dactyl "opener," the homologue of the leg muscle studied by Dudel and Kuffler (1961), the inhibition that accompanies closing is exclusively peripheral. Discharge in the inhibitor axon increases dramatically (Bush, 1962), while that in the single excitatory axon is not centrally suppressed and, in fact, may even increase (Wilson and Davis, 1965). Tension is probably controlled by the ratio between the discharge frequencies of inhibitory and excitatory axons. In the slow abdominal flexor muscles of crayfish and lobsters, on the other hand, a very different mechanism is employed. Reflex inhibition involves the prompt central suppression of discharge in the five excitatory axons that innervate the muscle. The peripheral inhibitor, which innervates less than half the fibers in the muscle, usually fires only during the silent period of the exciters (Kennedy and Takeda, 1965 *b*). In such a system, central mechanisms clearly bear most of the burden of reflex inhibition; and since the central program seldom allows the appropriate phase relation between inhibitor and exciter discharges, the presynaptic inhibitory mechanism cannot have functional significance.

The following account describes experiments on the nature of peripheral inhibition in the slow abdominal flexor muscles of crayfish and lobsters. The conductance change in muscle fibers as a result of inhibitory nerve stimulation at different frequencies has been estimated, and excitatory and inhibitory axons were stimulated at various frequencies and in different phase relations in order to determine whether reduction in the amplitude of ejp's could be attributed entirely to postjunctional conductance change. Finally, observations

on the electrical responses of slow abdominal flexor and—for comparative purposes—dactyl opener muscles were made under reflex conditions. The results show that although in the claw opener the dominant mechanism of peripheral inhibition is prejunctional, that in the abdominal flexors is almost entirely postjunctional. The distribution of inhibitory nerve endings in these neuromuscular systems, therefore, is consistent with the centrally imposed reflex program used to achieve inhibition.

METHODS

Crayfish (*Procambarus clarkii*) were maintained as previously described (Kennedy and Takeda, 1965 *a*). Atlantic lobsters (*Homarus americanus*) were shipped by air from the East Coast and held in cold sea water aquaria. Ventral dissection of the third and adjacent abdominal segments in both species was accomplished so as to leave the origins and insertions of the superficial flexor muscles intact. The thin bundle of motor axons innervating the superficial flexor muscles was raised into a drop of oil with a micromanipulated silver recording electrode for *en passant* recording of impulse activity. Intracellular recording from muscle fibers was accomplished with KCl-filled microelectrodes mounted on a flexible silver wire. Intracellular signals were amplified by neutralized-capacitance preamplifiers (Bioelectric Instruments, Inc.) and displayed and recorded by conventional oscillographic means.

Stimulation of the axon bundle was accomplished after drawing it up into oil, in this case onto bipolar platinum electrodes, with the proximal portion of the root crushed. Various means were employed to activate selected single fibers from this bundle. Since the axons are very much smaller than those in most crustacean motor nerves, conventional splitting of the bundle by fine dissection was not possible as a routine. Instead, a tandem pair of stimulating electrodes was used, with the root partially crushed between them; or a stimulating microelectrode was moved from place to place on the bundle so that it activated particular axons selectively. By combining these methods it was usually possible to achieve independent stimulation of the peripheral inhibitor axon and of one or more separate excitatory axons.

For measurements of membrane conductance changes, a second potassium chloride or potassium citrate–filled microelectrode was inserted into the muscle fiber and used to deliver hyperpolarizing or depolarizing current pulses of varying intensity and duration. No attempt was made to make precise measurements of membrane constants in these experiments, which were intended only to give a measure of the relative change in resistance resulting from inhibitory nerve stimulation.

RESULTS

It became apparent at the outset of these experiments that there was a substantial difference between the process of peripheral inhibition in the abdominal slow flexor muscles and that studied earlier in the claw opener by Fatt and Katz (1953) and by Dudel and Kuffler (1961). The nature of this difference is suggested by Fig. 1, which shows responses of claw opener muscle fibers and superficial abdominal flexor muscle fibers to normal, ongoing bom-

bardment from the central nervous system in intact preparations. In A, the response from the claw opener muscle, it may be seen that a constant rate of discharge in the excitatory axon is accompanied by discharge at almost equal frequency on the part of the inhibitor axon (*cf.* Bush, 1962; Wilson and Davis, 1965). In Fig. 1A, the frequencies are so nearly constant that the rather considerable facilitation characteristic of both types of junctional potential is not a factor. It can be seen that when there is an appropriate time-relationship (arrow) between hyperpolarizing inhibitory junctional potentials (ijp's) and depolarizing ejp's the reduction in the amplitude of the ejp is considerable.

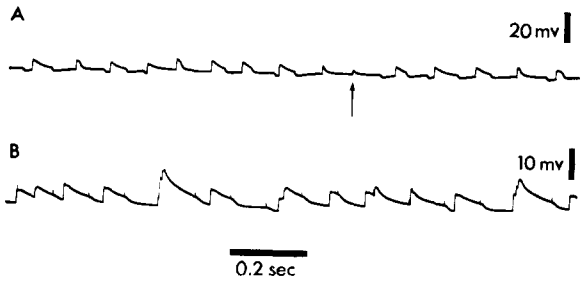

FIGURE 1. Spontaneous reflex activity recorded intracellularly from fibers of the dactyl opener muscle (A) and the slow flexor muscle of the third abdominal segment (B) in intact crayfish. In A, a section of record has been chosen in which the single excitatory axon and the inhibitory axon, which produce depolarizing and hyperpolarizing junctional potentials respectively, were active at approximately constant frequency but with shifting phase. The arrow marks a point of optimal interval at which the ejp is reduced to 25% or less of its normal amplitude by a nearly simultaneous ijp. In B, ejp's due to two different excitatory axons may be distinguished. Since normally the inhibitory axon is not active during excitatory reflex outflow, that axon was stimulated (artifacts preceding hyperpolarizing junctional potentials) at 10/sec. Although several appropriate intervals are found, there is no reduction of an ejp comparable to that seen in A.

This reduction is much greater than that found when the excitatory depolarization occurs at the peak of the inhibitory conductance change. Since the inhibitor in the slow flexor muscle system is normally not active during periods of excitatory bombardment (see below), ijp's were supplied in this record (Fig. 1B) by stimulating the inhibitory axon selectively at a frequency of 10 per sec. Thus ijp's were artificially interpolated into a train of ejp's elicited by two different motor axons. At no interval between an ijp and an ejp was there a marked reduction in the amplitude of the latter. This observation has been confirmed in a number of experiments in which inhibitory nerve stimulation at higher frequencies was superimposed on background excitatory bombardment. Though repetitive activity in the peripheral inhibitor can markedly accelerate the falling phase of ejp's, it has—at "physiological" discharge frequencies—much less influence than in the case of the opener muscle.

The relationship between the normal reflex discharge of the peripheral inhibitory axon and the several excitatory axons in the slow flexor muscle system is shown in Fig. 2. In both the records shown, an inhibitory natural stimulus (extension of the telson and uropods) was delivered at the point indicated by the arrows. In each case, the discharge of two or three excitatory axons stopped abruptly and was replaced by repetitive firing of a single fiber, the peripheral inhibitor. Fig. 2A, which illustrates simultaneous recordings of the motor branches on each side of the same segment, shows that the discharges of this axon on the two sides showed a close temporal relationship, a situation

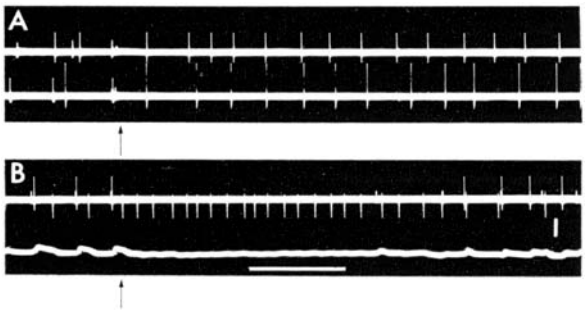

FIGURE 2. A. Simultaneous records from the posterior branches of the third root supplying the slow flexor muscles on the two sides of abdominal segment 3 (*Homarus*). B. Simultaneous records from the posterior third root branch on one side of abdominal segment 3 and (lower trace) from a microelectrode in one of the muscle fibers it supplies. At the arrow in each record, the telson and uropods were passively extended. The extracellular nerve records in both cases show repetitive discharge in a single axon and central inhibition of others. In B, it is seen that the activated axon produces hyperpolarizing junctional potentials, and the inhibited ones depolarizing junctional potentials. Time calibration, 0.5 sec; voltage calibration 30 mv, applies to intracellular trace only.

undoubtedly resulting from the fact that the inhibitory axons of the two sides are electrotonically coupled (D. Potter and M. Otsuka, personal communication). This coupling is never tight enough to produce a one-to-one discharge relationship, but does produce a tendency toward synchrony that will be the subject of future analysis. In Fig. 2B, a record from the bundle of motor axons is displayed with an intracellular record from a fiber in the slow flexor muscle that it innervates. This record shows that the unit which commenced firing at the delivery of the natural inhibitory stimulus was indeed the peripheral inhibitor, since it produced a train of small hyperpolarizing ijp's. Two of the axons which ceased firing during this period produced depolarizing excitatory junctional potentials. It is thus clear (*cf.* Kennedy and Takeda, 1965 *b*) that the central apparatus for driving this motor system includes arrangements for reciprocity between the discharge of the peripheral inhibitor and that of the five excitatory axons which innervate the same muscle. This

circumstance indicated that the presynaptic peripheral inhibitory mechanism shown to predominate in the claw opener system by Dudel and Kuffler (1961) could not serve a useful function in the slow flexor system, since the central nervous system seldom permits the required temporal relationship between inhibitory and excitatory events. The remainder of the experiments were directed to the question of whether the presynaptic mechanism was in fact absent.

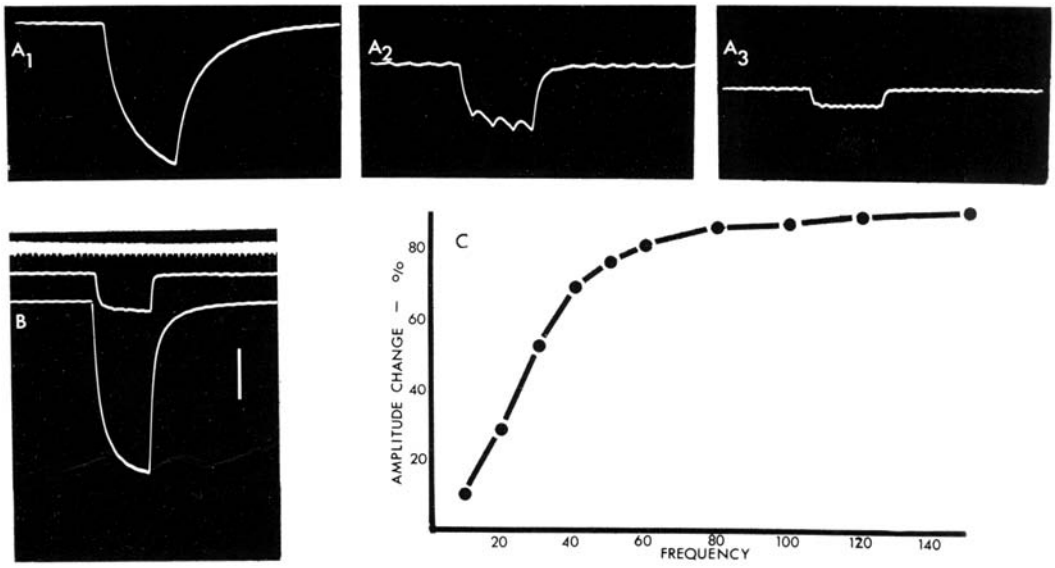

FIGURE 3. Effect of inhibitory nerve stimulation at various frequencies on hyperpolarizing current pulses. *Homarus* slow flexor fiber, K citrate-filled microelectrode used to pass current. A_1, current pulse alone; A_2, inhibitory axon stimulated at 30/sec; A_3, at 80/sec. The relative positions of the base lines in the three frames have been preserved. B. Effect on another preparation of stimulating the inhibitory axon at 120/sec; *Homarus*, KCl-filled current-passing electrode. Time marks, 10 msec; voltage calibration, 10 mv; these apply to both A and B. C. Plot of the per cent reduction in amplitude of a hyperpolarizing pulse as a function of inhibitory nerve frequency. Data from the muscle fiber shown in A.

It is quite clear that the peripheral inhibitory axon in the slow flexor system produces an impressive effect upon the conductance of the muscle fibers it innervates. Fig. 3 illustrates this change. In Fig. 3A, the effect of repetitive inhibitory nerve stimulation upon hyperpolarizing current pulses injected through a second microelectrode was assessed. A1 through A3 are the responses to inhibitory nerve stimulation at 0, 30, and 80 per sec. Fig. 3C shows a plot of the amplitude changes for these and other values of stimulation frequency in this preparation. It is of interest that the conductance change was nearly maximal at an inhibitory nerve frequency of 60 per sec; it appears that the

system is reasonably well adjusted to the physiological range of discharge frequencies, since we seldom observe the inhibitor axon to fire at higher rates than this under natural reflex conditions. Figure 3B is an example of the conductance change in a different preparation, in which a muscle fiber with a shorter time-constant was recorded from. In A, current was passed with a potassium citrate–filled microelectrode, and the ijp's—as was usual under these conditions—were consistently hyperpolarizing at the resting level and reversed their sign at a potential 10 mv or more above this. In B, a KCl-filled current-passing microelectrode was used and the ijp's were consequently depolarizing.

The postjunctional conductance change in these muscle fibers in response to inhibitory nerve stimulation (measured as the amplitude decrease of a hyperpolarizing current pulse) was comparable with that observed in the experiments of Dudel and Kuffler (1961) on crayfish opener muscle fibers, though precise quantitative comparisons are difficult because of possible differences in spatial decay. Dudel and Kuffler illustrate a drop to 30% of the resting value as a result of 130/sec stimulation of the inhibitor, and report other instances of tenfold changes in conductance. Our conductance increases were typically 80% or greater at stimulus frequencies of over 100/sec. Several experiments on crayfish indicate that their slow flexor muscle fibers are identical in this respect with those in the lobster. However, such effects are no guarantee that presynaptic mechanisms do not nevertheless predominate: even though Dudel and Kuffler's muscle fibers showed large conductance changes on inhibitory nerve stimulation, the quantitatively more important mechanism in attenuating ejp amplitude in that system is presynaptic.

Dudel and Kuffler demonstrated this in one set of experiments on a preparation in which the ijp's were depolarizing. These ijp's were combined with ejp's of sufficiently low amplitude that the sum of both depolarizations was still less than that necessary to reach the reversal potential for the ijp. Under such conditions, the two depolarizations should add together. In Dudel and Kuffler's experiments they did so at most intervals, but within a critical interval range—when the ijp preceded the ejp by 2 to 10 msec—there was a dramatic reduction in the amplitude of the latter, which could be attributed only to a prejunctional event. Fig. 4 shows an identical experiment on the slow flexor system. At all the sample intervals shown, approximately the predicted addition between the two depolarizations took place (the reversal potential for the inhibitory junctional potential in this fiber was approximately 10 mv depolarized from the resting level shown). The records shown in Fig. 4 are samples from an extensive interval series; at no point in the series was there a critical interval at which reduction in the ejp amplitude could be shown. The result was indicative of the fact that the presynaptic inhibitory mechanism is absent from this neuromuscular system.

This conclusion was confirmed by two other types of experiments. One of these, illustrated by Fig. 5A, involved a systematic interaction of inhibitory and excitatory stimuli in a number of different fibers in order to search for inhibitory actions that could not be accounted for simply on the basis of postsynaptic conductance change. In Fig. 5A, an excitatory and an inhibitory junctional potential were interacted at various intervals. The amplitude change in the ejp was about as large as any we have observed in such interactions; yet it had its maximum at a point where the peak of the ejp would coincide with the conductance maximum of the ijp. Similar experiments have

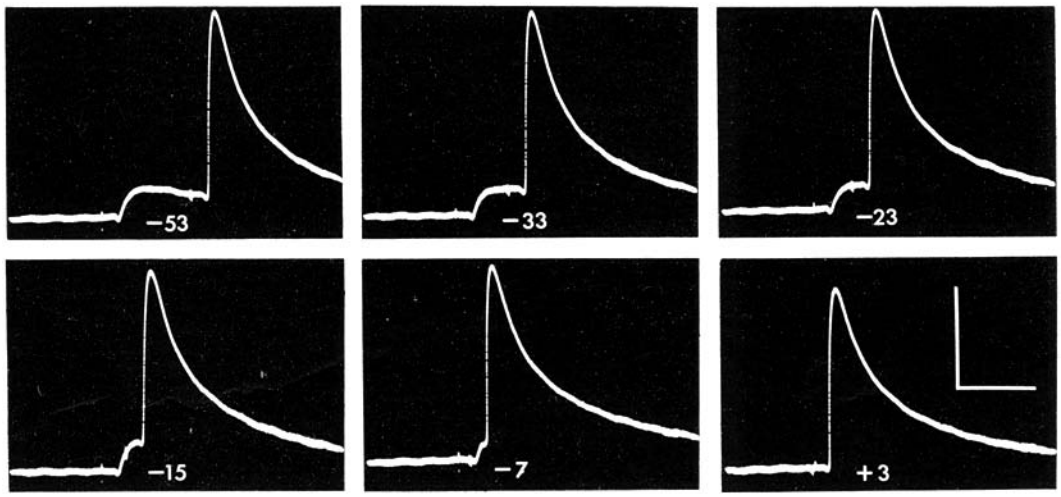

FIGURE 4. Addition of depolarizing ijp's and ejp's in a *Homarus* slow flexor fiber. The reversal potential for the ijp had been located, and was 10 mv lower than the resting potential. The intervals (I–E) are given below each record in milliseconds. Calibrations, 50 msec, 5 mv.

been performed on several occasions using two or even more of the different excitatory axons supplying a given muscle fiber, and the results have been similar for each excitatory input. That the attenuation is adequately accounted for by a purely postsynaptic mechanism is demonstrated by the parallel experiment shown in column B, where—instead of a real ejp—a depolarizing current pulse of approximately the same waveform was inserted in the same set of time relations with the ijp. As can be seen from the records, inhibitory nerve stimulation caused a reduction of the depolarizing pulse comparable to that of the neurally evoked ejp. A somewhat similar test was performed with repetitive inhibitory nerve stimulation. In Fig. 6 are shown the responses of a muscle fiber to an excitatory nerve impulse (early in the sweep) and to a hyperpolarizing current pulse (late in the sweep). The two responses

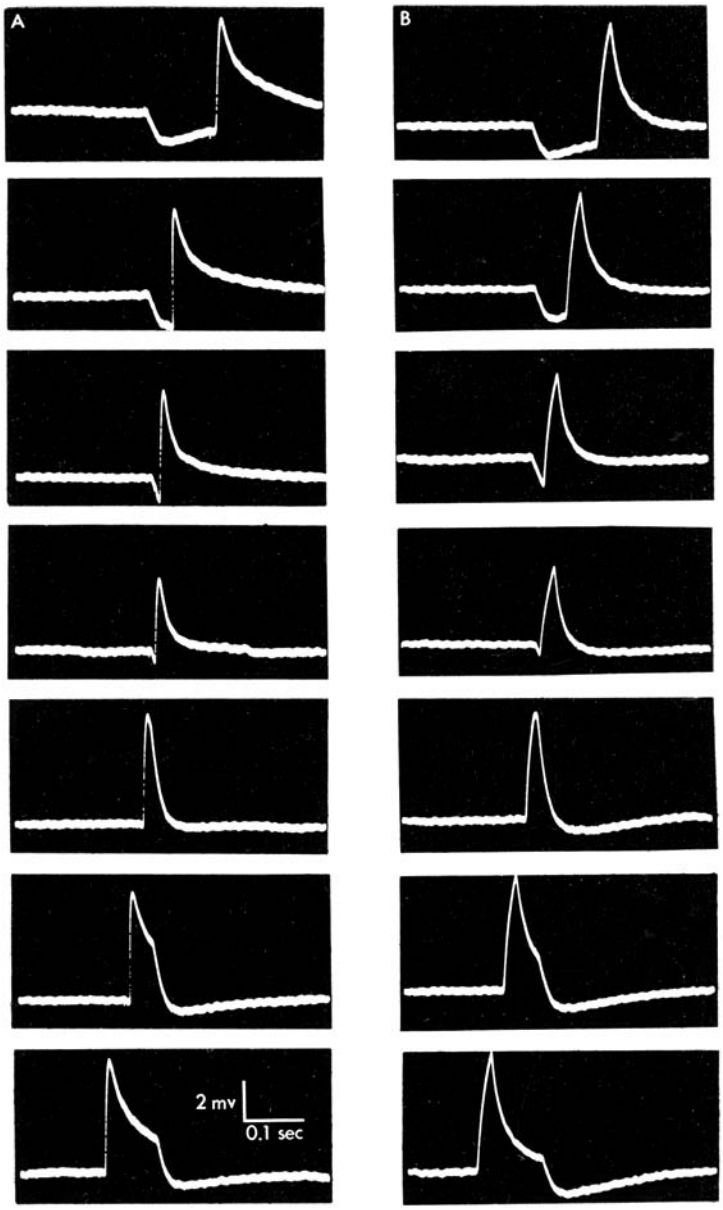

FIGURE 5. Interaction of ijp's with excitatory depolarizations in a *Homarus* slow flexor fiber. Left column, ejp evoked by stimulation of a single excitatory axon; right column, depolarizing current pulse delivered through a second, citrate-filled microelectrode.

were approximately equally reduced when the inhibitory nerve was concurrently stimulated at a frequency of 120 per sec.

DISCUSSION

These results are all in agreement in demonstrating that the entire range of inhibitory phenomena observable in the slow flexor muscle preparation can be attributed to the postsynaptic conductance increase evoked in muscle fibers by inhibitory nerve impulses. While the results do not rule out some very minor contributions by a presynaptic mechanism, they nevertheless are in sharp contrast to the situation at neuromuscular junctions of the claw opener, where properly timed inhibitory impulses reduce ejp's by 80% or more (*cf.* Fatt and Katz, 1953; Dudel and Kuffler, 1961).

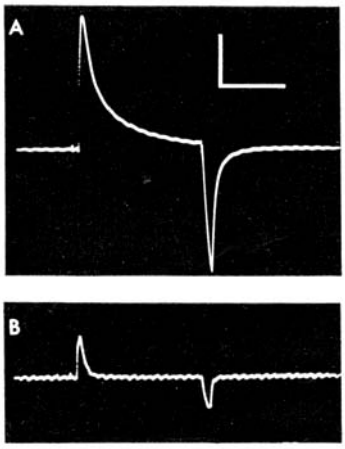

FIGURE 6. Comparison of the effect of repetitive inhibitory axon stimulation (120/sec) upon ejp's (first, depolarizing responses) and hyperpolarizing current pulses (second responses) delivered through a second, KCl-filled microelectrode. *Homarus* slow flexor fiber. Calibrations, 100 msec, 5 mv.

The absence of the presynaptic mechanism is in accord with the organization of reflex influence upon the system of efferent fibers that controls the slow flexor muscles. Whereas in the motor system of the claw opener excitatory and inhibitory nerve impulses concurrently bombard the muscle fibers in all possible phase relations, central mechanisms in the slow flexor system ensure almost total reciprocity between activity in the peripheral inhibitor and in the five excitatory motor axons. Our experiments have now shown that these two fundamentally different modes of reflex control have important peripheral as well as central aspects.

The function of the peripheral inhibitor in the slow flexor system would appear to be exclusively involved with reducing the long membrane time-constant of these large fibers. The slow decay of depolarization resulting from summating, facilitating, ejp's would be expected to produce a slow return of tension to 'resting" levels; this expectation has been substantiated by recordings of tension in small bundles of fibers under reflex conditions. The inhibi-

tory discharge that occcurs during the excitatory silent period in reflex inhibition would, in this view, have the function of speeding the termination of residual depolarization and, hence, of tension—in effect, of converting the system temporarily to a more phasic one. It is, however, not clear why this particular system of muscle fibers employs this reflex mechanism and that of the claw opener such a different one. One of the differences between the two systems is that the claw muscle receives only a single excitatory axon and the slow flexor muscle a total of five; within the latter muscle, individual fibers receive an average of two or three excitatory axons, and may be innervated in rare cases by all five. This multiplicity of excitatory innervation provides a delicate control over tension; it may be that a presynaptic inhibitory mechanism is impracticable in a situation in which the single inhibitory axon would have to "cover" a much larger number of excitatory endings with its own terminations. Also, in the claw, the excitatory axon is shared with the stretcher muscle of the carpo-propodite joint. The specific opener inhibitor is the only means of separate control of these two limb segments, and may thus require the extra measure of effectiveness provided by the presynaptic mechanism.

The results also naturally generate some speculation about the relationship between excitatory and inhibitory endings and the muscle fiber membrane. Dudel and Kuffler (1961) mentioned the two alternative possibilities that the presynaptic inhibitory effects could result either from the spread of transmitter from unspecialized inhibitory endings on muscle to adjacent excitatory terminals, or from the action of specific inhibitory branches upon excitatory terminals. The present results would suggest that the endings in the claw system are more likely to be specific. As judged by the magnitude of the conductance change, the inhibitory innervation of slow flexor muscle fibers is at least as rich as that of claw opener fibers. There would seem to be an equal opportunity for "random" spread of inhibitory transmitter from inhibitory to excitatory endings in this case as in the claw, yet no effect is found. To make this view completely convincing, however, it would be necessary to show that excitatory terminals in the slow flexor system possess pharmacological sensitivity to the inhibitory transmitter. The demonstration that certain compounds (*e.g.*, β-guanidinopropionic acid) selectively block the presynaptic inhibitory effect (Dudel, 1965 *a*) provides an opportunity to test this question directly.

The authors are grateful to Mrs. Philip C. Hanawalt for unfailing technical assistance.
This work was supported in part by grants from the Air Force Office of Scientific Research (AF-AFOSR G2-147) and the National Institute of Neurological Diseases and Blindness, United States Public Health Service (B-2944).
Received for publication 9 July 1965.

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