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INHIBITION AT NEUROMUSCULAR JUNCTIONS IN CRUSTACEA

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Inhibitory phenomena at neuromuscular junctions of skeletal muscle are to be regarded, in the present state of our knowledge, as the special prerogative of the Crustacea. For though in certain other invertebrates peripheral inhibition may occur, in no other instance is it yet proved that such inhibition truly affects the junctions and is not due to the inhibition of peripherally located motor neurones. For the Crustacea two classes of inhibition have been proposed, inhibition by attenuation of the electrical events which (presumably) lead to contraction, and uncoupling of the process interposed between membrane potential changes and contraction of the muscle substance. The latter, in which the electrical potentials are not reduced, was termed simple inhibition by Marmont & Wiersma (1938) and regarded by them as the more important physiological mechanism. They termed the other type, in which the electrical activity is attenuated, 'supplemented inhibition', preferring to regard the reduction of the electrical activity as a secondary event not related to the primary action of the inhibitory process. Kuffler & Katz (1946) and Katz (1949), in reviewing the processes of neuromuscular transmission in Crustacea, reversed the emphasis. They termed supplemented inhibition α-inhibition and simple inhibition β-inhibition.

The biophysical events associated with the attenuation of the electrical potentials were studied with the aid of intracellular electrodes by Fatt & Katz (1953). They did not study the mechanical concomitant of inhibitory action at the same time. They concluded that the results of their experiments threw considerable doubt on the interpretation of inhibitory action as a direct one on the contractile process.

Biedermann (1887) had found polarization of the muscle during stimulation of the inhibitory nerve fibre of the crayfish. Wiersma & van Harreveld (1935), Serkoff (1936) and Kuffler & Katz (1946) failed to confirm these findings. Nor did Fatt & Katz (1953) in the above series of experiments find any consistent inhibitory potentials, although they were using internal electrodes. They

did, however, make the important observation that inhibitory nerve impulses lead to changes in the permeability of the muscle-fibre membrane, because when the membrane potential was artificially raised by passing polarizing current across it, the inhibitory impulse gave rise to a depolarization of the membrane. Conversely, when the membrane potential was lowered, the potentials were reversed in sign, i.e. polarizing, although possessing exactly the same time course.

On the assumption that the normal resting potential is the result of a Donnan equilibrium potential for the system, and that the potential is at the potassium and chloride equilibrium potential level, any increase in permeability to K^+ or Cl^-, which is in effect a lowering of the resistance in series with the e.m.f., will not change the potential appreciably. But if the potential is displaced either above or below the potassium and chloride equilibrium potential level a decrease in the series resistance will result in the appearance of ionic current flow tending to restore this potential.

Fatt & Katz (1953) observed that since the inhibitory action caused such an increased permeability the excitatory junctional potentials decayed more quickly during inhibitory action. This resulted in a diminution of the (total) depolarization during inhibitory action, even at timing sequences in which the junctional potentials were not individually reduced in amplitude. In this way it might be possible to account for simple, or β-inhibition, for under these conditions the mean level of membrane potential may not be reduced to a level at which contraction occurs, although junctional potentials are present which, in the absence of inhibition, would cause a contraction

In this suggestion of Fatt & Katz there is implicit the hypothesis that contraction in crustacean muscle fibres is linked to the mean membrane potential, starting at a threshold level and presumably increasing with increasing extent of depolarization. In the taenia coli of the guinea-pig it has been shown that tension is inversely proportional to the mean membrane potential (Bülbring, 1956) and in the slow skeletal system of the frog, also, a similar relationship applies (Kuffler & Vaughan Williams, 1953). Thus it may be a common feature in muscle physiology. The hypothesis, for which certain findings in the preceding paper (Hoyle & Wiersma, 1958*a*) speak, is examined in the third paper (Hoyle & Wiersma, 1958*b*).

The aim of this second series of experiments was to study inhibitory actions with the aid of intracellular electrodes in a number of species, since it was known (e.g. Wiersma & Ellis, 1942) that large species differences are present concerning the types of inhibition which can be obtained. With outside leads many preparations fail, under any circumstances, to show supplemented (α) inhibition, in contrast to the preparations used by Fatt & Katz (1953). In addition, the events of inhibition have been studied during both slow and fast motor axon stimulation of doubly-motor-innervated muscles.

MATERIAL AND METHODS

The preparations used to study inhibition against single excitor axons were the openers of *Cambarus clarkii*, *Cancer anthonyi* and *Panulirus interruptus* and the stretchers of *Cancer antennarius* and *Panulirus*. Preparations used in the study of inhibition against both slow and fast excitor axons were the closers of *Cancer antennarius*, *Cancer anthonyi* and *Panulirus*, the benders of *Cancer antennarius* and *Panulirus* and the extensor of *Panulirus*. The opener and stretcher muscles of the crabs (*Cancer*) receive two inhibitor axons, a common inhibitor, which they share, and a specific inhibitor.

Single axons were isolated in the majority of preparations and stimulated separately. In some preparations small bundles of axons containing only one of the desired axons could be prepared, though only in the crayfish could this be done consistently for all axons wanted. In other preparations it depended upon the fortuitous grouping of axons in different bundles. A bundle is always to be preferred to an isolated nerve fibre since the latter is naturally very readily damaged. Other details were the same as those described in Hoyle & Wiersma (1958*a*).

RESULTS

Inhibitory potentials

We have found, in some muscle fibres of every muscle which we examined closely, electrical potentials associated with stimulation of the inhibitory axon (or axons) alone. These are too small to be visible at very low frequencies of stimulation in most instances, though a very few cases have been seen of measurable potentials occurring in response to stimulation by a single shock, e.g. potentials of not less than 0·1 mV. Some muscles showed responses in every fibre penetrated. Ordinarily we examined inhibitory potentials whilst stimulating the inhibitory nerve fibre at a frequency of 30/sec. Both negative (hyperpolarizing) and positive (depolarizing) potentials have been observed.

The time course of the inhibitory potentials is always slower than that of the excitatory junctional potentials. Individual differences in time course of inhibitory potentials are much less pronounced than those of excitatory ones. Likewise different muscles of different genera have inhibitory potentials of quite similar time course, although their excitatory ones may differ greatly in this respect.

Opener of Cambarus

We have studied inhibition intensively in the opener of the claw. In those animals examined in the autumn (October–December) most of the inhibitory potentials observed represented an increased negativity inside the muscle fibre, i.e. they polarized the membrane. This was the case in fibres of quite different resting membrane potentials, from 48 to 72 mV. The maximum magnitudes of individual inhibitory potentials at 30/sec were 0·7 mV and the minimum just at the limit of discernibility (*ca.* 0·1 mV). During repetitive stimulation the inhibitory potentials grew rapidly to their full height. The larger ones showed very little further increase in magnitude. They summed to polarize the membrane by various amounts. The extent of the total

polarization also varied with the frequency of stimulation, reaching its maximum at about 60/sec. The extent of this polarization was as little as 1 mV in some fibres, as large as 15 mV in others. The larger extents of polarization always occurred in fibres having an initially low resting potential, but apart from this there was no correlation between the magnitudes of the potentials. As the muscle fibre polarizes under the influence of the inhibitory stimulation, the individual inhibitory potentials become reduced in height.

Later in the autumn and in the early part of the new year we began to encounter fibres in which the inhibitory potentials were of the opposite sign, the internal lead going positive, the membrane depolarizing. There was also a small degree of summation in these fibres, leading to not more than 4 mV depolarization. In the early spring these depolarizing potentials were the only ones encountered and they were found in most of the muscle fibres penetrated. The range of resting potentials observed was very much narrower at this time, most fibres having potentials between 60 and 70 mV. Only in the early summer were polarizing potentials encountered again. Whether these variations represent a normally occurring seasonal event or whether they also depend on the condition of the laboratory-kept crayfish, which may well have been best during the early spring, is as yet undecided.

The inhibitory potentials were further investigated by inserting a second internal micro-electrode into the same muscle fibre and passing polarizing or depolarizing current across the membrane whilst observing the inhibitory potential changes.

Raising or lowering the membrane potential can lead to the abolition or reversal in sign of the potential or an increase in its magnitude (Fig. 1). A typical example of the relationship between membrane potential and inhibitory potential in a fibre having an inhibitory potential of large size is shown in Fig. 2. The membrane potential at which the inhibitory e.m.f. is zero we will term the 'change-over' potential. For crayfish examined in spring and early summer this was remarkably constant at about 58 mV.

Those fibres which did not show any potential change on inhibitory stimulation could sometimes be made to show one by passing polarizing current, as Fatt & Katz (1953) found in *Carcinus maenas* and *Eupagurus bernhardus*. But these fibres did not all have similar resting potentials. Also, the inhibitory potentials were very small in these fibres and it was not possible to determine the change-over potential with any degree of accuracy. Some muscle fibres showed no inhibitory membrane potential change even when polarized or depolarized by up to 60 mV.

Cancer antennarius

Inhibitory potentials have been observed in the opener, stretcher, closer, and bender muscles. These have almost all been polarizing. They were smaller

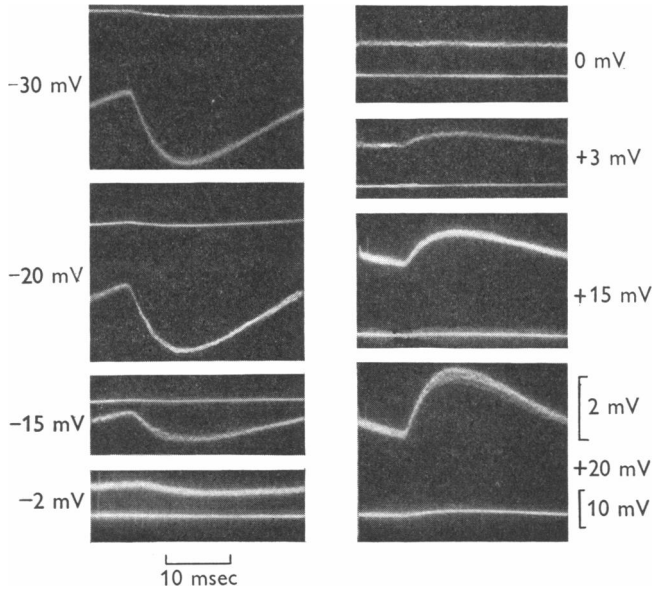

Fig. 1. The inhibitory potential recorded at different levels of membrane potential; opener of *Cambarus*. The resting potential of the muscle fibre was 58 mV; the figures beside each trace show by how much it was altered. One trace (with small deflexions) was recorded with direct-coupled amplification; the other with capacity coupling at higher gain. Amplitude indicated by the scales at lower right. Frequency of stimulation 30/sec.

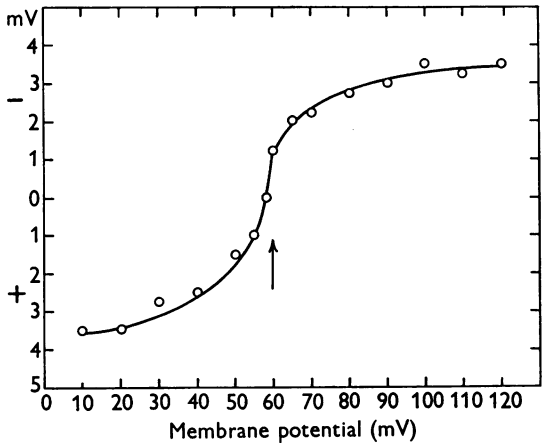

Fig. 2. Graph of the relationship between the magnitude of the inhibitory potential and the membrane potential in a muscle fibre of the opener of *Cambarus*. The arrow indicates the resting level of membrane potential. Frequency of stimulation 30/sec.

than those observed in *Cambarus* and did not achieve polarizing plateaux in excess of about 5 mV. Special interest is associated with the opener and stretcher muscles, since as in all crabs these receive two inhibitors. One of the two is a specific inhibitor which innervates only the one muscle. The other is a common inhibitor and it is this axon which also inhibits the closer and bender muscles. The specific inhibitor is slightly more efficient in causing mechanical inhibition than the common, i.e. a lower frequency of stimulation just suffices to inhibit the contraction completely (Wiersma & Ellis, 1942). Potential changes of the polarizing kind have been observed in response to stimulation of the common inhibitor but they were all very small. The majority of muscle fibres penetrated failed to show any potential change even when artificially polarized or depolarized by up to 40 mV. By contrast, the specific inhibitor usually showed a potential change, either normally, or during altered membrane potential, thereby indicating that the transmitter action was associated with an increased conductance of the membrane.

Cancer anthonyi

Both polarizing and depolarizing potentials were observed in this species (Fig. 3). These included the largest single responses observed in any of the muscles examined (Fig. 4). There was no facilitation when the responses were spaced close together, but the summation was complete. At higher levels of membrane potential, responses to both impulses were smaller and the second one appreciably smaller than the first. The maximum over-all polarization obtained was about 10 mV. The change-over potential was 60 mV.

The opener and stretcher muscles were examined in regard to both the common and specific inhibitors. Similar results were obtained to those in *C. antennarius*, namely that the common inhibitor evokes only minute or no conductance changes in these two muscles. It should be noted that this same inhibitor is reponsible for definite inhibitory junctional potentials in the closer and bender.

Panulirus

Inhibitory potentials of appreciable magnitude (0·2–0·6 mV) have been observed in closers, openers, stretchers and benders. Only very small potentials (0·1 mV) were found in the extensors. The change-over potential was determined for one stretcher muscle which had a mean membrane potential of 58 mV; it was also 58 mV. But fibres with a higher resting potential than 58 mV did not give positive (depolarizing) inhibitory potentials. We have found fibres with resting potentials of more than 80 mV and some of these had inhibitory potentials, but they were polarizing, and the change-over potential in these fibres must have been almost 85 mV. It would seem that in *Panulirus* the change-over potential is always a little higher than the membrane potential and that it can have a wide range, from about 50 mV in some fibres to 85 mV

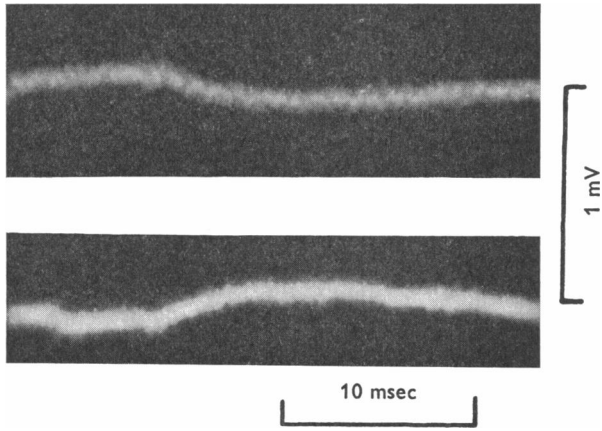

Fig. 3. Inhibitory potentials recorded from two adjacent muscle fibres of *Cancer anthonyi*. The two muscle fibres had similar resting potentials (60 mV).

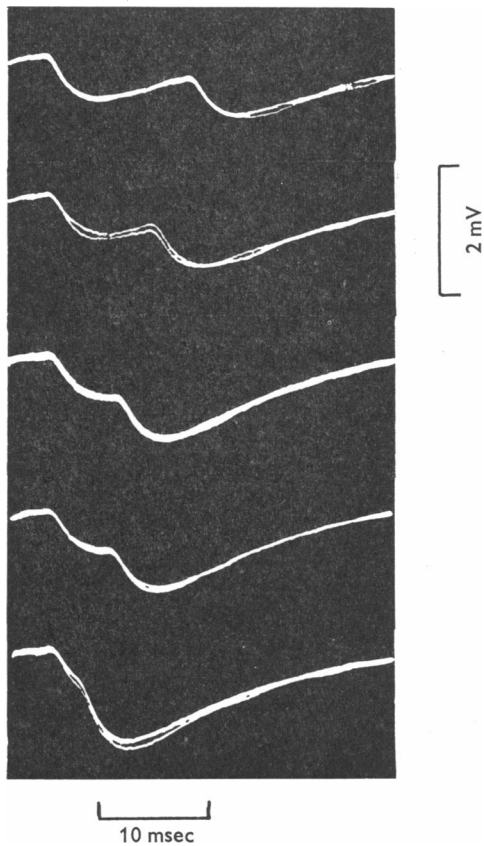

Fig. 4. Summation of inhibitory potentials. Paired stimuli were applied to the specific inhibitor axon of *Cancer anthonyi* opener muscle at progressively shorter intervals. Intracellular recording of unusually large potentials.

in others. In many cases, following repetitive inhibitory stimulation which raised the resting potential by several millivolts, the return to the original resting potential was very slow. In a few instances the higher level of membrane potential appeared to be retained indefinitely.

Simple or β-inhibition

Complete mechanical inhibition in the absence of reduction in the peak amplitude of intracellularly recorded junctional potentials has been observed in the openers and stretchers of *Cancer* for both common and the specific inhibitors; the opener, closer, bender and extensor muscles of *Panulirus* and the opener of *Cambarus* against the single or both slow and fast excitor axons. In some of these cases (common inhibitor of *Cancer*, all muscles of *Panulirus*) most fibres penetrated did not show any reduction, no matter what the 'timing' of the inhibitory against the excitatory impulses was; in the others absence of reduction occurred only when the inhibitory impulses arrived late.

In the extensor of *Panulirus* mechanical inhibition became incomplete when the fast motor axon and the inhibitor were stimulated at frequencies above 60/sec. Even increasing the inhibitory frequency above this value did not lead to a complete suppression of the contraction; in spite of the fact that there is no 'spiking' in the muscle responses. In other fast systems, like that of the closer of the claw of *Cambarus* or the 'closer' of the walking leg of *Pachygrapsus*, complete mechanical inhibition cannot be obtained at any frequency ratio. These are muscles which give some large spike responses. Evidently inhibition is effective against excitation afforded by junctional potentials and small spike potentials, but not against that afforded by large spike potentials. In one or two cases inhibitory action may not be able to work completely effectively against really large junctional potential excitation. This is probably an expression of the failure to reach a quantitatively great enough inhibitory action to suppress the excitatory one. By lowering the frequency of inhibitory impulses mechanical inhibition can always be made partial against a given excitatory frequency. In certain cases, however, the reason for the partial failure of inhibitory action is the long refractory period of the inhibitory nerve fibre (Wiersma & Ellis, 1942) rather than a quantitatively inadequate inhibitory action.

Supplemented or α-inhibition

The finding that mechanical inhibition is complete in the absence of any reduction in amplitude of the j.p.s means that when there is a reduction, as in supplemented inhibition, the action might be an incidental phenomenon lacking in functional significance. Inhibition in this peripheral system is a graded process, recognized by the degree of failure of the mechanical contraction during the continued excitation of the motor fibre. In order to be

interpreted as a second type of inhibition, the attenuation of the junctional potentials during supplemented inhibition would have to produce an additional effect above simple inhibition on the mechanogram. Such an influence could appear (*a*) by making it possible for just complete inhibition to occur at a lower ratio of inhibitory:excitatory impulse frequency or (*b*) by increasing through supplemented inhibition the rate of fall of tension at the onset of inhibitory action.

In our experience (see also Marmont & Wiersma, 1938) we have failed to find any evidence for either (*a*) or (*b*) in any of the systems examined. Attention should, however, be drawn to the experience of Kuffler & Katz (1946) on an

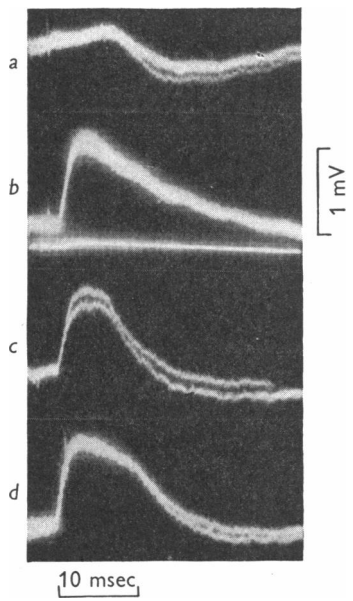

Fig. 5. Action of common inhibitor of *Cancer anthonyi* in muscle fibre of the opener. Large inhibitory potential (*a*) did not attenuate excitatory potential (*b*) but summed with it algebraically (*c*, *d*). Frequency of stimulation 30/sec.

Australian 'crayfish' opener. Although they found no effect of timing at higher frequencies, they did find one at 10 and 20/sec, when supplemented inhibition gave a more rapid decay of tension than simple inhibition.

Supplemented inhibition cannot be obtained, as stated in any of the muscles of *Panulirus*, which are as readily inhibitable mechanically as those of most other species. In most species where it is present it can be obtained only in the opener and stretcher muscles, although we have encountered it, occasionally, in other muscle fibres, e.g. in the closer of *Cancer antennarius*, in which a clear reduction of the junctional potentials, either fast or slow, did occur.

There is an interesting difference between the common and specific inhibitors of the opener muscles of crabs in regard to attenuation of excitatory potentials. It will be recalled that the common inhibitor has very little effect on membrane conductance in these muscles, as judged by the small size of the inhibitory potentials. The common inhibitor never attenuated the junctional potential by more than 5% at optimal timing (Fig. 5), whereas the specific

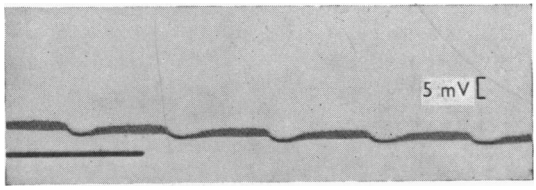

Fig. 6. Alternate simple (β) and supplemented (α) inhibition recorded from a muscle fibre of the opener of *Cambarus*. The excitatory and inhibitory stimulations were set at very slightly different stimulation frequencies to obtain a slow periodic change in the timing of the arrival of the impulses. The dark line gives the horizontal axis. Note that there is a slow polarization throughout. Stimulation frequency 30/sec. Mechanical inhibition was complete throughout.

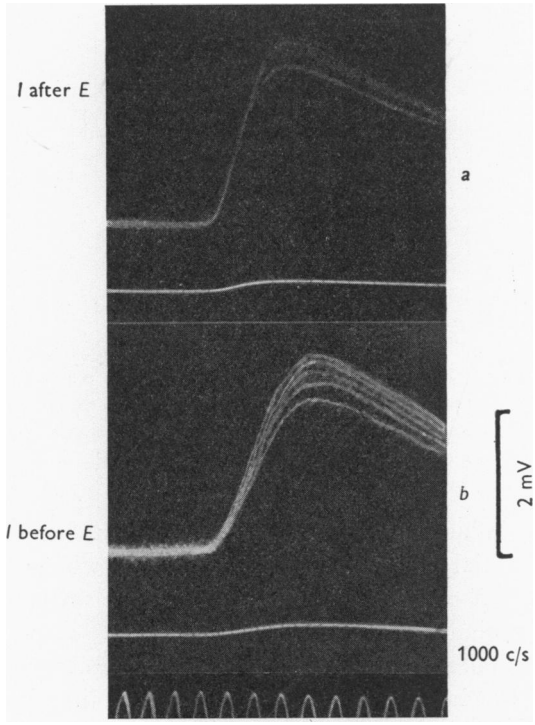

Fig. 7. Rates of rise of excitatory potentials during early and late supplemented inhibition. (*a*) late arrival cuts tops off the excitatory potentials; (*b*) early arrival causes slowing of rate of rise and introduces fluctuations in it. Opener of *Cambarus*.

inhibitor could attenuate the excitatory potential in the same fibres by as much as 70%.

As is well known, attenuation of the junctional potentials, where present, occurs only when the inhibitory impulse arrives at about the same time as the excitatory one (Fig. 6). It is maximal when the inhibitory impulse arrives just before the excitatory one. This phenomenon has been studied in detail by Fatt & Katz (1953) and we have little to add to their findings. We did, however, make a closer study of the effects, when the inhibitory impulse arrives too late to produce maximal reduction of the junctional potential,

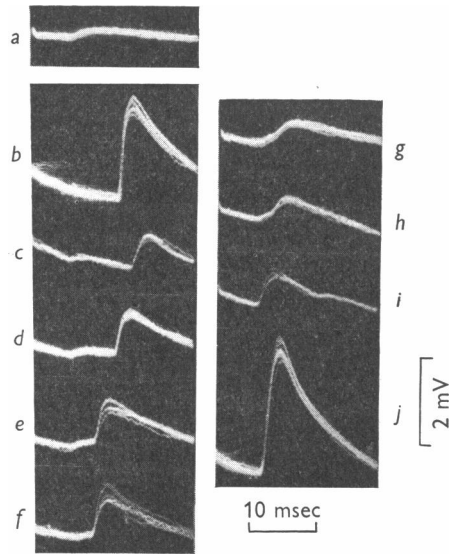

Fig. 8. Supplemented (α) inhibition in a muscle fibre of *Cambarus* opener which showed depolarizing inhibitory potentials. (*a*) Inhibitory potential alone; (*b*) excitatory junctional potential alone; (*c*)–(*h*), progressively closer timing of inhibitory and excitatory potentials; (*i*) late arrival of inhibitory potential—note summation of depolarizations; (*j*) excitatory potential alone at end of inhibition. This is larger than in (*b*) through the facilitatory process taking place notwithstanding the inhibitory stimulation.

compared to the influence when it arrives too early. In the first instance the junctional potential starts to rise at normal speed, which changes abruptly so that the top is cut off; in the second instance the rate of rise is reduced, especially at the beginning (Fig. 7).

In contrast to the observations of Fatt & Katz we did not always find that a late arrival of the inhibitory impulse caused the junctional potential to decay more rapidly. This does occur in some muscle fibres, but in others there is no effect and the decay may even be slowed down by the inhibitory impulse, though these fibres did not differ in the amount of attenuation at optimal

time interval. The decay was always slowed down in fibres which gave depolarizing inhibitory impulses (Fig. 8*i*). In these last fibres attenuation occurs in spite of the fact that the two potentials have the same sign.

Inhibitory transmitter substance

It was suggested by P. Fatt (1957) at the British Association meeting in 1957 that there is a common inhibitory substance in some crustaceans and that it may be γ-amino butyric acid. We have applied this substance in concentrations of 10^{-9}–10^{-3} to several preparations of crayfish and crab opener muscles. It did not produce mechanical inhibition in any of them, nor did it raise the resting potential in muscle fibres in which inhibitory action had this effect.

DISCUSSION

The presence of inhibitory potentials of opposite signs in different animals of the same species or even in different muscle fibres of the same muscle raises several problems. Polarizing inhibitory potentials were to be expected: they have been reported for a number of situations. Thus Brock, Coombs & Eccles (1952) found them present in mammalian motoneurones, Kuffler & Eyzaguirre (1955) in the sensory nerve cell of the stretch receptor of the crayfish, del Castillo & Katz (1955) and Hutter & Trautwein (1956) in the muscle fibres of the sinus venosus of the frog heart. Also, polarizing potentials in response to nerve stimulation have been found in muscle fibres of the jumping leg of the locust (Hoyle, 1955). By contrast, there is not the same precedence for depolarizing inhibitory potentials. Depolarizing potentials occurring naturally during stimulation of one of the inhibitory axons supplying a crustacean stretch receptor have been found (Kuffler & Eyzaguirre, 1955), and they can be produced artificially by changing the membrane equilibrium with polarizing current. This is also the case with the motoneurone of the cat (Coombs, Eccles & Fatt, 1955). But in both these instances the inhibitory potentials behave like excitatory ones when they are depolarizing, and give rise to spikes. Whether the transmitter action is excitatory or inhibitory depends on the membrane potential change which it evokes.

The situation is not so simple in crustacean muscle and it will be necessary to discuss it in some detail. Reversal of sign of the inhibitory potentials of crustacean muscle when the membrane potential is artificially raised was found by Fatt & Katz (1953). Reversal was obtained also in our investigations by raising the membrane potential in the case of polarizing potentials, and by lowering it in the case of depolarizing ones. This suggests that the inhibitory substance causes a decrease in the resistance in series with the e.m.f. of the membrane. But this has to be reconciled with the observation that the normal inhibitory potentials can have either sign. One possible explanation is that

there are two principal components of the membrane potential, both due to diffusion potentials, which differ in magnitude by a few millivolts only. At different times one or the other determines the membrane potential. Then if the inhibitory transmitter causes increased permeability to only one ion, or to both but to different extents, the potential evoked will be positive or negative according to whether the more freely moving ion is also the dominant or the minor component of the membrane potential at the time. The two most probable ions to be considered are potassium and chloride. A chloride ion dominance would, however, probably require the existence of a chloride ion pump, for which there is as yet no evidence in other systems.

Whatever the basis of the electrical changes in the membrane during inhibitory stimulation may be, it is apparent that the observed electrical effect is not proportional to the inhibitory action in all the cases examined. Nor is attenuation of the excitatory j.p.s, which in any case occurs in but a few muscles, a direct cause of inhibition. There is always, in inhibition, a reduction of the maintained depolarization produced by the excitatory stimulation, the phenomenon suggested by Fatt & Katz as a possible basis for inhibitory action. But it should be noted that the efficiency of inhibitory action is independent of whether the individual inhibitory potentials are polarizing or depolarizing, both in regard to mechanical action and in attenuating excitatory j.p.s. In this respect the inhibitory potentials differ from those in motoneurones of vertebrates and in crustacean stretch receptor cells, in which the action ceases to be inhibitory if it is depolarizing.

It may therefore be that the electrical effects are not the direct cause of mechanical inhibition. In attempting to explain inhibition in *Crustacea*, Marmont & Wiersma (1938) suggested that the inhibitory transmitter substance diffuses across the muscle-fibre membrane from the diffuse nerve terminals and acts within the fibre directly on the contractile elements. The main objection to this view is that the observable events are too quick to be explicable on the basis of ordinary rates of diffusion. But our present results require an explanation of a somewhat similar kind. By substituting a direct action on the coupling mechanism, which may be situated at or close to the surface membrane, rather than on the contractile mechanism, the time factor is taken care of. The problem is examined further in the following paper (Hoyle & Wiersma, 1958*b*).

There are appreciable differences in the inhibitory effects of the common and the specific inhibitors in the opener and stretcher muscles of crabs. The question arises then, as with slow and fast motor systems, whether they are due to differences in secondary factors, such as the location of the excitatory nerve endings relative to the inhibitory ones, or whether there are different inhibitory transmitter substances. At present one cannot decide between these alternatives.

SUMMARY

1. The nature of the electrical events associated with inhibition in crustacean muscle fibres has been examined with the aid of intracellular recording electrodes.

2. In many muscles stimulation of the inhibitory axon or axons results in the appearance of polarizing potentials in the muscle fibres. These sum to give a hyperpolarization plateau of up to 15 mV in some fibres during repetitive stimulation.

3. In 'spring' crayfish there were many muscle fibres which showed a depolarization during inhibitory stimulation. There was no loss of mechanical inhibitory effect.

4. In all cases the sign of the inhibitory potential could be reversed by changing the membrane potential artificially. The potential at which the sign reversed (change-over potential) was found to be constant in some fibres, whereas in others it depended on the actual value of the resting membrane potential of the fibre.

5. The greater proportion of muscles tested showed only simple (β) inhibition; inhibitory nerve impulses stopped the contraction without reducing the amplitude of the excitatory junctional potentials.

6. In those muscles which showed supplemented (α) inhibition, attenuation of junctional potentials as well as mechanical relaxation, no evidence could be obtained that appropriate timing for maximum electrical attenuation was also more effective mechanically.

7. Gamma-amino butyric acid did not mimic the inhibitory action.

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