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TONIC RELEASE OF TRANSMITTER AT THE NEUROMUSCULAR JUNCTION OF THE CRAB

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SUMMARY

1. Synaptic transmission was studied at the neuromuscular junction of the crab *Ocypoda cursor*, using conventional electrophysiological technique.

2. It was found that fibres of the extensor muscle and those composing the internal layer of the closer muscle have only post-synaptic inhibition (*S* fibres) while the fibres at the external layer of the closer muscle have in addition presynaptic inhibition (*R* fibres).

3. In *S* fibres, addition of GABA reduces input membrane resistance (R_m) and e.p.s.p. amplitude approximately to the same degree. The effect shows desensitization. In *R* type fibres, GABA reduces the e.p.s.p. much more than expected from changes in R_m. The post-synaptic effect of GABA on R_m shows desensitization, while the presynaptic effect does not show desensitization.

4. In about 50% of the cases, after desensitization occurred, R_m increased by about 10–30% above the control. Similar increase in R_m occurred after application of picrotoxin. These results suggest that initially the membrane resistance was lower due to tonic release of inhibitory transmitter.

5. The Q_{10} of R_m was found to vary between 2 and 3. In Ca^{2+} free media, Cl^- free media, or in picrotoxin the Q_{10} is about 1·3.

6. In *R* fibres, addition of picrotoxin increased the amplitude of the e.p.s.p. by 30–60% above the expected increase due to changes in R_m.

7. In *S* fibres the mean slope of log e.p.s.p. *vs.* log $[Ca^{2+}]$ was found to be 1·63, while in *R* fibres the slope was 0·93. These results suggest the presence of tonic release of the inhibitory transmitter which acts both post-synaptically and presynaptically.

INTRODUCTION

Release of transmitter is markedly dependent on extracellular Ca concentration in a great variety of synapses and neuromuscular junctions. In most of them the relation is sigmoidal with a non-linear start (Jenkinson, 1957; Dodge & Rahamimoff, 1967; Hubbard, Jones & Landau, 1968*a*, *b*; Katz & Miledi, 1970). Recently it has been shown, however, that the neuromuscular junction at the opener muscle of the crayfish leg is apparently different (Bracho & Orkand, 1970; Ortiz & Bracho, 1972). Here, the release of the excitatory transmitter follows almost linearly the changes in Ca concentration. Similar experiments, carried out in the present work on the closer muscle of the crab *Ocypoda cursor*, showed too that the amplitude of the excitatory post-synaptic potential (e.p.s.p.) varies approximately linearly with $[Ca^{2+}]$. However, both the opener muscle of the crayfish and the closer muscle of the crab *Ocypoda cursor* have presynaptic inhibition (Dudel & Kuffler, 1961; Sarne, 1974). This presynaptic inhibition offers a possible explanation for the apparent discrepancy; if there is a spontaneous release of inhibitory transmitter and if this release is a Ca^{2+}-dependent process, then changes in $[Ca^{2+}]$ could have a dual effect on the release of excitatory transmitter. First, changes in $[Ca^{2+}]$ will act directly on the electrosecretory coupling of the excitatory transmitter. Secondly, Ca ions would affect the release of the inhibitory one. The two modes of action of Ca will obviously have opposite effects.

To test this hypothesis, two sets of experiments were performed. In the first the e.p.s.p. *vs.* $[Ca^{2+}]$ relation was tested under normal conditions and after application of picrotoxin, which is known to suppress the action of the inhibitory transmitter (Grundfest, Reuben & Rickles, 1959). The second set of experiments was comparative and depended on the following observation. We found that in the walking leg of the crab there are muscle fibres with post-synaptic and presynaptic inhibition (external layer of the closer muscle – referred to as *R* fibres), and fibres having post-synaptic inhibition only (extensor muscle and the internal layer of the closer – referred to as *S* fibres). If the presynaptic inhibition has a role in the final action of Ca^{2+} on the release of excitatory transmitter, then it is expected that the e.p.s.p. will increase with $[Ca^{2+}]$ more markedly in *S* fibres than in *R* fibres, and it is expected that the e.p.s.p. *vs.* $[Ca^{2+}]$ relation will be steeper in the *S* fibres relative to the *R* fibres.

Such continuous outpour of inhibitory transmitter need not be confined to presynaptic inhibitory mechanisms only. It is feasible that similar tonic release of transmitter may participate in post-synaptic inhibition, thereby causing a continuous shunting of the muscle membrane. Thus, the main aim of this work was to test the possibility of tonic release of

inhibitory transmitter and its effects on the muscle membrane and on the excitatory axon's terminals.

Some of the results described here were presented in brief form to the Israel Physiological and Pharmacological Society (Sarne, Parnas & Rahamimoff, 1972; Parnas & Sarne, 1972).

METHODS

Animals. Crabs, *Ocypoda cursor*, were collected at the Mediterranean seashore. Each crab was kept separately in sea water, at controlled room temperature (19–21° C).

Preparation. Walking legs were removed by autotomy. The closer and extensor muscles (Wiersma, 1961) were exposed either by removal of a piece of cuticle (for recording from the external cell layers) or by the removal of the antagonistic muscles (for recording from the internal cell layer). The nerve was exposed in the meropodite after removal of the flexor muscle.

Stimulation and recording. The nerve was stimulated through a sharpened steel electrode or through a pair of silver hook electrodes with supramaximal square pulses of 50 μsec duration.

Glass micro-electrodes (with 5–10 mΩ resistance) for intracellular recording were filled with 2 M-KCl. Effective membrane resistance was obtained from the membrane potential change produced by current pulses injected intracellularly through a second micro-electrode. The current pulses were sufficiently long (100–400 msec) to achieve a steady level of membrane potential. Current micro-electrodes were filled with 2 M-K-acetate or 0·5 M-K_2SO_4. The current was measured by the potential drop over a 100 KΩ resistor.

Potentials were observed on an oscilloscope and photographed. In many experiments signals were also transmitted to an averaging computer.

Solutions. The standard bathing solution contained: NaCl (453 mM), KCl (12·8 mM), $CaCl_2$ (34 mM) and $MgCl_2$ (4·3 mM), Tris Cl buffer (2 mM). The pH was adjusted to 7·4–7·6 by titration with HCl (1 N). Calcium concentration changes were balanced by the appropriate change in Na concentration to maintain constant osmolarity. Calcium-'free' solution was composed of NaCl (453 mM), KCl (12·8 mM), $MgCl_2$ (34 mM) and Tris buffer (2 mM), EGTA (5 mM). Gamma-amino butyric acid (NBC) and picrotoxin (Sigma) were used.

The preparation was kept under a constant flow of bathing solution. A complete change of solution was accomplished in less than 5 sec. Experiments were done at room temperature (19–21° C) unless otherwise stated.

RESULTS

A. The effects of GABA and picrotoxin on membrane resistance and e.p.s.p.

One of the aims of the present study was to examine whether Ca ions affect differentially transmitter release from excitatory nerve terminals with and without presynaptic inhibition. In order to distinguish conveniently between muscle fibres with and without presynaptic inhibition, we compared the effect of GABA on muscle membrane resistance and e.p.s.p. amplitude. GABA is known to be the inhibitory transmitter in crustacean neuromuscular junctions (Otsuka, Iversen, Hall & Kravitz, 1966). Its

action can be either on the post-synaptic muscle membrane (Boistel & Fatt, 1958) or both the post-synaptic membrane and the presynaptic motor nerve terminals (Dudel, 1965; Takeuchi & Takeuchi, 1966*a*, *b*). In the case where GABA acts only post-synaptically, changes in e.p.s.p. amplitude should be consistent with the changes in membrane resistance (R_e). In the cases where pre-synaptic inhibition is also present, reduction in the amplitude of e.p.s.p. would be larger than that expected from the decrease in membrane resistance.

Fig. 1*A* illustrates an example of the effects of GABA on R_e and e.p.s.p. amplitude in an *S* fibre (from the extensor muscle). The relative changes in the muscle membrane resistance and e.p.s.p. amplitude are virtually the same. After the application of GABA (5×10^{-3} M, first upward arrow) the membrane resistance (R_e, filled circles) and the e.p.s.p. amplitude (open circles) reached a minimal value within 45 sec (28 and 30 % reduction respectively). Thereafter they increased gradually towards their initial value, even though the muscle was continually exposed to GABA. Short washout (downwards arrow) was not sufficient for recovery, and re-application of GABA (5×10^{-3} M, second upward arrow) produced no effect. Only after prolonged washout for 30–40 min was there a recovery and the membrane regained its sensitivity to GABA (not shown). Such a response to GABA was obtained only in the *S* type fibres. These fibres were found to compose the whole extensor muscle and the internal cell layer of the closer muscle.

The time course of the changes observed in R_e and e.p.s.p. depends on the concentration of GABA. The minimal values of R_e and e.p.s.p. were obtained after 30–120 sec with GABA varying between 10^{-2} to 10^{-5} M. At the high concentrations (10^{-2} to 10^{-3} M) complete desensitization occurred in less than 10 min (see Fig. 1), while in the lower concentration (10^{-4} to 10^{-5} M) significantly longer periods were required (20 min).

Fig. 1*B* illustrates the effects of GABA on R_e and e.p.s.p. amplitude in an *R* fibre (from the external cell layer of the closer muscle). Here, a dissociation between the effect of GABA on R_e and e.p.s.p. was observed. While R_e was initially reduced approximately by 50 % and desensitization occurred in the presence of GABA (within 5 min), the e.p.s.p. was completely abolished and remained blocked as long as GABA was present. Rapid (4 min) recovery of the e.p.s.p. took place after washing (downward arrow). At this stage the muscle membrane was still desensitized. Re-application of GABA (second upward arrow) produced no changes in R_e, while the e.p.s.p. disappeared again.

This experiment indicated that the reduction of e.p.s.p. amplitude after addition of GABA resulted both from an effect on the muscle membrane and on the excitatory nerve terminals. We can also conclude that the

effect of GABA on the muscle resistance shows desensitization, while its effect on the excitatory nerve terminal does not. A similar situation has been observed in another crab, *Cancer borealis* (Epstein & Grundfest, 1970).

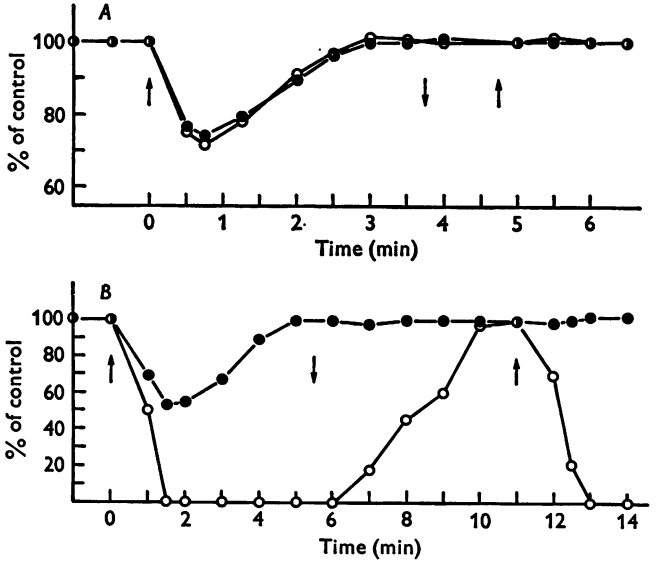

Fig. 1. Effect of GABA on e.p.s.p. amplitude and membrane resistance (R_e). E.p.s.p. amplitude (open circles) and R_e (filled circles) are expressed as percentage of the control values before GABA. Upward arrows, application of 5×10^{-3} M-GABA. Downward arrows, wash with Ringer. *A*, S-type fibre; note that the relative change in e.p.s.p. and R_e amplitudes is almost the same. *B*, *R* type fibre; note the dissociation between the e.p.s.p. and R_e. The effect of GABA on e.p.s.p. does not show desensitization.

Using these methods we found that the extensor muscle and the internal layer of the closer muscle are composed only of *S* type fibres. The external cell layer of the closer muscle is composed of *R* fibres, showing also presynaptic inhibition. To further confirm this finding, the inhibitory and excitatory axons were separated, and timing experiments (Dudel & Kuffler, 1961) were performed to check the effect of the inhibitory input on R_e and the e.p.s.p., when it arrives just before or after the excitatory input. We found that fibres which were classified as *R* type by the GABA method always showed both presynaptic and post-synaptic inhibition by the timing method. The *S* fibres showed only post-synaptic inhibition.

In about 50 % of the cells examined (twenty-eight out of sixty) R_e became higher than the control value by about 10–20 % after desensitization occurred. This was found both in *R* and *S* fibres. This finding suggests, that under 'normal' conditions there is a constant reduction in

R_e due to an action of spontaneously released inhibitory transmitter. If this assumption is correct, then application of picrotoxin, which antagonized the action of GABA, would be expected to cause an increase in R_e. This supposition was confirmed in eight out of twelve fibres tested. After the addition of 1·66 mM picrotoxin R_e increased by 10–30 %. It should be noted that the increase in R_e after addition of picrotoxin is rather similar in magnitude to the increase after desensitization.

In *S* fibres after application of picrotoxin, the evoked e.p.s.p. increases in amplitude by 10–30 %. This increase can be completely accounted for

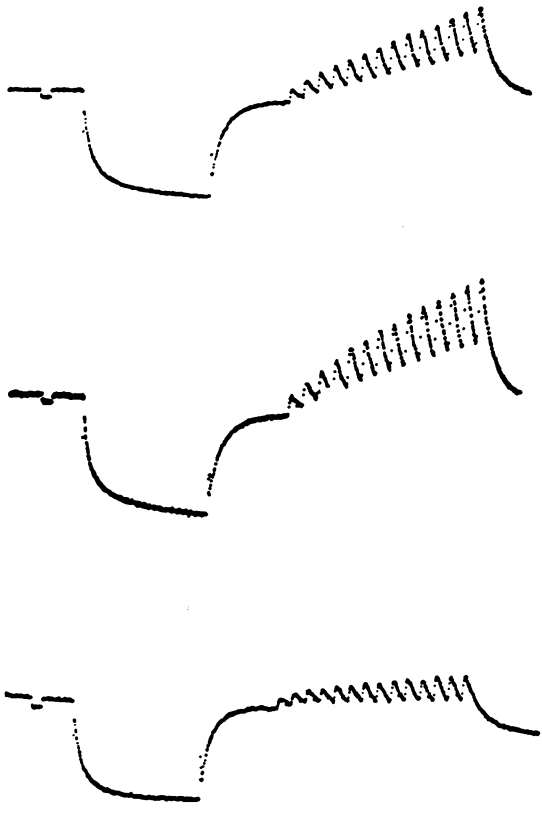

Fig. 2. Effects of picrotoxin and GABA on R_e and e.p.s.p. in an *R* type fibre. The records show an electrotonic potential produced by passing approximately 10 nA of current through a second intracellular micropipette, followed by e.p.s.p.s produced by the stimulation of the motor nerve. Each record was obtained by averaging thirty-two responses. Upper trace, control. Middle trace, in picrotoxin 1·66 mM. R_e is increased by 10 %, the e.p.s.p. by 45 %. Lower trace, after recovery from the picrotoxin and application of 2×10^{-5} M-GABA. R_e is reduced by 10 % while the e.p.s.p. is reduced by 50 %. Calibration pulses 0·1 mV, 20 msec.

by the increase in R_e. But in R fibres, where presynaptic action of GABA was observed, picrotoxin (10^{-3}, w/v) caused the e.p.s.p. to increase by 30–50 %, while the R_e in these cells was increased by 10–30 % only. Fig. 2 demonstrates an experiment performed in a cell from the external layer of the closer muscle. Here, R_e was increased by 10 % and the e.p.s.p. amplitude by 45 % (middle trace, Fig. 2). In this experiment GABA (2×10^{-5} M) affected the e.p.s.p. much more than R_e (50 % and 10 % respectively, lower trace, Fig. 2).

These results indicate that continuous release of inhibitory transmitter may occur and act both presynaptically on the excitatory nerve terminal and post-synaptically on the muscle membrane.

B. Effect of temperature on R_e

If R_e in an untreated preparation is constantly reduced due to a spontaneous release of the inhibitory transmitter, then factors which are known at other synapses to reduce transmitter release might cause an increase in R_e in the crab muscle. One such factor is a reduction of temperature. In the frog neuromuscular junction miniature end-plate potential (m.e.p.p.) frequency has a Q_{10} of about 3 (Fatt & Katz, 1952). It should be remembered, however, that changes in temperature affect also directly R_e of the frog sartorius, but with a Q_{10} of only 1·3 (del Castillo & Machne, 1953).

In the closer and extensor muscles of the crab lowering the temperature from 18 to 10° C increased R_e by 100 % (Fig. 3 left). This gives a value for Q_{10} of more than 2. In various experiments (ten cells) the value of Q_{10} ranged from 2 to 3. Under the same experimental conditions parallel control experiments were repeated on the frog sartorius muscle and showed that the Q_{10} is between 1·2 and 1·4 (Fig. 3 right).

If indeed the large Q_{10} for membrane resistance observed in the crab muscle was due to the tonic release of the inhibitory transmitter, then it might be expected that after treatment with picrotoxin the Q_{10} of the crab muscle would be smaller. After application of picrotoxin (10^{-3}, w/v) to the closer and extensor muscles of the crab, reduction of the temperature from 18 to 10° C caused the R_e to increase, in four experiments, only by 20–40 %.

The spontaneous transmitter release is known to depend on external Ca^{2+} concentration in some preparations (Hubbard, Jones & Landau, 1968*a*). In our experiments removal of external Ca^{2+} and application of 5 mM-EGTA resulted in reduction of the membrane resistance (Ortiz & Bracho, 1972), but also the dependence of R_e on temperature was decreased. In five experiments, the reduction of the temperature from 18 to 10° C caused an increase of R_e by 10–30 % only.

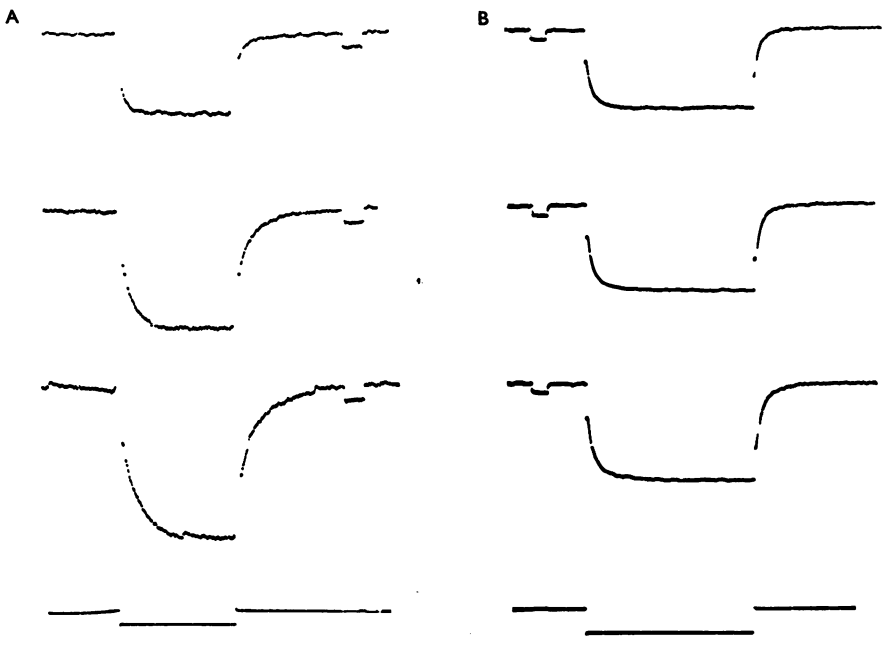

Fig. 3. Effect of temperature on R_e of a closer muscle fibre (left) and of a frog sartorius fibre (right). Each trace shows hyperpolarization produced by inward current pulses of 50 nA (*A*) and 10 nA (*B*), shown by the fourth trace. Temperature: 1st trace, 18° C; 2nd trace, 14° C; 3rd trace, 10° C. Hyperpolarization increases with decreasing temperature, indicating increase in membrane resistance. Calibration pulses: A, 1 mV, 50 msec. *B*, 1 mV, 20 msec.

C. Effect of Ca^{2+} on e.p.s.p. amplitude

In the extensor muscle cells and the internal cell layer of the closer muscle, only post-synaptic inhibitory effects of GABA were found. In the external cell layer of the closer muscle both pre- and post-synaptic effects of GABA were demonstrated. Thus, these two sets of muscle fibres offer convenient experimental systems to study the dependence of evoked excitatory potentials on Ca concentration.

In *R* fibres, where both pre- and post-synaptic inhibition were found, the mean slope of log e.p.s.p. amplitude against log Ca^{2+} concentration was 0·92 (fifteen cells 0·8–1·1), similar to findings of Bracho & Orkland (1970). However, in *S* fibres, where only post-synaptic effect of GABA was found, the slope of log e.p.s.p. against log Ca^{2+} was significantly higher; about 1·4–1·8 (mean = 1·63, in twelve cells). Fig. 4*A* illustrates the relations obtained in an *S* fibre (filled circles) and in an *R* fibre (open circles).

If Ca has a dual effect on excitatory transmitter release at terminals with presynaptic inhibition, then picrotoxin would increase the slope of the [Ca^{2+}]-e.p.s.p. relation in *R* cells. On the other hand, in cells with no presynaptic inhibition, the Ca^{2+} *vs.* e.p.s.p. relation should be very little affected by picrotoxin.

The experimental results confirm this prediction (Fig. 4*B*); while in *R* fibres of the closer muscle the slope before picrotoxin was about 1, after treatment with picrotoxin the mean slope was 1·47 (mean of five cells). In the *S* fibres of the extensor muscle the addition of picrotoxin had no effect on the Ca^{2+}– e.p.s.p. relation (four experiments).

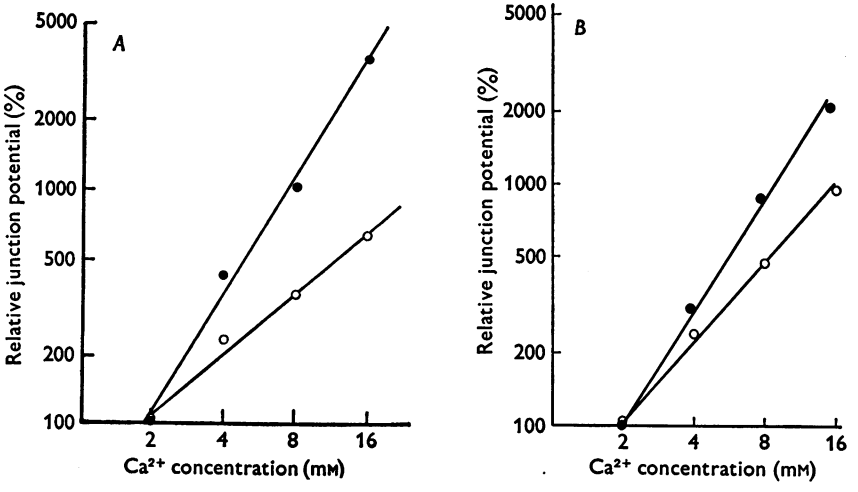

Fig. 4. The relation between log e.p.s.p. amplitude and log [Ca^{2+}]. The e.p.s.p. amplitude is given as percentage of the amplitude in 2 mM-Ca^{2+}. *A*, in an *S* type fibre (filled circles) and in an *R* type fibre (open circles). The slope for the *S* type fibre is 1·67; for the *R* type 0·86. *B*, the effect of 1·66 mM picrotoxin on the log e.p.s.p. – log Ca^{2+} relation, in an *R* type fibre. In control conditions (open circles) the slope is 1·07; after addition of picrotoxin (filled circles) the slope is 1·47.

DISCUSSION

The crustacean neuromuscular junction is a complicated system, with a number of components. In the relatively simple case of the *S* fibres two inputs influence the muscle membrane – an excitatory and inhibitory input. One of the questions examined in this work is whether in such fibres a spontaneous release of the inhibitory transmitter affects the muscle membrane. Three indirect lines of evidence suggest an affirmative answer to this question.

First, after complete desensitization with GABA the muscle membrane resistance is greater than the control value. This suggests that under normal conditions part of the membrane conductance is due to an action of the inhibitory transmitter released spontaneously.

Secondly, picrotoxin, which is known to counteract the action of the inhibitory transmitter, increases the muscle resistance. One might, of course, argue that the effect of picrotoxin is non-specific, and that it acts on the extra-synaptic membrane conductance. But then it would be difficult to explain why picrotoxin does not produce an increase in the membrane resistance of the desensitized muscle membrane (Sarne 1974).

Thirdly, there is the effect of temperature on membrane resistance. Reduction of temperature increases membrane resistance much more than expected by analogy with frog sartorius muscle. Furthermore, in the presence of picrotoxin or in the absence of Ca, crab muscle membrane resistance is affected by cooling to a similar degree as that of frog sartorius. Since it is known that temperature has a strong effect on spontaneous transmitter release (Fatt & Katz, 1952), it is plausible to assume that in the crab changes in R_e with temperature are partially due to changes in the liberation of the inhibitory transmitter.

The *R* fibres represent an even more complicated system. Here, in addition to the excitatory and the inhibitory inputs to the muscle fibre itself, there is also a branch of the inhibitory axon affecting the excitatory nerve terminals. When this input operates there is a presynaptic inhibition, that causes a decrease in the amount of the excitatory transmitter released (Dudel & Kuffler, 1961). The question examined here was whether this presynaptic input also operates spontaneously and is affected by Ca ions. If one assumes that Ca^{2+} ions affect the spontaneous release of the inhibitory transmitter, then its effects should be taken into consideration in the interpretation of the relation between the Ca^{2+} concentration and the e.p.s.p. amplitude. Since the direct experimental approach to this problem is difficult, we took the relation between e.p.s.p. amplitude and $[Ca^{2+}]$ as an indicator for the spontaneous activity of the presynaptic inhibitory fibre. The finding that at synapses with no presynaptic inhibition the slope of the log e.p.s.p. *vs.* log $[Ca^{2+}]$ is higher than the slope at synapses with presynaptic inhibition already suggests that such mechanism may take place. This suggestion is further strengthened by the fact that inactivation of the inhibitory mechanism by picrotoxin also increases the slope of the log e.p.s.p. – log $[Ca^{2+}]$ relation in *R* fibres, but has no effect on *S* fibres. It is possible that the finding of Bracho & Orkand (1970) and Ortiz & Bracho (1972), where the slope of log e.p.s.p. *vs.* log $[Ca^{2+}]$ in crayfish muscle is about 1, results from such indirect effect of Ca ions on the presynaptic inhibition. In another crab, *Chionoecetes*, Linder (1973)

found a slope of 3·1 in the plot of the log e.p.s.p. against the log [Ca^{2+}]. We do not know, however, whether the fibres examined had presynaptic inhibition.

Tonic release of inhibitory transmitter may be also of interest in the interpretation of neuromuscular facilitation in certain crustacean preparations. At the frog neuromuscular junction it was suggested that facilitation originates from residual Ca ions occupying critical sites at the time of arrival of a test stimulus (Katz & Miledi, 1968; Rahamimoff, 1968). Such an interpretation was based in part on the non-linear relation between [Ca^{2+}] and transmitter release, giving on a log–log plot a slope of more than 1. One of the consequences of the tonic release postulate would be the possibility that similar mechanism may operate at the crustacean neuromuscular junction.

We do not know at present whether the postulated tonic release of inhibitory transmitter occurs under normal physiological conditions *in vivo*. It is plausible that an inadequate solution composition and/or a relative hypoxia create this phenomenon *in vitro*. At the frog neuromuscular junction, for example, conditions which interfere with the normal energy production mechanisms cause an increase in the frequency of spontaneous miniature end-plant potentials (Rahamimoff & Alnaes, 1973; Alnaes, Meiri, Rahamimoff & Rahamimoff, 1974). Although the standard bathing solution perfusing the muscle was continuously oxygenated, it is still uncertain whether this procedure supplies enough oxygen and metabolites for normal function of the nerve terminal. However, most of the experiments on crustacean preparation are performed in this way. Therefore the spontaneous release of inhibitory transmitter may be of importance, at least in the interpretation of the experimental results, which may involve alterations in such release.

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