Inhibitory Synapses on Pacemaker

Neurons in the Heart Ganglion

of a Stomatopod, *Squilla oratoria*

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ABSTRACT The pacemaker neurons of the *Squilla* heart ganglion are innervated from the CNS through three pairs of extrinsic nerves. One of them, the α-nerve, is inhibitory to the heart beat. The effect of α-nerve stimulation on the pacemaker potential was examined with intracellular electrodes. Without extrinsic nerve stimulation the membrane potential of the pacemaker cell fluctuated spontaneously. On application of a tetanic train of stimuli to the α-nerve the membrane potential was shifted and fixed to a steady level, which with K_2SO_4-filled electrodes was near the peak of hyperpolarization after a spontaneous burst, but was less negative with KCl-filled electrodes. The shift of the membrane potential was due to the summated IPSP's. By changing the level of the membrane potential with injection of the polarizing current the IPSP could be reversed in sign, and the size of the IPSP was linearly correlated with the membrane potential level. During inhibition the membrane conductance increased. The increase depended on divalent cation concentrations in the outside medium. In Ca-rich saline the IPSP was greatly enhanced. In Mg-rich saline it was suppressed. The amplitude of antidromic spikes was reduced during inhibition especially when the spike frequency was high.

INTRODUCTION

Alexandrowicz (1934) found that the *Squilla* heart ganglion is innervated by neurons from the CNS through three pairs of regulator nerves, which are called α, β, and γ, respectively, starting from the rostral side. Applying electrical stimuli to the α-nerves causes inhibition whereas the β- and γ-nerves cause acceleration of the heart beat. Thus the *Squilla* heart ganglion with its regulator nerves supplies a typical preparation for the synaptic regulation with antagonistic effects of inhibition and acceleration displayed. Similar systems are also found in decapod hearts (Alexandrowicz, 1932), and much physiological work has already been devoted to elucidating the

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mechanism of their action (Maynard, 1953; Terzuolo and Bullock, 1958; Otani and Bullock, 1959; Florey, 1960). In decapod hearts, however, intracellular recording has been possible only from follower cells, since the pacemaker cells are not visible in living preparations. In stomatopods, pacemaker cells are visible in living preparations, and intracellular recording proved to be feasible (Watanabe, Obara, Akiyama, and Yumoto, 1967; Watanabe, Obara, and Akiyama, 1967). Another anatomical advantage is that in stomatopods the three regulator nerves separately enter the heart ganglion, allowing separate stimulation to the inhibitor and accelerator nerves.

In the present paper, the effects of inhibitor nerve stimulation on the pacemaker activity will be described. The inhibition of the heart beat is mediated by inhibitory postsynaptic potentials (IPSP) which clamp the membrane potential and suppress the spontaneous depolarization in the pacemaker neurons.

METHODS

The anatomy and the method of dissection of the *Squilla* heart are described in a previous paper (Watanabe et al., 1967). The three regulator nerves are found under the fourth thoracic tergum (Alexandrowicz, 1934), which had to be gently removed, at the same time trying not to apply tension to the nerves. They were cut at the inner edge of the dorsal muscles and were freed from the inner surface of these muscles. The whole heart was isolated from the animal and mounted on the experimental trough, as described previously. The length of the nerves available for stimulation was only 1–3 mm.

At the initial stage of the experiments, the regulator nerves were stimulated with an insulated silver wire, the cut end of which was pressed against a regulator nerve, which lay on the heart muscle. At the later stage, one of the regulator nerves was sucked into a glass capillary (about 0.2 mm in diameter) and the stimulating pulse was applied between the inside of the capillary and the bath (Furshpan and Potter, 1959).

The intracellular electrodes were filled with either 3 M KCl or 0.6 M K_2SO_4. With the KCl-filled electrode the reversal potential of the IPSP shifted to the depolarized level (Coombs, Eccles, and Fatt, 1955). With the K_2SO_4-filled electrode, the shift did not take place, but the higher electrode resistance created some recording difficulty due to increased rate of noise, distortion, or drift. Since we had to select electrodes with very fine tips for penetrating the pacemaker cells, the resistance used for the K_2SO_4-filled electrode was 100–200 MΩ.

Except as otherwise noted, normal saline with the following composition was used for the outside medium (mM): NaCl, 450; KCl, 15; $CaCl_2$, 10; $MgCl_2$, 20. Its composition is based on that of the animal serum (Lee and McFarland, 1962). Compared to seawater, the normal saline has a higher K^+ and a lower Mg^{++} concentration. Reduced Mg^{++} concentration in the outside medium was essential for the study of inhibitory and acceleratory effects from the regulator nerves. In seawater, many attempts have been made to discover the effects of nerve stimulation, but a very

slight acceleratory effect of γ-nerve stimulation was found in only one instance. This effect was small and appeared in only one series of tetanic stimulations. In normal saline, most preparations responded to regulator nerve stimulation with good acceleration or inhibition.

In normal saline the frequency of the spontaneous discharge was larger (about ⅔ sec) than in seawater (about ¼ sec). Experiments showed that the difference is due mainly to the difference in concentration of Mg^{++} ions, whereas the difference in K^+ concentrations between the two salines produced no clear effects on the burst frequency.

The burst duration was usually shorter in normal saline than in seawater. As shown by Brown (1964), the burst duration is positively correlated with the interval between bursts.

In some experiments, solutions with only one type of divalent cations were used. They will be called Mg^{++} saline, Ca^{++} saline, etc., according to the species of the divalent cations. Thus Mg^{++} saline contains 450 mM NaCl, 15 mM KCl, and 30 mM $MgCl_2$.

The experiments were performed at a room temperature of 18–22°C.

RESULTS

Inhibition of the Spontaneous Discharge   Fig. 1 A_1–A_3 shows the effect of tetanic α-nerve stimulation on the spontaneous discharge of a pacemaker neuron. When the stimulation started, the membrane potential shifted from the usual periodic time course towards a fixed hyperpolarized level, and after about 150 msec, it became almost steady. The burst discharge stopped completely during stimulation. The extracellular records also failed to pick up any burst discharge, showing that the inhibition was taking place in all the primary pacemaker cells. When the secondary pacemaker at the caudal end of the heart (Watanabe, Obara, and Akiyama, 1967) was active, it was not inhibited by α-nerve stimulation.

In the following sections, the shift of the membrane potential is explained as being caused by summated IPSP's produced by inhibitory fiber stimulation.

The steady level during inhibition was different among preparations, but with the K_2SO_4-filled intracellular electrode, it was usually near the peak of hyperpolarization after the burst. When a KCl-filled electrode was used, the level of the membrane potential during inhibition was more positive and near the firing level of the burst discharge. The shift of the steady level with the KCl-filled electrode has been reported in many kinds of nerve cells (Coombs et al., 1955).

With 100/sec stimulation, the effect of inhibition was almost always complete regardless of the phase of spontaneous discharge. In Fig. 1 A_2, stimulation started just before initiation of a burst, and the inhibition was quite effective. In Fig. 1 A_3, stimulation started during a burst, and the burst

duration was curtailed. In Fig. 8 of a previous paper (Watanabe et al., 1967) it was shown that the brief tetanic stimulation of the inhibitory fiber can abolish the tail of the plateau.

During tetanic stimulation, the maximum hyperpolarization was reached in the early stage. Afterwards there was a small drift in the depolarizing direction. In Fig. 1 A, the rate of spontaneous depolarization between bursts was 5.4 mv/sec, and during inhibition it decreased to 0.12 mv/sec (less than one-fortieth).
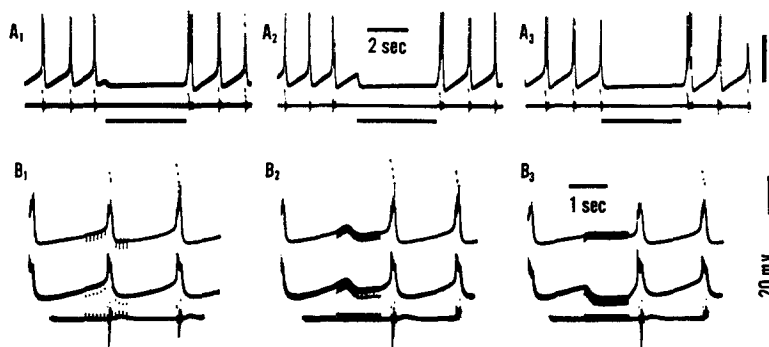

FIGURE 1.   Effects of α-nerve stimulation on spontaneous activity in a pacemaker neuron. A, repetitive stimulation at 100/sec applied for about 4 sec occurred at different phases with respect to the spontaneous bursts of the neurons. Upper beam, Gc. 4, cell 131, K_2SO_4 electrode. Lower beam, external recording. The period of stimulation occupies the middle third of each record, as is indicated by a bar at the bottom. B, repetitive stimulation at various frequencies. Upper beam, Gc. 5, cell 180. Middle beam, Gc. 6, cell 181. Lower beam, external recording. Stimulus frequency, 10/sec in B_1, 40/sec in B_2, and 100/sec in B_3. KCl electrodes.

After stimulation the membrane was depolarized rapidly and the following burst discharge usually had a longer duration with a larger number of spikes. This is a kind of rebound phenomena, and may be called postinhibitory facilitation (Kuffler and Eyzaguirre, 1955). In some preparations, the membrane potential was hyperpolarized following inhibitory nerve stimulation (see p. 912).

Effect of Stimulus Frequency   When the stimulus frequency is reduced, the effect of inhibition is decreased. Fig. 1 B_1–B_3 shows one such example. With a frequency of less than 20/sec, the inhibitory effect was not clear. With 40/sec, a burst could be completely suppressed, but the spontaneous depolarization continued for a time before inhibitory hyperpolarization developed. With 100/sec, the inhibitory effect started immediately and attained a steady level after about 0.3 sec. The rate of generation of the inhibitory effect is thus dependent on the frequency of stimulation, and this is probably due to potentiation of individual IPSP's with high frequency

stimulation. Usually further increase in stimulus frequency did not increase the inhibitory effect, and the summated IPSP's took an irregular form, apparently due to the failure of response of the presynaptic fiber to each stimulus.

As seen in Fig. 1 B₁, single IPSP's were barely recognizable in response to individual stimuli in normal saline. The individual IPSP's could be observed in solutions with higher Ca^{++} and lower Mg^{++} concentration, as will be described later (p. 917).
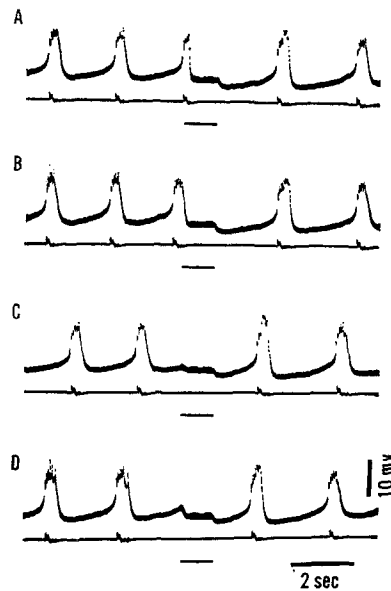

FIGURE 2. An example of the postinhibitory polarization. Upper beam, Gc. 4, cell 126. K_2SO_4 electrode. Lower beam, external recording. A bar below each record shows the period of tetanic stimulation applied to the α-nerve with a frequency of 100/sec. The spontaneous burst discharge was suppressed during stimulation, and the postinhibitory polarization appeared after the period of stimulation.

Postinhibitory Polarization   After the period of tetanic stimulation, the membrane potential started to return to its original level, and although the time constant of the shift of membrane potential was slightly longer (about 0.1–0.2 sec) than that of the electrotonic potential (less than 0.1 sec), no standstill was observed in most preparations. On several occasions, however, we encountered preparations in which the membrane potential was further hyperpolarized after the period of stimulation. Fig. 2 shows one example. During tetanic stimulation of the α-nerve, the membrane potential was fixed at a steady level. After stimulation, the membrane potential was further hyperpolarized by 3–5 mv. The interval between the cessation of stimulation and the following burst was very much prolonged. The effect sometimes lasted for several seconds and during that period interburst intervals were prolonged and the afterhyperpolarization was enhanced. The effect was observed with both KCl and K_2SO_4 electrodes.

At present we do not know the conditions for the appearance of this phenomenon, and the detailed analysis has not yet been performed. A very

similar phenomenon is described in the crustacean stretch receptor and is called postinhibitory polarization (Kuffler and Eyzaguirre, 1955). In molluscs, Tauc (1960) and Frazier et al. (1968) describe a pronounced postexcitatory polarization (inhibition of long duration following excitation), which seems to be very similar to the postinhibitory polarization in the crustacean nervous system. Until now no definite interpretation has been presented for this long-lasting inhibitory effect. Kuffler and Eyzaguirre (1955) consider that some "active" process is going on underneath this inhibitory effect.
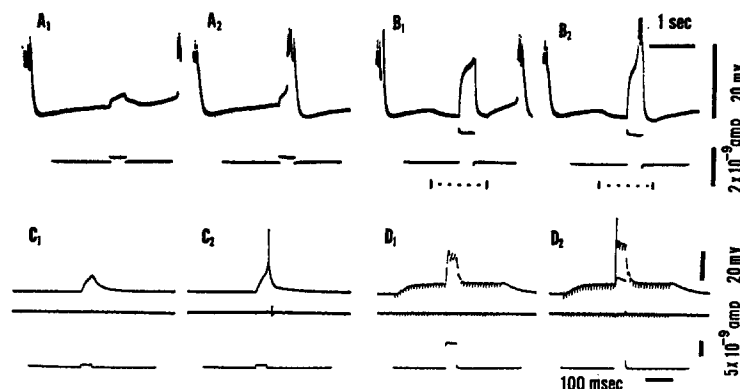

FIGURE 3.   Increase in threshold for direct stimulation during inhibition. A and B, in normal saline. The cell was spontaneously active. Upper beam, Gc. 5, cell 221. K_2SO_4 electrodes. Lower beam, injected current. A, without α-nerve stimulation. B, tetanic stimulation at 100/sec was applied during the interval shown by the dotted line at the bottom of the records. Current was subthreshold in A_1 and B_1, and suprathreshold in A_2 and B_2. C and D, in Ca^{++}-rich saline (NaCl, 435; KCl, 15; $CaCl_2$, 80; $MgCl_2$, 20 in mM). No spontaneous activity. Upper beam, Gc. 5, cell 235. KCl electrodes. Middle beam, external recording. Lower beam, injected current. C, without α-nerve stimulation. D, with stimulation at 100/sec. Current was subthreshold in C_1 and D_1, and suprathreshold in C_2 and D_2.

Firing Level during Inhibition   By injecting current pulses into a soma, the firing level has been examined with and without inhibitory fiber stimulation, and it has been found that this level increased markedly during inhibition. The example in Fig. 3 A shows that the firing level without inhibition was about 7 mv (measured from the peak of the preceding afterhyperpolarization), whereas it was about 15 mv during inhibition (Fig. 3 B). In terms of the stimulating current, the threshold increase was six times, because of the reduced membrane resistance during inhibition (see p. 914). A similar but stronger effect was also observed on application of γ-aminobutyric acid to the preparation.

The effect of inhibition is greatly enhanced when the outside Ca^{++} ion is rich in comparison with Mg^{++} ions (see p. 917). Fig. 3 C and D show one experiment in which the outside medium contained 80 mM $CaCl_2$ and 20 mM

$MgCl_2$. Without inhibition the threshold depolarization was about 11 mv. During inhibition only a graded response could be elicited with direct stimulation. The threshold for such a response was about 30 mv. With stronger current injection, the graded response could attain an amplitude comparable to that of the soma spike without inhibition (Fig. 3 D_2). However, extracellular electrodes failed to pick up any axon spikes, indicating that excitation was confined within the soma. Therefore, when inhibition is most effective, the soma is electrically isolated from other parts of the neuron. This shows that the receptor sites for inhibitory transmitter distribute not only on the soma but also along the proximal part of the axon. Because of increased membrane conductance, local current produced by the soma spike must be attenuated for a short distance, and cannot elicit spikes in the distal part of the axon.

Reversal Potential of IPSP   Since in the pacemaker neurons the membrane potential is spontaneously fluctuating, the reversal potential (which is commonly called the equilibrium potential; for this terminology, see Bennett, 1964) for the IPSP can be assessed without introducing the second electrode. When the membrane potential was at a depolarized level, the effect of inhibitory fiber stimulation for a brief period was to increase the membrane potential. When the membrane potential was at a hyperpolarized level, the effect was to decrease the membrane potential. At the intermediate stage, the effect was at first to depolarize, then to hyperpolarize the membrane. With the K_2SO_4-filled electrode, the reversal potential usually falls within several millivolts of the peak of afterhyperpolarization.

More quantitative information can be obtained by using a separate current electrode to move the membrane potential at will. In Fig. 4, records from one experiment are presented, and the size of the IPSP is plotted against the change in membrane potential. The size of the IPSP is linearly correlated with the change in membrane potential over a wide range. The IPSP changes its direction when the membrane is hyperpolarized, as was found in other materials (Fatt and Katz, 1953; Coombs et al., 1955). In this particular example, the reversal potential for the IPSP is slightly more negative than the average level of the peak of afterhyperpolarization.

The linear relationship between the membrane potential and the size of the response provides good evidence that the response to inhibitory nerve stimulation is a summated IPSP. It is well-known that postsynaptic potentials show a linear relationship with the membrane potential (e.g., Hagiwara, Kusano, and Saito, 1960), and especially on application of polarizing current (Fig. 4 A) the IPSP may reverse its sign (Fatt and Katz, 1953).

Resistance Change during α-Nerve Stimulation   Fig. 5 shows one experiment in which the effective resistance at the soma was measured with and without
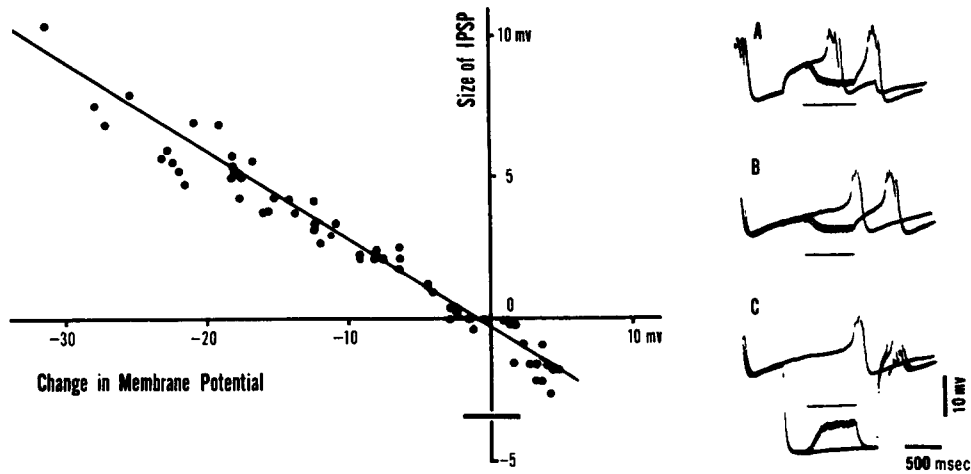
FIGURE 4. The relationship between the membrane potential and the amplitude of summated IPSP's. A and B, two traces with and without α-nerve stimulation were superimposed. In A, depolarizing current pulses were injected. C, three traces were superimposed: first, control; second, with hyperpolarizing current; third, with hyperpolarizing current and α-nerve stimulation. The bar in each record shows the period of nerve stimulation at 100/sec. Results of the complete experiment were plotted in the graph (left). The origin of the abscissa is the mean of the peak of afterhyperpolarization, which varied within the range shown by the bar below the origin. Gc. 5, cell 140. K_2SO_4 electrodes.

α-nerve stimulation. Without stimulation, the resistance was 10.6 MΩ (only hyperpolarizing current was used for the estimation; depolarizing current produced burst discharges which hindered measurement of the mem-
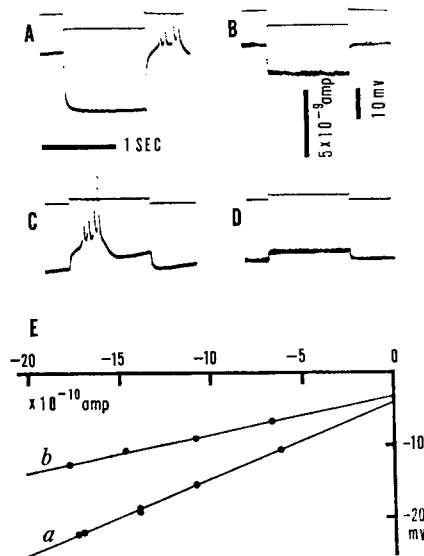
FIGURE 5. Conductance increase of the soma membrane during α-nerve stimulation. A–D, upper beam, injected current; lower beam, change in membrane potential. A and C are controls, and B and D are with α-nerve stimulation at the frequency of 100/sec. Results with hyperpolarizing current are plotted in E; *a*, without stimulation. *b*, with stimulation. Gc. 4, cell 129. K_2SO_4 electrodes.

brane resistance). On α-nerve stimulation the amplitude of the electrotonic potential decreased to about one-half (Fig. 5 B). The time constant was about 50 msec before stimulation, and about 20 msec during stimulation. In terms of the conductance, α-nerve stimulation increased the conductance of the soma by about 1.1×10^{-7} mhos. The conductance increase (Δg) of five somata examined fell within a range of 0.1 to 0.2 μmho in normal saline. The resting conductances of those somata were 0.1–0.2 μmho, and on α-nerve stimulation the increase of conductance was about 70–110%. The value depends on concentrations of divalent cation species in the outside medium, as will be described later (p. 917).

Effect of α-Nerve Stimulation on Antidromic Spikes Antidromic spikes were produced by delivering stimuli at the caudal part of the heart. When tetanic stimulation was applied to the α-nerve, the antidromic spike changed its form. The change depended on the rate of firing of the antidromic spike. When the antidromic spike was produced with stimuli of low frequency, effects of α-nerve stimulation were relatively slight. With application of tetanic stimulation to the α-nerve, the spike amplitude was slightly decreased, and the rate of fall was accelerated. These effects can be explained as the result of the conductance increase of the postsynaptic membrane. When the spike frequency is low, the soma is excited by the invading impulse, and on α-nerve stimulation the spike-generating membrane is short-circuited by the postsynaptic membrane, which is represented as a low resistance channel with an emf at the reversal potential (see, e.g., Fatt and Katz, 1953). The result is to bring the membrane potential towards the reversal potential for the IPSP, although the effect is not drastic during the spike due to the increased conductance of the electrically excitable part of the soma membrane (Grundfest, 1959).

When the antidromic spike was produced with stimuli of higher frequency, the effect of α-nerve stimulation was more drastic. In one example on α-nerve stimulation when the frequency of the antidromic spike was 2/sec, spike amplitude was decreased to about 90% of the control. With a frequency of 8/sec, however, some spikes showed an amplitude about 40% of the control. In another example, the spike amplitude was decreased to about 50% of the control during inhibition with a spike frequency of 2/sec, but to about 10% with a spike frequency of 5/sec.

Such a strong decrease in spike amplitude indicates that with higher frequencies of antidromic stimulation the soma membrane is in a state of refractoriness, and the safety factor for the axon-soma conduction is reduced. (The refractory period of the axon membrane seems to be far shorter than that of the soma membrane. See Watanabe, Obara, and Akiyama, 1967, Fig. 6.) On α-nerve stimulation, therefore, invasion of the soma is easily

blocked, and the recorded potential change becomes simply the electrotonic spread of the axon spike. Because the effective membrane resistance of the soma is reduced to about one-half, a decrease of the spike up to 50% is thus expected. Since, however, the decrease sometimes becomes more drastic, one has to assume, again, that the receptor for transmitter substance distributes not only at the soma but also along the proximal part of the axon. During inhibition the antidromic spike stops at a point on the axon and the local current of the spike spreads towards the soma with only a high rate of attenuation, producing a very small spike potential at the soma.

Even when the size of the antidromic spike was extremely small at the soma, its latency did not change appreciably during inhibition. This indicates that the effect of inhibition is only produced within a part of the axon. The conduction along the ganglionic trunk (which is composed of axons far from their somata) is barely affected by α-nerve stimulation.

In one experiment, a simultaneous recording was successfully made from a soma (Gc. 6) and from an axon in the trunk. Stimulation to the α-nerve produced clear IPSP's in the soma, but no detectable potential change in the axon, confirming the above inference.

Effects of Mg^{++} and Ca^{++} on IPSP   As has already been mentioned, the outside divalent cations have profound influence on the size of the IPSP. When Mg^{++} is the only divalent cation species in the medium, the IPSP is completely abolished. When Ca^{++} is the only divalent cation species, the IPSP is enhanced compared to that in normal saline. In an example shown in Fig. 6, the total divalent cation concentration was kept constant at 30 mM. A, B, and C show the IPSP when all divalent cations were Ca^{++}. As will be seen, single stimuli to the α-nerve produced clearly recognizable individual IPSP's. This does not usually occur in normal saline. The summated IPSP's in Ca^{++} saline, produced by tetanic stimulation with higher frequencies, attained a larger size, and a more rapid rate of rise than in normal saline. Fig. 6 D, E, and F show the absence of any recognizable IPSP's with Mg^{++} as the sole divalent cation species in the external medium. This absence was not due to a coincidence between the resting level and the reversal potential for the IPSP, because polarization of the membrane did not reveal any response either. Even with tetanic stimulation with a frequency of up to 100/ sec, the response could not be produced at all. The effect was reversible; when Ca^{++} was added to the outside medium the IPSP's reappeared promptly. It is not probable that the presynaptic axon stops firing in the Mg^{++} saline; because (a) the postsynaptic neuron fires in this medium and (b) even with a small amount (3–5 mM) of Ca^{++} in the outside medium, the small IPSP is produced with high frequency stimulation to the α-nerve showing that conduction along the presynaptic fiber is intact. The response then does not

resemble the presynaptic block; observed with high frequency stimulation the presynaptic block manifests itself as a dropping off of individual IPSP's, producing many notches at the top of the summated IPSP's.

Conductance increase (Δg) of the postsynaptic membrane during tetanic α-nerve stimulation is a continuous function of the outside Ca^{++} concentration. When the Mg^{++} concentration was kept constant, Δg was zero when Ca^{++} concentration was zero, and increased with increasing Ca^{++} concentration. The increase of Δg was large when the Ca^{++} was below 10 mM, but was smaller when the outside Ca^{++} was above 10 mM. With 80 mM Ca^{++}, Δg was about twice that in the normal saline.
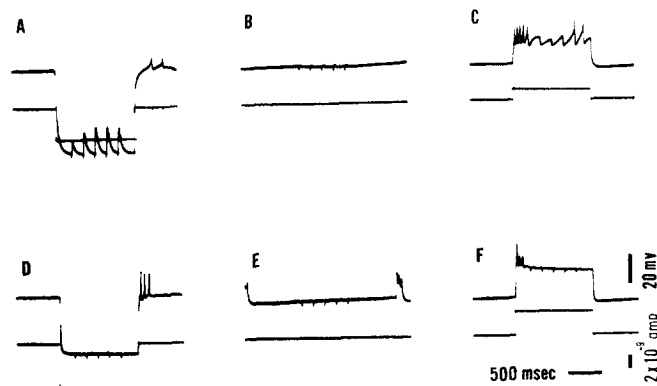

FIGURE 6. Effects of Ca^{++} and Mg^{++} on the IPSP. All records are from Gc. 4, cell 224. Upper beam, membrane potential. Lower beam, injected current. A–C, medium contained 30 mM Ca^{++} but no Mg^{++}. D–F, medium contained 30 mM Mg^{++} but no Ca^{++}. The α-nerve was stimulated five times with about 0.16 sec interval. KCl electrodes.

Effects of Sr^{++} on IPSP   When the usual divalent cations were replaced with Sr^{++}, the IPSP was enhanced. A prominent feature was that the falling phase from the summated IPSP became slower compared to that in normal saline (Fig. 7). It is possible that the action potential in the presynaptic terminal is prolonged and the release of transmitter by one impulse continues for a longer time.

In Sr^{++} saline, the membrane potential fluctuated through a larger amplitude, and the spontaneous activity was at a higher frequency. But the membrane resistance did not change appreciably, when measurement was made with relatively strong hyperpolarizing currents that suppressed completely the spontaneous fluctuation. The conductance increase on α-nerve stimulation was larger in Sr^{++} saline than that in the normal saline. The reversal potential was almost the same in the two types of saline.

The effect of Sr^{++} saline was stable and reversible; on returning to normal saline the IPSP returned to the original one.
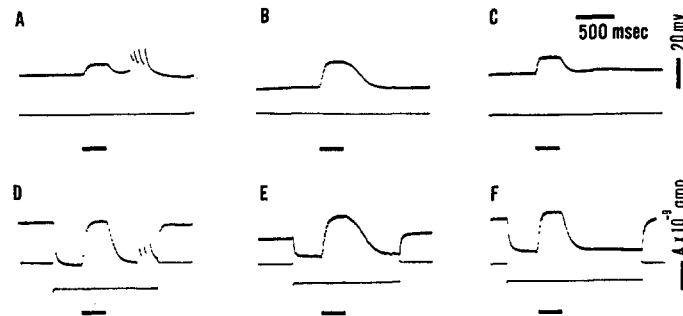
FIGURE 7. Effects of Sr^{++} saline on summated IPSP's. Upper beam, Gc. 5, cell 230. Lower beam, injected current. A and D, in normal saline. B and E, in Sr^{++} saline. C and F, after returning to normal saline. Tetanic stimulation was applied to the α-nerve at 100/sec for a period indicated by a bar below each record. KCl electrodes.

The Effect of Ba^{++} on IPSP When the divalent cations were replaced with Ba^{++}, a characteristic change took place in the responses to α-nerve stimulation (Fig. 8). The membrane resistance rose transiently, and the rate of rise of the summated IPSP became larger (Fig. 8 B). The falling phase of the summated IPSP became markedly slower, and eventually (Fig. 8 F) the IPSP persisted for at least 1 sec after stimulation. The membrane resistance fell again, and small fluctuations of the membrane potential took place on the steady level of the electrotonic potential (this is probably due to an irregular release of inhibitory transmitter from the presynaptic terminals). Then the IPSP disappeared suddenly, suggesting conduction block in the presynaptic element (Fig. 8 G). On returning to the normal saline, the usual IPSP appeared again (Fig. 8 H).
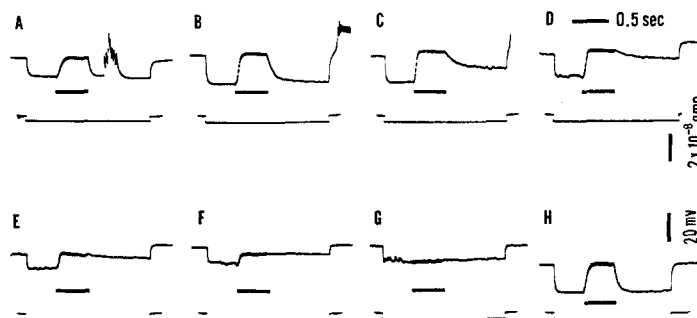
FIGURE 8. Effects of Ba^{++} saline on summated IPSP's. Upper beam, Gc. 5, cell 232. Lower beam, injected current. The bar below the current beam indicates the period of tetanic stimulation. In all records some hyperpolarizing current was applied to show the IPSP and to indicate the membrane resistance. A, the response in normal saline. B-G, time sequence after changing to Ba^{++} saline. Times after replacement: B, 1 min 10 sec; C, 1 min 30 sec; D, 2 min 10 sec; E, 4 min; F, 5 min 10 sec; G, 5 min 20 sec. Record H was taken after 5 min of washing with the normal saline. KCl electrodes.

The experiment shows that the presynaptic terminal can release transmitter in Ba^{++} saline as long as conduction persists in the presynaptic axon. Ba^{++} exerts a strong depolarizing action on the postsynaptic membrane. A similar depolarizing process probably takes place in the presynaptic axon and causes blockage of conduction. The very slow falling phase of the IPSP could probably be due to enhanced afterdepolarization of the presynaptic spike.

Effect of γ-Aminobutyric Acid (GABA) on the Cell Activity   GABA is known as a depressant of the activity of crustacean hearts (Enger and Burgen, 1957; Florey, 1957). At a concentration of 10^{-4} M it produced profound effects on the activity of the soma membrane of the *Squilla* heart ganglion. The spontaneous activity stopped completely. The membrane conductance more than doubled within 3 min. This was observed also in Mg^{++} saline, with almost the same amount of conductance increase as that in Ca^{++} saline. This is consistent with the view that GABA works on the postsynaptic membrane, whereas the divalent ions influence transmitter release from the presynaptic terminal. The effect of GABA on the inhibitory postsynaptic membrane in arthropods has now been extensively studied (Kuffler and Edwards, 1958; Boistel and Fatt, 1958; Grundfest, Reuben, and Rickles, 1959; Takeuchi and Takeuchi, 1965; Usherwood and Grundfest, 1965).

On application of GABA the antidromic spike was so reduced that the response finally became a barely noticeable deflection. Surface electrodes, however, picked up a conducted spike. This indicates that the receptors for GABA are distributed only on the soma and the proximal part of the axons, but not elsewhere on the axons.

Direct stimulation produced a graded spike in the soma. With stronger current application the amplitude attained as much as 60 mv. In spite of it, surface electrodes on the axon failed to pick up any observable response. Thus the soma activity is completely isolated from the other part of the neuron. This is analogous with the effect of α-nerve stimulation in Ca^{++}-rich saline (Fig 3 C and D).

Effects of Picrotoxin on the IPSP and on the Action of GABA   Picrotoxin is known to block effects of inhibitory nerve stimulation of the crayfish heart (Florey, 1957). Picrotoxin at a concentration of 10^{-4} M strongly reduced the amplitude of the summated IPSP of the *Squilla* heart ganglion. Without applying a hyperpolarizing pulse, the IPSP disappeared almost completely. On applying a hyperpolarizing pulse, some electrical effect of α-nerve stimulation was observable, but it was very small. In one experiment, the conductance increase (Δg) of the cell membrane with tetanic α-nerve stimulation was about 0.15 μmho. On adding picrotoxin, Δg was decreased to about 0.01 μmho (less than one-tenth). Recovery with normal saline was usually excellent.

Picrotoxin also inhibits the effect of GABA on the membrane. The reduced membrane resistance in GABA saline recovers almost completely on application of 10^{-4} M picrotoxin.

Thus picrotoxin is a blockader of the inhibitory transmitter action as well as of the GABA action on the soma membrane. This is in common with other arthropod inhibitory synapses (Van der Kloot, Robbins, and Cooke, 1958; Grundfest et al., 1959; Usherwood and Grundfest, 1965; Ozeki, Freeman, and Grundfest, 1966).

DISCUSSION

The intracellular potential change of the crustacean heart ganglion during inhibitory fiber stimulation was first recorded by Terzuolo and Bullock (1958). They found that the response was different according to the types of cells penetrated. When the cell was spontaneously active, the response was mostly biphasic. An initial depolarization was followed by a long-lasting hyperpolarization. With repetitive stimulation, the hyperpolarizing component summated and the over-all effect for tetanic stimulation was a shift of the membrane potential towards a hyperpolarized level. Only rarely was a purely hyperpolarizing response obtained.

When the cell was a pure follower, the response was a pure depolarization which might lead to excitation of the neuron.

The above behavior is not necessarily similar to that observed in the *Squilla* heart ganglion. Hagiwara (1961) points out that use of the KCl-filled intracellular electrodes might complicate the above observation, since intracellular accumulation of Cl^- ion changes a hyperpolarizing postsynaptic potential to a depolarizing one (Coombs et al., 1955). This does not, however, explain the biphasic time course of the inhibitory response observed in spontaneously active cells in the lobster heart. One possible explanation is that the postinhibitory polarization, described in crustacean stretch receptors (Kuffler and Eyzaguirre, 1955) and in the present material (see p. 912), is larger and more common in the lobster heart ganglion, and that the initial depolarization, the reversed IPSP, is followed by the hyperpolarizing postinhibitory polarization.

Our results on the membrane potential change during inhibition are more closely similar to those observed in the crustacean stretch receptor (Kuffler and Eyzaguirre, 1955; Hagiwara et al., 1960; Eyzaguirre, 1961). Inhibition of the discharge is produced by a shift of the membrane potential towards a steady level near the peak of afterhyperpolarization, and the shift is due to summated IPSP's. The antidromic spike may be reduced in amplitude, or may be blocked at some point from the soma during inhibition. The nature of the IPSP observed in this material is similar to that observed in other ar-

thropod nerve or muscle, including its pharmacological properties (see Edwards, 1960; Curtis and Watkins, 1965; Grundfest, 1966).

It has been reported many times that divalent cations in the external medium play an important role in transmission at excitatory synapses (Del Castillo and Katz, 1954; Takeuchi and Takeuchi, 1962). It has been shown here that the balance of Mg^{++} and Ca^{++} plays a crucial role also at the inhibitory synapse. Otsuka, Iversen, Hall, and Kravitz (1966) recently showed that the neuromuscular inhibitory process in the lobster is blocked by deficiency of Ca^{++} in the external medium.

In conclusion, the effects of inhibition in the *Squilla* heart ganglion by stimulation of the α-nerve are mediated mostly by the IPSP which has many features in common with other crustacean inhibitory synapses. In contrast, the acceleratory process in the *Squilla* heart ganglion is not associated with prominent production of EPSP's, as far as the observation is made at the neuron soma (Watanabe, Obara, and Akiyama, data to be published). An analogous asymmetry between acceleratory and inhibitory effects is observed in the vertebrate heart pacemaker, where stimulation of the vagus nerve produces a discrete hyperpolarizing response, but stimulation of the sympathetic nerve produces an increase in rate of rise of the pacemaker depolarization, but no discrete EPSP's (Hutter and Trautwein, 1956).

REFERENCES

ALEXANDROWICZ, J. S. 1932. The innervation of the heart of the crustacea. I. Decapoda. *Quart. J. Microscop. Sci.* 75:181.

ALEXANDROWICZ, J. S. 1934. The innervation of the heart of crustacea. II. Stomatopoda. *Quart. J. Microscop. Sci.* 76:511.

BENNETT, M. V. L. 1964. Nervous function at the cellular level. *Ann. Rev. Physiol.* 26:289.

BOISTEL, J., and P. FATT. 1958. Membrane permeability change during inhibitory transmitter action in crustacean muscle. *J. Physiol. (London).* 144:176.

BROWN, H. F. 1964. Electrophysiological investigations of the heart of *Squilla mantis*. I. The ganglionic nerve trunk. *J. Exptl. Biol.* 41:689.

COOMBS, J. S., J. C. ECCLES, and P. FATT. 1955. The specific ionic conductances and the ionic movements across the motoneuronal membrane that produce the inhibitory postsynaptic potential. *J. Physiol. (London).* 130:326.

CURTIS, D. R., and J. C. WATKINS. 1965. The pharmacology of aminoacids related to gamma-aminobutyric acid. *Pharmacol. Rev.* 17:347.

DEL CASTILLO, J., and B. KATZ. 1954. Quantal components of the end-plate potential. *J. Physiol. (London).* 124:560.

EDWARDS, C. 1960. Physiology and pharmacology of the crayfish stretch receptor. *In* Inhibition in the Nervous System and Gamma-Aminobutyric Acid. E. Roberts, editor. Pergamon Press, New York. p. 386.

Enger, P. E. S., and A. S. V. Burgen. 1957. The effect of some amino acids on the perfused lobster heart. *Biol. Bull.* **113**:345.

Eyzaguirre, C. 1961. Excitatory and inhibitory processes in crustacean sensory nerve cells. *In* Nervous Inhibition. E. Florey, editor. Pergamon Press, New York. p. 285.

Fatt, P., and B. Katz. 1953. The effect of inhibitory nerve impulses on a crustacean muscle fibre. *J. Physiol. (London).* **121**:374.

Florey, E. 1957. Further evidence for the transmitter function of Factor I. *Naturwissenschaften.* **44**:424.

Florey, E. 1960. Studies on the nervous regulation of the heart beat in decapod crustacea. *J. Gen. Physiol.* **43**:1061.

Frazier, W. T., E. R. Kandel, I. Kupfermann, R. Waziri, and R. E. Coggeshall. 1968. Morphological and functional properties of identified neurons in the abdominal ganglion of *Aplysia californica. J. Neurophysiol.* **30**:1288.

Furshpan, E. J., and D. D. Potter. 1959. Transmission at the giant motor synapse of the crayfish. *J. Physiol. (London).* **145**:287.

Grundfest, H. 1959. Synaptic and ephaptic transmission. *In* Handbook of Physiology. Sect. 1. Neurophysiology. I. J. Field, editor. American Physiological Society, Washington, D. C. p. 147.

Grundfest, H. 1966. Heterogeneity of excitable membrane: Electrophysiological and pharmacological evidence and some consequences. *Ann. N. Y. Acad. Sci.* **137**:901.

Grundfest, H., J. P. Reuben, and W. H. Rickles, Jr. 1959. The electrophysiology and pharmacology of lobster neuromuscular synapses. *J. Gen. Physiol.* **42**:1301.

Hagiwara, S. 1961. Nervous activities of the heart in crustacea. *Ergeb. Biol.* **24**:287.

Hagiwara, S., K. Kusano, and N. Saito. 1960. Membrane changes in crayfish stretch receptor neuron during synaptic inhibition and under action of gamma-aminobutyric acid. *J. Neurophysiol.* **23**:505.

Hutter, O. F., and W. Trautwein. 1956. Vagal and sympathetic effects on the pacemaker fibers in the sinus venosus of the heart. *J. Gen. Physiol.* **39**:715.

Kuffler, S. W., and C. Edwards. 1958. Mechanism of gamma-aminobutyric acid (GABA) action and its relation to synaptic inhibition. *J. Neurophysiol.* **21**:589.

Kuffler, S. W., and C. Eyzaguirre. 1955. Synaptic inhibition in an isolated nerve cell. *J. Gen. Physiol.* **39**:155.

Lee, B. D., and W. N. McFarland. 1962. Osmotic and ionic concentrations in the mantis shrimp *Squilla empusa* Say. *Publ. Inst. Marine Sci.* **8**:126.

Maynard, D. M. 1953. Activity in a crustacean ganglion. I. Cardioinhibition and acceleration in *Panulirus argus. Biol. Bull.* **164**:156.

Otani, T., and T. H. Bullock. 1959. Effects of presetting the membrane potential of the soma of spontaneous and integrative ganglion cells. *Physiol. Zool.* **32**:104.

Otsuka, M., L. L. Iversen, Z. W. Hall, and E. A. Kravitz. 1966. Release of gamma-aminobutyric acid from inhibitory nerve of lobster. *Proc. Natl. Acad. Sci.* **56**:1110.

Ozeki, M., A. R. Freeman, and H. Grundfest. 1966. The membrane components of crustacean neuromuscular systems. II. Analysis of interactions among the electrogenic components. *J. Gen. Physiol.* **49**:1335.

Takeuchi, A., and N. Takeuchi. 1962. Electrical changes in pre- and postsynaptic axons of the giant synapse of *Loligo. J. Gen. Physiol.* **45**:1181.

Takeuchi, A., and N. Takeuchi. 1965. Localized action of gamma-aminobutyric acid on the crayfish muscle. *J. Physiol. (London).* **177**:225.

Tauc, L. 1960. Evidence of synaptic inhibitory actions not conveyed by inhibitory postsynaptic potentials. *In* Inhibition in the Nervous System and Gamma-Aminobutyric Acid. E. Roberts, editor. Pergamon Press, New York, p. 85.

Terzuolo, C. A., and T. H. Bullock. 1958. Acceleration and inhibition in crustacean ganglion cells. *Arch. Ital. Biol.* **96**:117.

Usherwood, P. N. R., and H. Grundfest. 1965. Peripheral inhibition in skeletal muscle of insects. *J. Neurophysiol.* **28**:497.

VAN DER KLOOT, W. G., J. ROBBINS, and I. COOKE. 1958. Blocking by picrotoxin of peripheral inhibition in crayfish. *Science.* **127**:521.

WATANABE, A., S. OBARA, and T. AKIYAMA. 1967. Pacemaker potentials for the periodic burst discharge in the heart ganglion of a stomatopod, *Squilla oratoria. J. Gen. Physiol.* **50**:839.

WATANABE, A., S. OBARA, T. AKIYAMA, and K. YUMOTO. 1967. Electrical properties of the pacemaker neurons in the heart ganglion of a stomatopod, *Squilla oratoria. J. Gen. Physiol.* **50**:813.