Sodium- and Calcium-Dependent Spike Potentials in the Secretory Neuron Soma of the X-Organ of the Crayfish

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ABSTRACT Membrane characteristics of neuron somata in the medulla terminalis ganglionic X-organ of crayfish have been investigated with intra-cellular glass microelectrodes. The soma membrane developed action potentials with 10–20 mv of overshoot. Delayed rectification appeared at 10–20 mv above resting membrane potential. In 50% of the neuron somata examined, action potentials were observed in Na-free medium or TTX medium. The peak potential level of the spike in these media depended on the extracellular concentration of Ca ion. It increased with the Ca concentration. In low calcium media, the peak potential level of the spike varied with Na concentration. Action potentials of the X-organ–sinus gland tract disappeared after bathing in Na-free or TTX medium, suggesting that the conductive action potential was dependent on Na ions. From these results, it is concluded that there are two systems in the neuron soma, one of which responds to the Na ion and the other, to the Ca ion. Inhibitory innervation of the X-organ by the cerebral ganglion was manifested by IPSP's when the optic peduncle was stimulated. A postulated connection between the Ca-dependent spike and the release of hormone in X-organ neuron somata is discussed.

INTRODUCTION

The discovery of glandlike nervous elements in the eyestalk of crustacea initiated the physiology of neurosecretion of crustacea (Bellonci, 1882; Hanström, 1931, 1933). The cell cluster which is located on the medulla terminalis of the eyestalk of crustacea was found by Welsh (1941) and was named medulla terminalis ganglionic X-organ (M.T.G.X.) by Carlisle and Knowles (1959) in order to avoid confusion with other neurosecretory cell groups in the eyestalk. Cytological and electron microscopic studies (Enami, 1951; Passano, 1951a; Carlisle and Passano, 1953; Bliss, Durand, and Welsh, 1954; Potter, 1958; Hodge and Chapman, 1958; Knowles, 1959; Fingerman and Aoto, 1959) revealed that the M.T.G.X. is composed of several types of neuro-
secretory perikarya containing granules of from 300 to 3000 Å in diameter. The axons of these neurosecretory neurons terminate in the sinus gland in close association with the blood sinus. Physiological investigation of the X-organ with special attention to the function of chromatophorotropic, molt-inhibiting, and metabolic hormones led to the conclusion that the hormones are synthesized in the perikarya and passed along M.T.G.X. neuron axons to the sinus gland where they are stored and later released into the body fluid. Also, the fact that only the destruction of both the M.T.G.X. and the sinus gland causes the complete acceleration of molting or the change in the metabolism is suggestive that the site of release is close to the X-organ in addition to the sinus gland (Passano, 1951a; Bliss, 1951; Travis, 1951; Havel and Kleinholz, 1951).

Cytological changes in the X-organ perikarya of crayfish showing a molting cycle (Durand, 1956) strongly suggest that this cell group functions in the synthesis and release of the molt-inhibiting hormone in this animal.

In the nerve terminals of the pars nervosa and the chromaffin cells of the adrenal medulla in vertebrates, Douglas (1968) and his colleagues proposed a role for Ca ion. For hormone release Ca ions flow into the nerve terminals or chromaffin cells before the release of hormone in association with the electrical events at the nerve terminals or the chromaffin cell membrane. At any synapse, the necessity for influx of Ca ions shortly before the release of transmitter substance has been inferred (Katz and Miledi, 1967) and the electrical potential responsible for the regenerative movement of Ca ions at the nerve terminals has been clearly demonstrated (Katz and Miledi, 1969).

It is generally accepted that neurosecretory cells are neurons which early in their differentiation also acquire the characteristics of glandular cells (Scharrer and Scharrer, 1945). The neuronal characteristics of neurosecretory cells have been investigated with special attention to their electrical excitability (Potter and Loewenstein, 1955; Bennett and Fox, 1962; Ishibashi, 1962; Cooke, 1964; Kandel, 1964; Yagi and Bern, 1963; Ishikawa, Koizumi, and Brooks, 1966; Yagi, Azuma and Matsuda, 1966; Bennett, Gimenez, and Ravitz, 1968). From the work of these authors it has become clear that the neurosecretory neuron may generate and conduct action potentials in the same way as do ordinary neurons. Furthermore the work by Fridberg et al. (1966) indicated that the action potentials may be associated with the transport and release of hormone.

In the present paper, the ionic mechanism of spike initiation by the neurosecretory soma in the medulla terminalis ganglionic X-organ of crayfish was investigated with special reference to hormone release mechanisms. In the soma, a spike potential with overshoot can be initiated either by current applied to the soma membrane or by the intrinsic automatic mechanism. In half of the neurons examined, the spike height depended not only on Na ion
but also on Ca ion concentration around the neuron soma. There seem to be two systems in a neurosecretory neuron soma. The role of the inflow of Ca ion into the neuron soma is discussed in the light of hormone release mechanisms involved in this neurosecretory system, the X-organ–sinus gland complex. A preliminary account of the present study has appeared elsewhere (Iwasaki 1968; Iwasaki and Satow, 1969, 1970).

METHODS

The eyestalk of crayfish, Procambarus clarkii, was isolated from the male or female animal body. Average length of the experimental animal was 10–15 cm. After the exoskeleton surrounding the eyestalk was shelled off, the muscles around the stalk and the inner sac of the eyestalk were carefully removed. The medulla terminalis ganglionic X-organ, the axons running from the X-organ to the sinus gland, and the sinus gland were visible under the binocular microscope. After peeling off the thin connective tissue covering the X-organ, all the cell bodies of the X-organ became visible. The diameter and the color of the cell bodies differed from preparation to preparation. Often the cells were whitish in color but sometimes they were transparent. The diameter of neuron soma in the ganglionic X-organ ranged from 30 to 50 \( \mu \). The variability might depend on the season or on the stage of the hormonal cycle of the animal. Seasonal changes or sex differences in the electrical characteristics of the cell membranes were not detectable in the present experiments. The experiments were carried out from December, 1967, to July, 1968, and during the same season of 1968 to 1969. In our laboratory, molting behavior during which the carapace of crayfish becomes soft and/or the gastrolith in the stomach becomes more prominent, was conspicuous in the early spring to early summer. In this stage the cell body of the X-organ appears larger and more whitish in color. The relation between the molting cycle and the morphological change was not further investigated. Characteristic grouped discharge of single cells was observed, and this phenomenon may have some connection with the molting cycle (Iwasaki and Satow, 1969).

The eyestalk preparation was mounted in a pool (Fig. 1) which contained 0.7 ml of saline. The solution ran into the pool from a reservoir at the rate of 1.3 ml/min and drained off at the same rate through a filter paper (F) placed at the end of the pool. The test solutions contained in reservoirs (R) were diverted to the pool with stopcocks. The solutions share a common path 2 cm before the inlet of the pool, so that the new test solution arrived at the preparation within 3 sec after switching. The cluster of neuron somata of the ganglionic X-organ was placed facing the stream of solution, so that the new test solution hit and washed the surface of the neuron cluster.

**Electrical Measurements** Microelectrodes filled with 3 M KCl or 0.6 M K\(_2\)SO\(_4\) (E), resistance between 20 and 40 megohms, were used for recording the membrane potential change and for injecting the stimulating direct current by means of a bridge circuit (Araki and Otani, 1955). For recording the junction potentials a K\(_2\)SO\(_4\) electrode was used instead of KCl. The series resistance of the bridge circuit was 200 megohms. From one arm of the bridge the injected currents were monitored through
a 1 megohm resistor. A high input impedance amplifier with capacity compensation (MZ-3B, Nihon Koden Co., Tokyo, Japan) was used to record the membrane potential change.

In order to record the action potential of the axon extracellularly, a microsyringe suction electrode (M) was used. The needle of the microsyringe was covered with a polyethylene tube (P) 10 mm long and 200-300 μm in tip diameter. The sinus gland (S) with axons which come from several parts of the protocephalic organ, including the medulla terminalis ganglionic X-organ, and terminate in the sinus gland, was dis-
sected out of the medulla. The sinus gland–axon complex was sucked into the polyethylene tube and was lifted away from the medulla. With this device, spontaneous and evoked electrical activities from the axons could be recorded continuously for more than 24 hr.

**Stimulation** The tract from the protocephalic ganglion to the X-organ in the eye runs through the optic peduncle. This peduncle and consequently the tract was stimulated with a fluid electrode (Fig. 1A). The peduncle was passed through a slit in a partition plate in order to insulate the stimulating pools. After it was covered with the mixture of petroleum jelly and cotton wool, stimulating current was passed through an Ag-AgCl electrode (St). The responses to this stimulation were recorded either on the X-organ or from the sinus gland axons.

**Solutions** Van Harreveld solution was used as the standard saline. It was weakly buffered to pH 7.2–7.3 by sodium bicarbonate. In the Na-free solution, Tris was used instead of sodium bicarbonate (Table I). Tetrodotoxin (Sankyo Co. Ltd., Tokyo, Japan) $10^{-7}$–$10^{-8}$ g/ml or picrotoxin $10^{-5}$ g/ml was added to the standard or the modified solution in some experiments.

### Table I

<table>
<thead>
<tr>
<th>Solution Type</th>
<th>NaCl</th>
<th>CaCl₂</th>
<th>KCl</th>
<th>MgCl₂</th>
<th>Choline-Cl</th>
<th>NaHCO₃</th>
<th>Tris</th>
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<td>Standard</td>
<td>205.0</td>
<td>13.5</td>
<td>5.4</td>
<td>2.6</td>
<td>0</td>
<td>1-3</td>
<td>0</td>
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<tr>
<td>Na-free</td>
<td>0</td>
<td>13.5</td>
<td>5.4</td>
<td>2.6</td>
<td>205.0</td>
<td>0</td>
<td>2-3</td>
</tr>
<tr>
<td>Na-deficient (1)</td>
<td>10.0</td>
<td>13.5</td>
<td>5.4</td>
<td>2.6</td>
<td>205.0</td>
<td>0</td>
<td>3.0</td>
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<td>Na-deficient (2)</td>
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<td>13.5</td>
<td>5.4</td>
<td>2.6</td>
<td>195.0</td>
<td>1.0</td>
<td>0</td>
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<td>Ca-rich in Na-deficient (1)</td>
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<td>27.0</td>
<td>5.4</td>
<td>2.6</td>
<td>184.8</td>
<td>3.0</td>
<td>0</td>
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<tr>
<td>Ca-rich in Na-deficient (2)</td>
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<td>54.0</td>
<td>5.4</td>
<td>2.6</td>
<td>144.3</td>
<td>3.0</td>
<td>0</td>
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<tr>
<td>Low Ca in Na-deficient (1)</td>
<td>0</td>
<td>3.4</td>
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<td>2.6</td>
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<td>2.6</td>
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<td>1.0</td>
<td>0</td>
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<td>Low Ca in Na-deficient (3)</td>
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<td>2.6</td>
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<td>1.0</td>
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**RESULTS**

**General Characteristics of the Spike Initiation in Neuron Soma of the X-Organ**

When the microelectrode was inserted into the cell soma, visible under the binocular microscope, a resting potential of $-50$ to $-70$ mV appeared. When the membrane was depolarized with an outward current and the potential reached a certain level (Fig. 2), a spike developed with an overshoot of 10–20 mV. Accordingly, the spike height measured from the resting potential level ranged from 60 to 90 mV. Maximum rate of rise of the spike potential was
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32 ± 13 (SE) v/sec. Spikes that developed during the depolarizing current exhibited a rapid return toward the base line of the resting potential, indicating a terminal phase of high conductance for an ionic battery with an emf close to the resting potential and presumably caused by K activation. The half-

![Figure 2: Records of action potentials and currents injected into X-organ cell soma. In each pair of records except the lowest, the upper trace shows the current and the lower the potential induced in the cell soma. The frequency of the spikes, which had overshoots of 15 mv, increased with the current intensity. Because of the bridge circuit, a fraction of the spike potentials is seen on the current record.]

duration of the spike was defined as the duration at the level of 50% spike amplitude as measured from the summit of the spike to this trough. The half-duration ranged from 3.0 to 8.8 msec (mean 5.4 ± 1.7 msec) in the standard solution. No noticeable inflection in the rising phase of the spike was observed, but in 1 neuron out of more than 50 investigated, an inflection at which a component of the spike sometimes failed to develop was observed in a series of firings. Increasing the strength of the current injected caused the discharge
frequency to increase as shown in Fig. 2. Further increase of the strength resulted in a cessation of spike initiation after several spikes but the membrane potential fluctuated around the depolarized level. Further excitation was not possible. There was regular spontaneous firing or spontaneous grouped firing in some preparations. The nature of these spontaneous potentials was reported elsewhere (Iwasaki and Satow, 1969).

The point at which the electrode was inserted was verified in some experiments by using an electrode filled with fast green FCF (Chroma Gesellschaft, Germany) in addition to the 3 m KCl. After the electrical responses were recorded the dye was injected into the cell electrophoretically. Under the binocular microscope, a change in color of the cell body to bluish green was clearly observed, indicating that the microelectrode was in the cell soma rather than in the axon hillock or in another part of the X-organ.

**Current-Voltage Relationship in the Neuron Soma of the X-Organ**

Current-voltage relationships were measured in preparations bathed in solutions containing TTX (10^-7 g/ml) or in Na-deficient solutions. As described in the following section, solutions do not block spike initiation completely. The first one or two spikes occurred with applied depolarizing current but further repetitive spikes disappeared within a few minutes after introduction of the new solution. In a few preparations repetitive spikes during the applied current persisted in the TTX medium. The voltage corresponding to injected currents of 500-1000 msec duration was measured at the end of the current. In the hyperpolarizing direction, the current-voltage relationship was linear until the level of -110 mv of membrane potential. Delayed rectification was prominent for depolarizations >15-20 mv (Fig. 3). Membrane resistance calculated from the slope of Fig. 3 was 22 megohms in the hyperpolarizing direction and 4.4 megohms in the depolarizing direction at the fully rectified level. Membrane resistance ranged from 13 to 30 megohms with an average of 20 ± 5 se megohms with hyperpolarizing currents. The solid arrow indicates the level of spike initiation in tetrodotoxin and the dotted one indicates that in the normal solution. Measurements of membrane depolarization in normal solution utilizing currents which ordinarily produced repetitive spikes, were taken from cells in the overexcited state (refer to General characteristics), in which the membrane ceased to fire due to the excess depolarization.

**Spike Initiation in Na-Deficient or Na-Free Medium**

In about half the neurons after the standard solution was switched to Na-deficient solution, within 1 or 2 min the threshold for firing increased to some extent and the repetitive firing with sustained depolarizing current disappeared. Only the initial spike was evoked by the applied current but sometimes oscillatory potentials followed the spike. The amplitude of the spike
became smaller in many cases and the maximum rate of rise decreased in general. In the case shown in Fig. 4, the spike height changed from 93 to 58 mv and the maximum rate of rise decreased from 33 to 8.8 v/sec. Thethresh-

old increased without exception. The spontaneous potentials mentioned in the first section of the Results disappeared within a few minutes after the application of Na-deficient solution. Since the cells were exposed to the bath and the new solution streamed to the cell soma directly (Fig. 1 and Methods), the
Figure 4. Spike initiation in a Na-deficient medium. Left column, spike potentials (upper trace) in the normal medium (206 mM Na) induced by injected current (lower trace). Frequency of the repetitive spikes increased as the strength of the stimulating current increased. The first spike of a train is shown at a faster sweep at the bottom. Right column, 18 min after application of Na-deficient solution (3 mM Na). The first spike remained but it was of smaller amplitude, while the repetitive spikes disappeared. Increase in the threshold is evident on comparison of the two top records. At the faster sweep, the first spike is superimposed on the hyperpolarizing potential. Time and current calibration (T, \(I\)), 500 msec (25 msec for the bottom) and 5.3 namp. Voltage calibration (V), 56 mv for the left and 50 mv for the right column.

Medium surrounding the soma must be exchanged within a few seconds. The time for the replacement of the solution after the Na-free solution was switched in was tested by measuring the Na concentration near the cell body every minute by means of a flame photometer or with a selective ion electrode (Beckman). Na concentration near the cell body declined from 208 to 2 mM in the first minute and it took only 5 min to reach 0.6 mM. The records in
Fig. 4 were taken 18 min after the solution was changed. It is unlikely that any significant concentration of Na ions remained to play a role in generating so large an action potential. The decrease in the number of spikes responding to the sustained depolarizing current in the Na-deficient medium or in TTX (following section) might be due to the difference in some accommodation process involved in the spike initiation. The alternative explanation would be an increase in the refractory period which was often observed in Na-deficient or TTX media.

In the Na-free solution, additional changes occurred. The effective membrane resistance decreased within 10–20 min after Na was replaced either by choline or by sucrose to less than 50% of that in the standard solution (208 mM Na). Consequently, a strong depolarizing current was necessary to excite the membrane. Because of imbalance of the bridge circuit after the insertion of the electrode and also because of rectification in the electrode during the strong current, quantitative experiments could not be performed in the Na-free solution after 10–20 min. However, before the change in the membrane resistance appeared, enough records were taken in several preparations to corroborate the results obtained in Na-deficient solutions. The quantitative change in the threshold, decrease in the maximum rate of rise of spike, and
decrease in the number of discharges were the same as in Na-deficient solutions. The mechanisms involved in this reduction of the resistance are not clear.

**Spike Initiation in TTX Medium**

Tetrodotoxin (TTX), puffer poison, is an agent which selectively blocks the increase in permeability for Na ions and consequently spike development.

![Soma spike initiation comparison](image)

**Figure 6.** Comparison between effects of TTX and Na-free medium on soma spike initiation. From left to right in succession: normal, TTX $10^{-7}$ g/ml, normal, Na-free, and normal. Stimulating current injected in B was stronger than in A and records with 20 times faster speed are shown in C. The hyperpolarizing potential is superimposed on the spike (C) of Na-free medium. In D, hyperpolarizing potentials are shown in each medium to demonstrate the conductance change in Na-free medium. Extracellular action potentials of the X-organ–sinus gland tract are shown in the lower trace in each record of A, B, and C. Although the spike potentials corresponding to the soma spike were hard to recognize because of the spontaneous discharges in A and B, they can be seen in the middle row (normal medium) of C. Larger spikes in the extracellular record were not the spikes in question. Voltage calibration, 50 mv for soma potentials and 0.7 mv for extracellular tract potentials. Time calibration is 500 msec except for the C record and either end of the D record. Calibration for C is 25 msec and for both sides of D is 2.2 sec.

(Narahashi, Moore, and Scott, 1964). An application of TTX $10^{-7}$ g/ml to the X-organ cell soma brought about an increase in the threshold, decrease in the spike amplitude, and decrease in the number of spikes during a depolarizing current, as in the case of Na-free solutions. One example is shown in Fig. 5, where the intensity of stimulating current was increased from top to bottom. The records taken at the same stimulus strength are arranged in the same row. In TTX, increase in threshold was evident in this figure and in Fig. 3. In Fig. 6, the effect of TTX is compared with the effect of a Na-free solution.
The records in row C show the first spike potential taken with expanded sweep speed. The records in D are the hyperpolarizing potentials elicited by inward currents, which indicate the effective resistance in each medium. The sweep speeds of the first and the last records in D are slower than the others. The decrease in the effective resistance in the Na-free solution, mentioned in the previous section, is clearly shown in this figure. Withdrawal of Na ions from the medium brought about a similar effect on spike initiation as did the application of TTX. Although a slight difference in the delayed rectification was seen between the TTX and Na-free records, it is probably not significant considering the time (approximately 2 hr) that elapsed between the two series of experiments. It usually takes more than 40 min for the cells to recover from TTX $10^{-7}$ g/ml.

The spike potentials appearing in the Na-free medium were reversibly blocked by the application of 10 mM MnCl$_2$. Examination of the effect of divalent cations remains to be performed in the future.

Not all neurons develop action potentials in Na-deficient (or Na-free) or TTX media. 17 neurons out of 36 in Na-deficient (or Na-free) media and 7 out of 15 neurons in the TTX medium did exhibit action potentials. In the rest of the cells, the action potential disappeared with the change of the solution. Small extracellular axon potentials in the normal medium are shown in Fig. 6 under each soma potential (see the third column). They correspond to the soma spike in one-to-one fashion. Often large spikes were superimposed on the small spikes which corresponded to the soma action potentials. The relation between the extracellular axon potentials and the spike potentials of soma will be demonstrated in a later section.

Role of Ca Ions in the Spike Initiation in Na-Free Medium

Ca ions can be a charge carrier for spike electrogensis when the major cation, Na, is omitted from the medium in amphibian sympathetic ganglion cells (Koketsu and Nishi, 1969), in squid giant axons (Watanabe et al., 1967), and in molluscan ganglion cells (Geduldig and Junge, 1968). In some invertebrate muscle fibers and vertebrate smooth muscle fibers, Ca ion is the principal ion carrying the charge and it initiates the spike potential regardless of the presence of Na ion (Nonomura et al., 1966; Bennett, 1967; Brading, Bülbring, and Tomita, 1969; Fatt and Katz, 1953; Fatt and Ginsborg, 1958; Hagiwara, Chichibu, and Naka, 1964; Hagiwara and Naka, 1964).

The effect of Ca ion on spike initiation was investigated in the present experiments utilizing the soma of the X-organ cells which can initiate action potentials in the Na-free or in the TTX medium. After the preparation had been poisoned by TTX $10^{-7}$ g/ml, the Ca ion concentration of the medium was changed successively to 54, 27, and 3.4 mM and restored to the normal
Ca concentration (13.5 mM), keeping the concentration of other ions constant (Fig. 7). The membrane potential and the effective resistance of the cell membrane were -56 mv and 26 megohms, respectively, in this particular cell in the normal solution. Change in the membrane potential was not conspicuous but reduction in the effective resistance always occurred in the low Ca medium. Because of the delayed rectification in the direction of depolarization (see the section on I-V relation), no noticeable change in the effective resistance could be seen in Fig. 7. The spike height increased with higher Ca concentration and it decreased with lower Ca concentration.

![Figure 7](image_url)

**Figure 7.** Effect of Ca ion concentration on the spike potentials in TTX (10⁻⁷ g/ml). The calcium concentration was as indicated at the top of each column. The current is shown on the lower trace in each record. The membrane was held during the experiment at -66 mv by the injecting current. The resting potential of this neuron was -52 mv. The spike in 13.5 mM Ca is shown at a 20 times faster sweep in the lower right.

The same kind of experiment, in which the Ca concentration of the medium was changed, was carried out in Na-deficient solution (Fig. 8). In this preparation a Na concentration of 3 mM was constant throughout the experiment in order to keep the membrane resistance unchanged (see the preceding section). In many cells the peak potential level of the spike in Na-deficient or TTX medium increased with the stimulus current. In standard medium the peak potential of the spike does not change with the stimulus current (indicating larger conductance during the spike potential compared with the resting conductance). It can be inferred that in the Na-deficient or TTX medium the increased conductance responsible for the spike potential is not so much larger than the conductance of the resting membrane that the IR drop caused by the passed current is wiped out at the peak of the spike potential. In Fig. 9 the peak potential level of the spike during the stimulating
current of 2.5 namp was plotted against the Ca concentration in a TTX medium. While with the stronger stimulating current the peak potential level became higher, the slopes with change of Ca concentration were nearly the same. They were 25–28 mV per 10 times the change in Ca concentration. Although the peak potential level of the spike with a given stimulus strength would depend on the ratio of the conductance during the spike to the conductance of the resting membrane, from this figure a certain contribution of Ca ions to the spike potential could be inferred.

**Effect of Na Concentration on the Spike Potentials in Low Ca Medium**

As already shown in the previous section, reduction of the Na concentration in the medium brought about only a slight change in the spike height, as long as a certain amount of Ca ion was present. In the presence of Na ion, likewise, the amplitude and peak potential of the spike did not change after the Ca concentration of the medium was reduced to 2–4 mM from the stand-
ard (13.5 mM). When the Na ions were varied (replaced by choline, see Methods) in a low Ca medium, the peak potential level of the spike changed within 3 min after the change of the Na concentration. The peak potential level of the spike in relation to the Na concentration is plotted in Fig. 10. Ca concentration was lowered to 4 mM in these preparations. In the lower Na concentration media, the peak potential level did not decrease linearly, and sometimes increased, suggesting the Ca ion component and the competitive action of Na ion vs. Ca ion (see next section). The slopes of a 10-fold change of the Na concentration were estimated from the points of 200 and 100 mM of Na concentration. They ranged from 33 to 51 mv. In Fig. 10, the slope was 50 mv for A and 51 mv for B.

**Na Concentration and Spike Potential in TTX Medium**

The Na concentration was changed from normal (205 mM) to 10 mM in the TTX medium as shown in Fig. 11. The lower beam in every record is the extracellular axon potential. After the application of TTX 10\(^{-7}\) g/ml, threshold increased as mentioned previously. The depolarizing current shown in the second row brought about delayed rectification instead of spike initiation. Further increase in the stimulus strength (the third row)
resulted in spike initiation. With expanded sweep speed, depolarizing and hyperpolarizing potentials are shown with the same stimulus strength as in row three. Because the unbalanced bridge circuit occurred between the normal and TTX solution, surge potentials appeared in the initial part of the record and the peak potential did not indicate the absolute potential value.

**Figure 11.** Effect of Na ion on the spike initiation in the TTX medium. Na concentration was decreased from 205 to 10 mM in the TTX $10^{-7}$ g/ml medium. In the lowest middle record, hyperpolarizing potential was superimposed on the depolarizing potential with spike. Because of imbalance of the bridge circuit after the insertion of the electrode, an initial surge was prominent in the figure. Action potentials of X-organ-sinus gland tract (lower beam of each record) were seen in the normal medium. Voltage calibration of 80 mV is for the soma potentials and 0.1 mV for the tract potentials. Time calibration of 25 msec is for the lowest record.

In the Na 205 mm–TTX medium, 95% of the sodium ions were replaced by choline (right column). A very slight increase in the rate of rise and the amplitude was often observed. This figure clearly shows dissociation of development of delayed rectification from spike initiation in the TTX medium.

**Axon Spike in the Na-Free or TTX Medium**

The axon spike corresponding to the soma action potential could be recorded among the spontaneous action potentials (Fig. 12). This correspondence is
recognizable, although it was often not easy to distinguish the particular spike when the spontaneous firing was very frequent (Fig. 6). In Fig. 6, the axon potentials were much smaller than those of the frequent spontaneous spikes so that only the axon potentials in the first and the second rows of the middle column (normal solution) can be recognized in the figure.

Figure 12. Soma and tract potentials. The soma spike and the corresponding extracellular action potentials of the tract were recorded in three preparations (A, B, and C). In C₁, spike potentials corresponding to the soma spike are marked by dots; the spontaneous spikes of another fiber are also seen in the trace. Between C₁ and C₃, TTX $10^{-7}$ g/ml was applied. After this procedure, spike potentials corresponding to the soma spike as well as the spontaneous one disappeared whereas the soma spike was still prominent. Two records before and after the application of TTX are superimposed in C₃. Note the increase in threshold after TTX. Calibration, 50 mv and 20 msec for A and C, 50 mv and 10 msec for B.

The difference in latency between the soma action potential and the axon potential was not clear. In the preparation of Fig. 6, the axon potential appeared a little after the beginning of the rise of the soma action potential when the stimulating current was passed through the electrode inserted into the soma. In the case of Fig. 11, the peak of the axon spike arose close to but clearly before the peak of the soma spike. The axon potential was seldom observed to appear after the summit of the soma spike. From this fact it appears unlikely that the axon spike is triggered by the action potential evoked in the soma by an applied current.
In TTX and in Na-free solutions, the axon potential disappeared immediately and recovered when the standard solution was restored (Fig. 6). The axon potentials of Figs. 11 and 12 C also disappeared in TTX or in Na-free medium as in Fig. 6. Disappearance of the axon potential in TTX or in the Na-free medium occurred without exception, while the action potential in the soma remained.

**Innervation of the X-Organ by the Nerves through the Optic Peduncle**

Stimulation of the optic peduncle evoked a small depolarizing junction potential in the soma. The latency was about 5 msec indicating a conduction velocity roughly of the order of 1–1.5 m/sec since the distance between the stimulus point and the recording site was 5–7 mm. The amplitude of the junction potential changed with the membrane potential when polarizing current was passed. The reversal potential was very close to the resting potential as shown in Fig. 13 A and B, and would appear to be an inhibitory postsynaptic electrogenesis. No excitatory junction potentials from the optic peduncle were observed.

Picrotoxin supplied evidence for the inhibitory nature of the junction potential. It has been known as an agent to block Cl and/or K ion permeability, and especially to block the inhibitory junction potentials. This agent (10⁻⁵ g/ml) blocked the junction potentials of the X-organ evoked by the stimulation of the optic peduncle and also the miniature potentials which are often seen when the membrane potential is hyperpolarized.

**Figure 13 A.** Junction potentials evoked by the stimulation of the optic peduncle and recorded with a K₂SO₄ electrode in the three membrane potential levels, -60, -75, and -88 mv. The resting potential was -78 mv. Calibrations, 5 mv and 1 sec. Fig. 13 B. Junction potentials are plotted against membrane potentials shifted by the application of the current through the recording electrode. Hyperpolarizing junction potentials are plotted as negative.
DISCUSSION

In the neurosecretory systems, initiation of the action potential in the cells has been reported in invertebrates and vertebrates (Yagi and Bern, 1963, in leech; Frazier et al., 1967, in Aplysia; Cooke, 1964, in crab; Yagi et al., 1966, in rat; Ishikawa et al., 1966, in cat; Bern and Yagi, 1964, in fish). Membrane characteristics of neurosecretory neuron soma have been investigated in vertebrates (Kandel, 1964; Bennett and Fox, 1962; Ishibashi, 1962). In these papers it has been reported that neuroendocrine cells seem to have electrical membrane properties that are similar to those of neurons in the central nervous system. In invertebrates, membrane characteristics in neurosecretory neurons have been definitely demonstrated in the present paper. The secretory neuron soma of the X-organ of crayfish has electrical properties similar to those of the ordinary excitable neuron in the resting state (resting membrane potential, membrane resistance) and in the active state (spike initiation with overshoot, delayed rectification, and repetitive firing by a dc depolarizing current, etc.). However, in the active state, the secretory neuron seems to have a mechanism which responds to the Ca ion content of the medium in addition to the one responding to the Na ion.

It is evident that the neuron soma of the X-organ of crayfish initiates the action potential in Na-free or TTX media in which Na spike electrogensis is abolished and that the spike is modified by the Ca ion concentration of the medium, indicating the increase in permeability for Ca ions. The system which is responsive to the Ca ion concentration can exist together with the Na system in the same cell. Two such components of the spike potential have been found recently in the invertebrate neuron soma (Geduldig and Junge, 1968) and in the vertebrate ganglion cell soma (Koketsu and Nishi, 1969) as well as in the cardiac muscle fiber (Hagiwara and Nakajima, 1966; Reuter, 1967; Rougier et al., 1969).

Although the soma of the neurons in the X-organ can generate a spike in the absence of Na, relying upon a gradient for Ca, in all the axons the spike electrogensis appears to require the presence of Na. The axon spikes are always blocked by TTX, whereas the Ca-dependent spike electrogensis of the soma is not blocked. Thus, it seems that a gradient for Na is essential for the electrogensis of the conducted response in the axon.

Ca ion movement to the inside of the cell and a critical reaction with vesicles or granules which discharge their transmitter or hormone content have been proposed by investigators of both neurotransmitter release and of neurosecretion (Katz and Miledi, 1967, 1969; Douglas and Rubin, 1961; Douglas and Poisner, 1964; Douglas, Kanno, and Sampson, 1967; Douglas, 1968). In the X-organ–sinus gland complex, the sinus gland is considered to be the site of storage and release of neurosecretory materials (review by Gabe,
Axons which arise out of the cell somata of the X-organ run, for 1 mm in the crayfish, to the sinus gland. The existence of a Ca component in the soma spike implicates the entry of Ca ions during the spike potential in the soma or near the soma. Since the potentials recorded by the electrode inserted in the soma do not distinguish the spikes initiated at the soma itself from the spikes initiated near the soma, it is difficult at present to decide the spike at which the regenerative Ca entry takes place. Electron microscopy reveals an abundance of capillaries in the region of the neck of the cell body (Uchizono, unpublished data). If this be true, together with the electrical evidence it can be deduced that the Ca entry takes place at or near the soma and that this might be responsible for the release of hormone in the X-organ as well as in the sinus gland. Further study of this problem is necessary.

Nearly 50% of the neuron somata examined in the X-organ did initiate the action potential in a Na-free or TTX medium and were affected by the Ca concentration of the medium. The rest of the cells responded only to Na ions. Identification of neurons responding to Ca ions was not possible by morphological or topographical criteria; differences might depend on season or the stage in the molting cycle. There were somata which did not show action potentials in a Na-free medium and which sent their axons to the sinus gland where the axon potential corresponding to the soma spike in the normal medium was recorded. Moreover, there were cases in which no action potentials were recorded from the sinus gland tract while the somata fired vigorously. One cannot consider, however, that the latter neurons do not send their axons to the sinus gland, because the recording system used for the axons might easily miss a spike of a particular axon. Further topographical study is necessary in connection with the endocrine function of these cells.

Competitive action between the Na ion and the Ca ion for the spike potential could be surmised from the experiment in which the Na concentration was changed in the TTX medium (Fig. 11). The greater rate of rise and the greater amplitude in the lower Na concentration are phenomena indicative of the competition between Na and Ca ions. The competitive action between Na and Ca is also noted in the process of transmitter release at the neuromuscular junction (Colomo and Rahamimoff, 1968; Kelly, 1965).

In the normal medium there were no definitive differences in the latency between the axon spike and the soma spike. The axon spike arose in many preparations slightly after the foot of the soma action potential and reached the peak before the peak of the soma spike. Preparations which showed an axon spike after the peak of the soma action potential were seldom observed. The distance from the soma to the sinus gland was about 1 mm. It would take 1 msec for the spike propagation, if one surmises the conduction velocity to be 1 m/sec. Since the conduction time is short compared to the time
course of the soma action potential, it might be hard to decide where the axon spike originated. However, it can at least be safely said that the axon spike starts much before the point at which the peak of the soma potential develops. There are some neurons in which the spike potential develops initially in part of the axon rather than in the soma (Coombs, Curtis, and Eccles, 1957; Edwards and Ottoson, 1958; Tauc and Hughes, 1963).

Stimulation of the optic peduncle induced inhibitory junction potentials on the X-organ of crayfish exclusively. Although picrotoxin blocks the junction potentials as well as spontaneous miniature potentials, it cannot be deduced at present that we are dealing with a transmitter mechanism similar to those already demonstrated for this animal (Boistel and Fatt, 1958; Kuffler, 1960; Takeuchi and Takeuchi, 1965, 1967), because there are definite differences between the equilibrium potentials for IPSP's and GABA potentials (unpublished data). Excitatory synaptic mechanisms probably are also present because some spontaneous synaptic noise remained after the application of picrotoxin.

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