Effects of Some Organic Cations on Generator Potential of Crayfish Stretch Receptor

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ABSTRACT The generator potential of both slowly and rapidly adapting crayfish stretch receptor cells can still be elicited by mechanical stimuli when all the Na of the bathing medium is replaced by various organic cations. In the presence of tris(hydroxymethyl)aminomethane (Tris), the generator potential is particularly large, about 30–50% of that in the control saline, while spike electrogenesis of the cell is abolished. Persistence of the generator response is not due to retention of Na by a diffusion barrier, and ionic contributions to the electrogenesis by Ca and Cl can also be excluded. Thus, whereas the electrogenesis of the generator membrane must be due to an increased permeability to monovalent cations, the active receptor membrane appears to be less selective for different monovalent cations than is the receptor component of some other cells, or the conductile component of the stretch receptor neuron.

INTRODUCTION

The stretch receptor neuron of crustacea is the only biological mechanotransducer system that allows intracellular observation of receptor or generator potential (Eyzaguirre and Kuffler, 1955 *a*). Ionic requirements for the generator potential were investigated by Terzuolo and Washizu (1962) and by Edwards et al. (1963), who concluded that the generator potential is produced by a conductance increase to Na and to either K or Cl, or to both. In other types of receptors only Na has been proved to be indispensable for electrogenesis (Ottoson, 1964; Diamond et al., 1958).

The present study stemmed from the work described in a previous paper (Obara and Grundfest, 1968), in which it was found that the stretch receptor of crayfish could still produce a considerable depolarization in response to stretch after all the Na in normal saline was replaced by Tris as well as Li. As will be described, several organic cations which are not effective substitutes for Na in spike electrogenesis of this cell can substitute for Na in producing a

generator potential. The anionic contribution to generator potentials appears to be negligible. Some of the relevant characteristics of the stretch receptor were also reinvestigated. A preliminary report has appeared (Obara, 1967).

METHOD

The material and the experimental arrangements were almost identical with those described in the previous paper (Obara and Grundfest, 1968).

Intracellular microelectrodes were filled with 3 M KCl and had a resistance of 10–40 MΩ. In the series of experiments in which the chloride concentration of the bathing media was modified, the microelectrodes were filled with 3 M potassium acetate. Recording and stimulating equipment was standard for the laboratory. In order to pass current intracellularly, the cell was penetrated with a separate microelectrode. In most cases, a constant current was obtained with the aid of a feedback circuit (Nakajima, 1964).

Preliminary experiments showed that a change in the fluid surface level could modify sensitivity of the cell to stretch, sometimes to a considerable extent. Therefore, in changing the outside solution a procedure was adopted which would maintain the fluid surface always at about the same level, while keeping the electrodes inside the cell. The chamber formed two pools that were connected by a narrow central platform on which the cell body was placed. A test solution in volume at least 10 times that of the chamber was introduced into one pool, and the chamber was drained simultaneously by suction applied at the other pool. Complete flushing took 5–8 min, and a steady state in the response was obtained within this period (see Results). In some cases, the complete exchange of a solution with respect to Na and K was confirmed by flame photometry.

Solutions

The standard saline was the same as that used in the previous work, except that magnesium was omitted without discernible difference in the results, the modified version being, in mmoles/liter, NaCl 205, KCl 5.4, $CaCl_2$ 13.5, and Tris HCl buffer 2.0 (adjusted to pH 7.4).

In test solutions, sodium was replaced on an equimolar basis by one of several monovalent organic cations, other constituents being unchanged. Tris saline was prepared from a stock solution which contained 1.0 mole/liter tris(hydroxymethyl) aminomethane (Tris), neutralized with HCl to pH 7.4. In this stock solution about 84 % of the Tris was neutralized (Bates, 1961), and accordingly the osmolarity should be less than 10 % lower than that of an equimolar NaCl solution. Hydrazine saline was prepared to pH 6.8, because of lower ionization of hydrazine chloride at higher pH. Variation of pH in the range between 6.8 and about 8.0 in the normal saline was without effect on the cell.

Sucrose saline had 364 mM sucrose in place of NaCl in the normal saline. In Ca saline, NaCl was replaced by 147 mM $CaCl_2$. Intermediate concentrations of a given Na substitute were obtained by mixing a test solution with either the control saline or sucrose saline.

In one series of experiments, the chloride of the normal saline was replaced by acetate or isethionate, and these solutions are designated as acetate saline and isethionate saline, respectively. A particular combination of a cation and an anion was designated according to the major substitutes in the saline, e.g., Tris acetate saline. The buffer was added also as 2.0 mM Tris acetate.

Control Responses in Normal Saline and General Criteria

Average values in the membrane and action potentials of the receptor were identical with those in the previous study (Obara and Grundfest, 1968). The afterpotential following the spike in the completely relaxed cell was generally depolarizing.

Since, during most of the experiments, a cell was usually subjected to a series of solutions with the microelectrode kept inside the cell, the change in membrane potential on changing the solution was followed by recording differentially between the intracellular and the low-resistance external microelectrodes. The sequential changes of the steady-state membrane potential in test solutions, if there were any, were then compared with the membrane potential level during an intervening period in control saline, and also with the final measurement when the electrode was withdrawn from the cell.

As the level of the afterpotential depends on the membrane potential (Eyzaguirre and Kuffler, 1955 *b*) and also on external potassium concentration (Nakajima and Takahashi, 1966), both the afterpotential and the spike amplitude serve as helpful measures of the membrane potential in the intervening periods in control saline. This method leaves some uncertainty as to the measurements of the resting potential in the test solutions, while confirming the reversibility of the responses. In some cases, such as that shown in Fig. 6, the electrode was withdrawn with the cell still in the test solution to verify the observed change in the potential.

The definition of the *resting length* of the receptor organ and the restriction as to stimulus range adopted in the previous paper similarly apply to the present work. The control generator potential was obtained in the presence of TTX (tetrodotoxin) (Loewenstein et al., 1963).

In view of the presence of an equilibrium level for the generator potential and also because the current voltage characteristic of the membrane is nonlinear (Fig. 2), the linear relation of the generator response vs. stretch (Terzuolo and Washizu, 1962) must be considered an approximation. However, it holds fairly well within the range of stretches used in the present work.

As a routine, a series of stretches was applied, and the stretch-response curves in various solutions were compared in order to minimize an effect of possible fluctuation of the response to a given stretch. This method is tedious and often tends to lead to a gradual or sudden deterioration of the cell, but it was preferred in order to exclude any possibility of the so-called "movement artifact," which is not related linearly to the stretch and is mostly irregular and irreversible (see Eyzaguirre and Kuffler, 1955 *a*).

Unless otherwise stated, all the data described in this paper were collected only from the cases which showed completely reversible responses.

RESULTS

Effect of Tris on Generator Potential

In the course of the studies with lithium described in a previous paper (Obara and Grundfest, 1968), Tris Cl was tentatively used as an inert substitute for sodium chloride, primarily in order to obtain a rough estimate of the time that was necessary to allow complete replacement of the chamber by a test solution.

The spike, as a rule, was abolished within the first minute after starting perfusion with Tris saline. The membrane potential increased transiently by 5–8 mv, but it had returned to the original level by the time perfusion was completed. When the Tris saline was replaced by the standard saline, return of the spike was similarly rapid. There was also a transient depolarization which induced spontaneous firing. Thus, the new steady states following change of the bathing medium appeared to be reached within a few minutes, even less than the time required for completion of the perfusion.

However, it was surprising to find that the cell gave a small but definite response to stretch while in the Tris saline (Fig. 1 C). The amplitude of the stretch response in Tris saline varied from cell to cell, but in more than 15 completely reversible cases, the response could be one-half to one-third of that in normal saline. Its linear dependence on the amount of stretch as well as the time course appeared practically identical with those in the control, though the after-hyperpolarization phase frequently observed in Na saline (Eyzaguirre and Kuffler, 1955 *a*) was absent or was converted to a small depolarization (Fig. 1 C).

1. ROLE OF A DIFFUSION BARRIER Workers studying the receptor electrogenesis of muscle spindles (Ottoson, 1964) and Pacinian corpuscles (Diamond et al., 1958) have suggested that the persistence of the potential long after removal of Na is due to a diffusion barrier which prevents complete removal of Na from the vicinity of the cell membrane. In order to explore the possibility of such a barrier at the generator membrane in the present material, the following experiments were carried out.

a) *Current-Voltage Relation after Various Times in Tris Saline* The block of the antidromic spike described above merely indicates that sodium concentration has become too low to maintain conduction along the axon. When a part of the sodium chloride in normal saline was replaced by either sucrose or Tris Cl, the antidromic spike in the soma became progressively smaller and was delayed. Invasion of the soma was blocked at a sodium concentration of less than 20% of normal, leaving electrotonic spread of the axon spike, which, in turn, was completely abolished with less than 10% sodium outside. Since

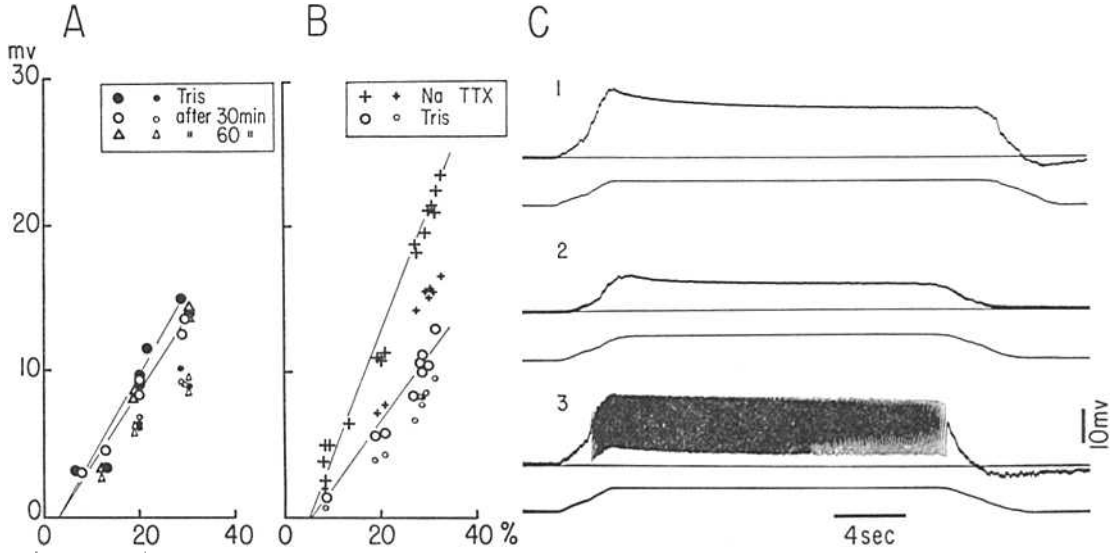

FIGURE 1. Effect of Tris saline on the generator potential; slowly adapting cell, in this and other figures, except where otherwise stated. A, B: abscissa, per cent stretch; ordinate, generator potential. In this and subsequent stretch-response graphs, the large symbol in a given solution shows the initial peak of the generator potential and the small one the quasi-steady state level.

A. The first set of measurements (filled circles) was made immediately upon completing the perfusion with Tris saline. The chamber was then again perfused with this solution. Measurements made after 30 min and 60 min in Tris saline showed no decrease in generator potential.

B. Comparison of the amplitudes of generator potentials in Na saline with TTX and after replacement by Tris saline.

C. Sample records in the order of changing solutions from the series graphed in B: 1, Na saline with TTX; 2, Tris saline; 3, Na saline. Spike electrogenesis was restored, but peaks of the spikes are not seen at the high amplification of these recordings. Note also that the after-hyperpolarization which was present in the slowly adapting cell in Na saline (1 and 3) was absent in the Tris saline. The upper trace in each record shows the response to the stretch monitored in the lower trace, in this and similar recordings in the subsequent figures.

the perfusion period exceeded the time required to abolish the antidromic spike by more than 10 times, the sodium concentration must have been further reduced greatly, at least around the axon. Any possible retention of sodium, if it occurred at any part of the preparation, e.g. cell body, dendrites, or muscle strand, should then be detected by a change in the response to current injected directly into the soma.

The I-V relation in Tris saline was followed after completion of flushing the chamber. In the experiment shown in Fig. 2, measurements were made at 30 min intervals up to 2 hr, after flushing the chamber with test solution of 12 times the chamber volume. The zero current level in Tris saline was tentatively

set at the peak of the transient hyperpolarization mentioned before, although the gradual return of this transient presumably brought the membrane potential to within a few millivolts of the original level.

As shown in Fig. 2, the effective resistance increased immediately in Tris saline by about 30% over the control, and there was no further change during a period of 90 min. Spikes were abolished. Thus the cell appeared to have reached a new steady state immediately on completion of the change of solution, so far as the resting and spike-generating properties were concerned.

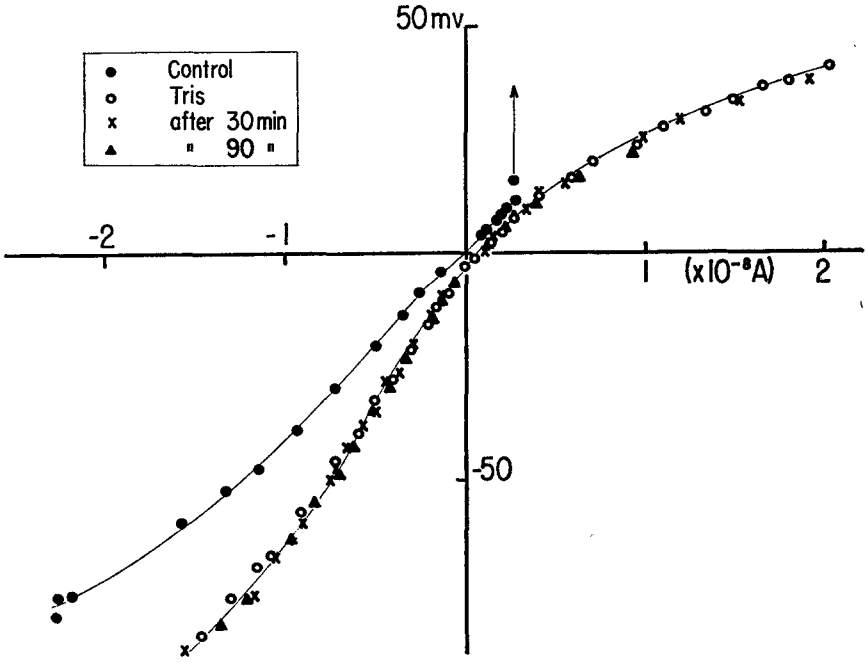

FIGURE 2. Effect of Tris saline on the current-voltage (I-V) characteristics. Effective resistance was increased by about 30% immediately after completion of perfusion with Tris saline, but there was no further change up to 2 hr in this solution. Spikes were abolished completely within the first minute. Only three sets of measurements in Tris saline, made immediately after the change, at 30 min, and at 90 min in this solution, are shown.

b) *Stretch Response at Various Times in Tris Saline* It might be argued that the above data on I-V relations could not provide any evidence against the existence of a diffusion barrier around finer branches of dendrites which are supposed to be responsible for the generator electrogenesis. This was examined by studying the stretch response in Tris saline followed in time.

In the case shown in Fig. 1 A, after the chamber was flushed with 20 times its volume of Tris saline, the first measurements that were obtained are shown as filled circles. Additional flushing with the same volume of the test solution

was repeated before the subsequent measurements It will be noted that the measurements made at 30 and 60 min in the Tris saline are almost identical with one another and differ very little from the first set. On reintroduction of the normal saline, the resting potential and the antidromic spike were completely restored.

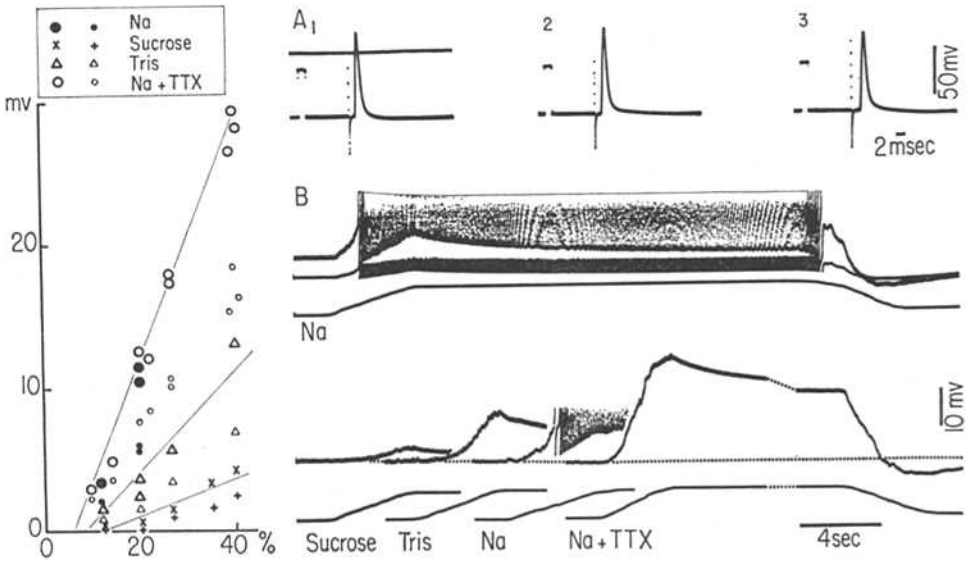

FIGURE 3. Comparison between the responses to stretch in various media. Solution changes are in the order shown in B.

A_1, an antidromic spike evoked in the first Na saline. A_2, a similar response during the second exposure to Na saline. A_3, the antidromic spike after TTX was removed from the bathing medium.

B. Stretch responses evoked in various media indicated under each record. The whole response including spike activity is shown only in the middle trace of the first recording for the initial exposure to Na saline, at a lower amplification which corresponds to that in A. The uppermost trace in the first Na and the upper traces in the subsequent records are made at higher amplification as shown by the voltage calibration in B. The bottom traces in each record monitor the stretches, the durations of which are approximately the same. A final recording, made in the control saline after removal of TTX (corresponding to A_3), is not shown.

Graph: The stretch vs. generator potential relation in the various media.

In this experiment, a direct comparison between the amplitudes of the generator potentials in the normal and Tris saline was made after repenetration of the cell, and is shown in Fig. 1 B and C. The generator potentials to a moderate stretch in Tris saline were slightly smaller than in the preceding series of measurements (Fig. 1 A). The decline may have been due to a small change in the resting length of the preparation or, more likely, to an injury caused by repenetration. At any rate, the generator potential evoked in the Tris saline was about half that in the Na saline containing TTX (Fig. 1 B, C_2).

In all the experiments of this series, it was found that after an initial flushing of the chamber with a test solution 5–10 times the chamber volume, additional flushing with the test solution did not further alter either the generator potential (Fig. 1) or the I-V relation (Fig. 2). Accordingly, in all experiments to be subsequently described, the chamber was flushed with test solution 10 times the chamber volume. Edwards et al. (1963) found flushing with a volume 6 times their chamber volume adequate.

c) Change in the Generator Potential during a Sequence of Changes of Various test solutions In this series of experiments, a comparison was made between the generator potentials in various media.

As illustrated in Fig. 3, a cell was brought into sucrose saline and showed

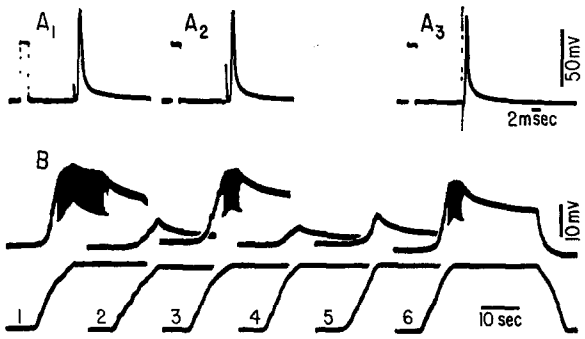

FIGURE 4. Changes in amplitude of the generator potential of a rapidly adapting cell during a sequence of changes of the bathing medium. A_{1-3}, antidromic spikes recorded with cell in Na saline, corresponding to 1, 3, and 6, respectively, in the sequence shown in B (see text). During 2 and 5, the cell was in Tris saline, and during 4 it was in choline saline. Note that the response in Tris saline was about as large after exposure to choline (4 to 5) as after exposure to Na (1 to 2).

only a small response (cf. Edwards et al., 1963), in this case about one-tenth of the control, to a moderate stretch. Then the chamber was flushed with Tris saline. Clearly, the cell gave a much larger response to the same stretch (42–47% of the control). The control response was obtained in the presence of TTX, after the cell was returned from Tris saline to normal saline, thus confirming recovery of the response both in spike amplitude and in the pattern of response to stretch.

Since both sucrose saline and Tris saline contain the same amount of potassium and calcium as the normal saline, it is necessary to conclude that the difference in the responses on changing from sucrose to Tris is due to a contribution of Tris ion to the generator potential. The effect of differences in chloride concentration as well as of lower conductivity of sucrose saline will be discussed in the following sections.

That the response amplitude is independent of the order of exposure to

different solutions is illustrated in Fig. 4 B, which also represents an experiment in a rapidly adapting cell. The sequence of changes in media in this case was: Na saline (B_1), Tris saline (B_2), Na saline (B_3), choline saline (B_4), Tris saline (B_5), and Na saline (B_6).

Large control responses were easily observed owing to the higher threshold of spike initiation in the rapidly adapting cells (Eyzaguirre and Kuffler, 1955 *a*). The amplitude of the responses in Tris saline was almost identical in B_2 and B_5, regardless of the preceding steps.

d) Effects of Gamma Aminobutyric Acid (GABA) These experiments were designed to serve a twofold purpose. Firstly, GABA is known to act on the

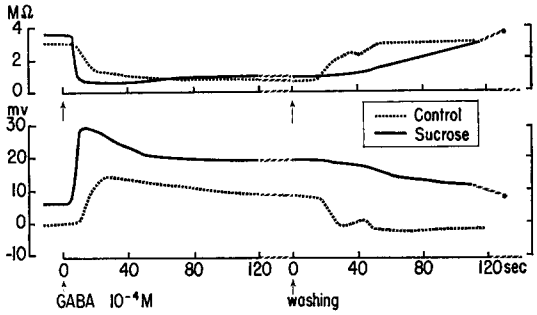

FIGURE 5. Chloride potential levels determined during application of gamma aminobutyric acid (GABA), in control and Cl-deficient media (sucrose saline). *Upper graph:* effective resistance measured by applying small hyperpolarizing current pulses. *Lower graph:* membrane potential change. The substitution of sucrose for NaCl in this cell caused an increase in the effective resistance by about 20% and an irreversible depolarization of about 6 mv. At time zero in the graphs, GABA was applied. It caused a marked increase in conductance, and depolarization which was much larger in sucrose saline. Note that generator potential in sucrose saline is vanishingly small (Fig. 3), suggesting that the contribution of the chloride ionic battery to the generator potential is negligible.

inhibitory postsynaptic membrane in the same way as the inhibitory transmitter does, causing a conductance increase to chloride (Hagiwara et al., 1960), and perhaps also to potassium (Kuffler and Edwards, 1958; Edwards and Hagiwara, 1959). Since the inhibitory synapses in the stretch receptor occur on the fine branches of dendrites at which the generator potential is initiated (Florey and Florey, 1955; Kuffler and Eyzaguirre, 1955), the time of onset of the GABA effect should give some estimate of the time required for diffusion around the generator membrane. As described in detail in the next section, when GABA was introduced into the perfusate, its effect developed very rapidly (Fig. 5), reaching a maximum in the first 20–30 sec, after which followed a gradual decay. The result is in agreement with that reported by Hagiwara et al. (1960).

That a molecule as large as GABA can reach the synaptic space in a con-

centration sufficient to exert a marked effect within the short time mentioned above would be additional evidence against the diffusion barrier around the dendrites, provided GABA does not affect the soma membrane directly. At the concentration applied, conduction along the axon was not impaired.

2. CONTRIBUTION OF IONS OTHER THAN NA TO THE GENERATOR POTENTIAL

a) Chloride The second type of information deduced from the preceding experiment is as follows: With a microelectrode filled with 3M KCl, the inhibitory postsynaptic potential (IPSP) and the GABA-induced potential in this cell are both depolarizing responses (Hagiwara et al., 1960). Since this type of electrode has been used for the most part in the present work, it might be argued that the generator potential observed in Tris saline could be an indication of an ionic contribution of chloride, as suggested by previous workers (Edwards et al., 1963). Although this seems unlikely in view of the difference between the responses observed in Tris and choline salines (Fig. 4), in which the Cl concentration is almost identical, this possibility was also examined.

In the absence of sodium chloride, e.g. in sucrose saline, the above suggestion should imply a shift of the stretch-response toward a level to which the conductance increase to chloride or to both chloride and potassium would bring the membrane potential. Owing to the decrease of chloride outside, this level should be more depolarized. The possibility was subjected to an experimental test, by determining the GABA-induced potential level in normal and chloride-deficient solutions.

GABA was introduced into the perfusate at concentrations of 5×10^{-5} to 10^{-4} mole/liter, after the cell was equilibrated in the test solution. An example in Na saline and sucrose saline is shown in Fig. 5. The cell was penetrated with electrodes filled with 3M KCl. Changes in the membrane potential and resistance in response to GABA were observed, applying small constant current pulses. As was expected, in the normal saline the response to GABA was a depolarization of 12–14 mv, which was attained within the first 20–30 sec. Effective resistance was decreased to 20–26% of the control. Similar values were obtained when the cell was bathed in Tris saline. In sucrose saline, GABA evoked more marked depolarization, up to 26–32 mv in the same cell, within an even shorter time. The resistance change appeared to be more abrupt, but the steady-state value reached was about the same as that in control saline. Recovery in sucrose saline was characteristically slow, and upon repeated application of GABA the response became smaller, approaching that in normal saline. A similar effect of GABA in sucrose saline has been reported by Hagiwara et al. (1960).

Since the larger GABA response in sucrose saline is attributable to the change in the chloride concentration gradient, and since the stretch response

in this medium is negligibly small (Fig. 3), a contribution of a chloride battery to the generator potential appears to be unlikely.

Further evidence on this point was obtained in a series of experiments in which the chloride in the bathing medium was replaced by acetate or isethionate. The microelectrodes were filled with 3M K acetate. Cells exposed to the organic anions gave spontaneous repetitive discharge for long periods of time. The spontaneous discharge occurring even in completely relaxed cells was previously noted by Edwards et al. (1963). This activity could be suppressed

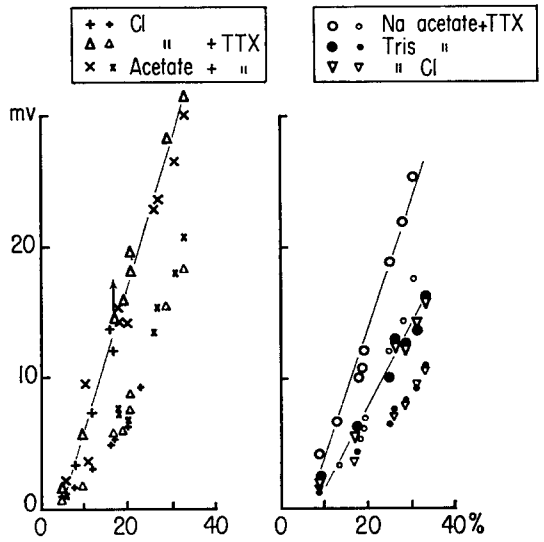

FIGURE 6. The absence of Cl has no effect on the generator potential. *Left:* replacement of NaCl saline by Na acetate saline. In the first set of measurements without TTX, spikes were generated at the level shown by the arrow. The resting potential was 70 mv initially and 72 mv in Na acetate saline. *Right:* another cell equilibrated for 12 hr in Na acetate saline. The resting potential was 75 mv. Changes to Tris acetate saline and to Tris Cl saline reduced the generator potential, but the substitution of Cl for acetate had negligible effect.

by soaking the cell overnight in the Cl-free saline at low temperature. When the preparation was returned to room temperature, the resting potential, the spike, and the pattern of response to stretch all appeared to be essentially identical with those observed in the presence of chloride.

In the cell represented in Fig. 6 left, the generator potentials evoked in Cl saline and acetate saline were compared in the presence of TTX, and were almost identical. The resting potential was 70 mv in Cl saline and 72 mv in acetate saline. Another cell (Fig. 6 right) was transferred from Tris acetate saline to Tris Cl saline. The resting potential changed by only 1–2 mv, and the stretch-response curves in the two solutions were superimposable.

The change of chloride concentration gradient across the membrane was again evidenced by observing the IPSP. In acetate saline the IPSP was a depolarizing potential large enough to trigger a spike (Hagiwara et al., 1960), even after the cell was soaked overnight in the Cl-free medium. When the chamber was perfused with Cl saline, the resting potential underwent a transient hyperpolarization and returned toward the original level within the perfusion time. The IPSP rapidly changed its polarity even during the transient hyperpolarization and became a large, steady hyperpolarizing potential within a few minutes.

These data are in keeping with the conclusion derived from the experiments on the effect of GABA. Therefore, the generator membrane in its active state can be considered a cation-sensitive membrane.

b) *Calcium* Reduction in calcium concentration invariably led to an unstable resting potential accompanied by a drop in the membrane resistance, regardless of whether it was done in Na saline, in the presence of TTX, or in Tris saline.

A fivefold increase in the calcium concentration did not appreciably change the peak amplitude of the generator potential, despite the fact that the concentration of sodium was decreased in order to maintain isotonicity. Perhaps this is due to the smaller rectification of the membrane, which will be discussed in a later section. A marked change, however, was observed in the decay of the generator potential from its peak (Fig. 7). Clearly, the decay was faster in the high-Ca solution. This change was fairly reversible (Fig. 7 C). As will be shown later, accommodation for spike electrogenesis also became faster. Edwards et al. (1963) have already reported the latter change, by comparing firing frequency in response both to a stretch and to a current pulse, in normal and high-Ca solutions.

The resting potential was little changed by increasing Ca. The antidromic spike was smaller and prolonged. A notch appeared on the rising phase (Fig. 7 A_3) and the antidromic invasion of the soma was easily blocked on slight increase in stimulation frequency (Fig. 7 A_2). Though the change in the spike was partly due to the decrease of sodium concentration in this medium, it was more marked than when a comparable decrease in sodium was made through substitution of sucrose.

On further increase of calcium, the generator potential decreased progressively. However, the higher the calcium concentration in the outside solution, the worse was the recovery of the responses on return to normal saline. Although there was usually a small response to stretch in Ca saline where Na was completely replaced by Ca, the data were not pursued further because of the poor reversibility. The present data thus do not rule out a possible contribution of this ion to the generator potential. However, they do suggest that the

ionic contribution of calcium, if any, seems to be very small, as compared with that of monovalent cations.

c) Reversal Level of the Generator Potential This series of experiments was designed to serve a twofold purpose: Firstly, the ionic contribution of potassium was suggested by measuring the reversal level of the generator potential (Terzuolo and Washizu, 1962). Secondly, since it has been concluded that the active generator membrane appears to be a relatively nonselective cation-

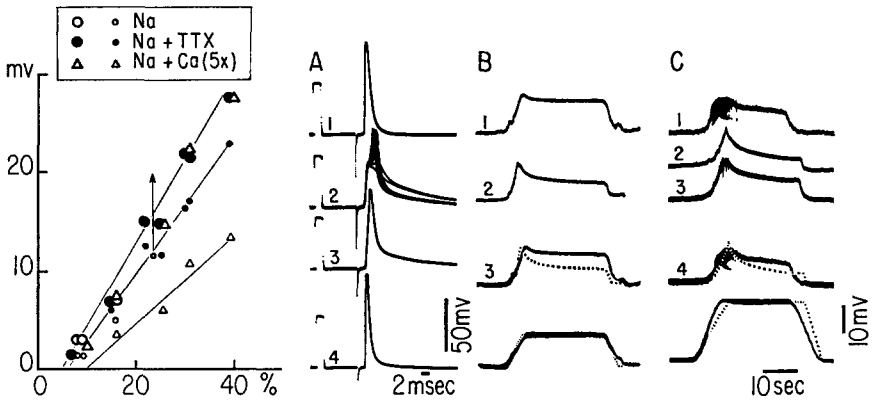

FIGURE 7. Effects of high-Ca solution on spike and generator potentials. A and B are from a slowly adapting cell. The antidromic spikes are shown as A_1 in control saline, A_2 and A_3 in high-Ca saline, and A_4 after return to normal saline. Stimulation was given at 1 per sec in all records except A_2, where stimulation at 3 per sec enhanced the block of invasion to the soma, which was also suggested by the notch in the rising phase in A_3. B_1, the generator potential in Na saline with TTX; B_2, in high-Ca saline; B_3, superposition of B_1 on B_2. C, similar recordings in a rapidly adapting cell: C_1, in Na saline; C_2, in high-Ca saline; C_3, in Na saline; C_4, superposition of C_1 on C_2. Bottom traces in B and C show representative stretches in each column.

Graph: Complete series of measurements from the slowly adapting cell shown in B. Note that the initial peak of the generator potential was little changed between normal and 5× Ca solutions, whereas the level of the later quasi-steady state was much smaller in high Ca (small triangles) than in the normal Ca (small circles).

sensitive membrane, it was thought worthwhile to determine the reversal level and the conductance increase for the generator potential in the presence of various Na substitutes.

Measurements of the reversal level of the generator potentials in the control saline and in test solutions were compared with the aid of dual penetration. The membrane potential was set by a separate current electrode, and the change in amplitude of the generator potential was recorded at various membrane potentials. Owing to the rectification for depolarizing currents, an interpolation has not been possible in the normal saline.

Reproducible measurements were obtained from four cells, in two of which results could be compared between the control saline and Tris saline (Fig. 8). In these two cells with a mean resting potential of −70 mv, the reversal level determined by extrapolation was −20 to −30 mv in the normal saline. This suggests a mechanism similar to that of the end plate potential of frog muscle, namely, conductance increase to both Na and K (Takeuchi and Takeuchi, 1960). Making a number of simplifying assumptions, it is possible to calculate the ratio of conductance increase for these ions, $\Delta g_{Na}/\Delta g_K$, which would account for measured reversal level, and the ratio at the active generator membrane is estimated as roughly unity, or less.

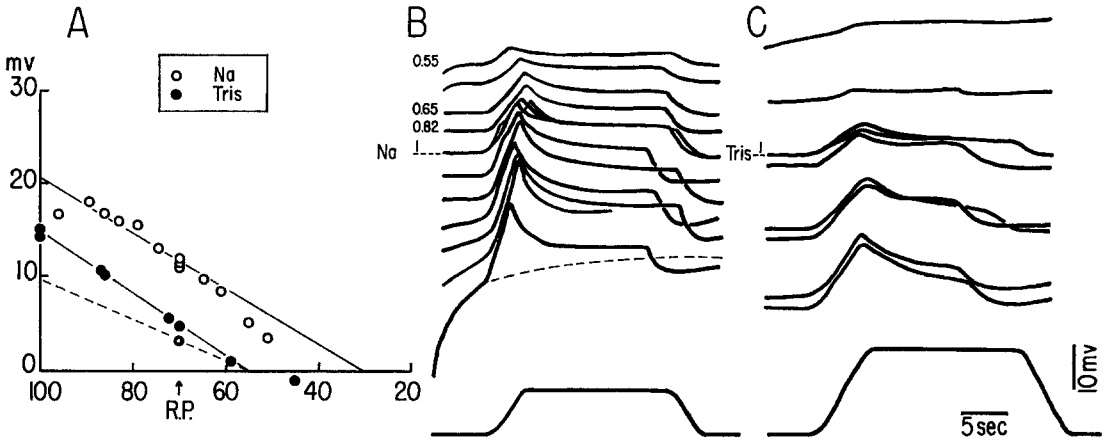

FIGURE 8. Equilibrium level of the generator potential in normal and Tris salines measured with intracellularly applied currents. A: ordinate, peak amplitude of the generator potential; abscissa, membrane potential. The resting potential (*R.P.*) is indicated by the arrow. Broken line shows normalized curve in Tris saline. B, tracings from the records in the normal saline; C, similar records in Tris saline. The resting level of membrane potential is indicated by *Na* in B, and by *Tris* in C, respectively. Small numbers in B and C show changes in the relative slope resistance. A representative record of stretch is shown at bottom in each series. Note that the stretch was increased in order to obtain a larger response in Tris saline.

Terzuolo and Washizu (1962) studied the reversal level of the generator potential of the stretch receptor with the aid of a single microelectrode connected to a bridge circuit, and showed that the reversal potential is close to zero membrane potential. The experimental conditions as well as the method were, however, somewhat different, since their resting potential was about −50 mv on the average, as compared with the value (−70 mv) in the present work.

The reversal potential was shifted to −45 to −55 mv in Tris saline. The relative conductance increase of the generator membrane in the two media could be compared from the curves, after normalizing the response amplitude

for stretches of the same magnitude. The relative increase in conductance in Tris saline was estimated as only about one-half of that in the control, taking into account the fact that the effective resistance of the resting cell increased in Tris saline, as is shown in the following section.

An experiment in sucrose saline was done reversibly in one cell, but the response was barely above the noise level and did not change appreciably on application of polarizing currents.

It should be noticed that in all these measurements, the polarizing and recording electrodes were far from the site of the generator potential, so that the reversal level at the generator membrane proper should be more negative and the conductance increase larger than in the actual measurements (cf. Burke and Ginsborg, 1956). The discrepancies may be less enhanced in the present case than those which were estimated in the distributed synapses, since the time course of the generator potential is far slower than the effective time constant of the cell, thus approximating a steady state during the response.

On the other hand, a change in the effective space constant in a test solution may introduce a further complication. Particularly, the low conductivity of sucrose saline probably reduces the effective space constant along the finer branches of the dendrites. Therefore, the small response in this low-conductivity solution must be, at least in part, due to a greater attenuation of electrotonic spread.

Effects of Some Other Organic Cations

CHOLINE Choline has been widely used as a supposedly inert substitute for sodium. Choline appears to be inert so far as the spike electrogenesis of the stretch receptor is concerned (Figs. 9 and 12). The resting potential did not change appreciably after an initial transient hyperpolarization on changing to choline saline. However, the cell still gave some responses to stretch (Fig. 9). The response amplitude in this solution was quite small, one-fourth to one-fifth of the control in a cell that showed a stretch response of about one-third in Tris saline (cf. also Fig. 4). It is interesting to note that the ratio given above is similar to the residual amplitude of the receptor potential in a muscle spindle that has been soaked in choline saline for 60 min (Ottoson, 1964).

Thus far, a direct comparison between sucrose saline and choline saline has not been successfully carried out with reasonable accuracy, mainly because of the small amplitude of the responses in both solutions, and partly because of an irreversible decrease of the resting potential which was frequently encountered in sucrose saline, as will be described later. However, it seems necessary to conclude that choline ion can contribute to the stretch response to some extent, in view of the data that the average response amplitude was 20–25% in choline saline and less than 10% in sucrose saline. Essentially the same experiments,

using sucrose and choline as the sodium substitutes, have been performed by Edwards et al. (1963), but no quantitative description was given, perhaps for the same reason.

Because of the small but definite contribution of choline toward generator electrogenesis, it was interesting to test effects on the generator potential of other onium ions, some of which have been reported as effective substitutes for sodium in spike electrogenesis in various cells (cf. Lorente de Nó, 1949). Of the onium ion series, TMA (tetramethylammonium), TEA (tetraethylammonium), NH_4, and guanidinium and hydrazinium ions were tested. All except TMA and TEA were found to be strong depolarizing agents. Total replace-

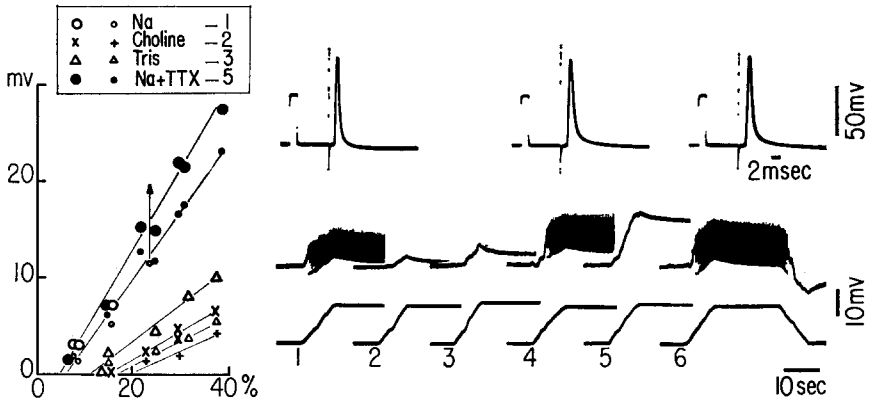

FIGURE 9. Generator potentials in Na saline, choline saline, and Tris saline. Sequence of changes is shown in box in graph, except 4 and 6 (return to Na saline from Tris saline and from Na saline with TTX, respectively). Spikes were evoked in Na saline at depolarization of about 14 mv (arrow in the graph). The hyperpolarization after release of stretch in 6 was unusually large and irregular, suggesting that it may have been partly due to an artifact.

ment of sodium by one of them generally gave a large immediate depolarization which was usually poorly reversible.

TETRAMETHYLAMMONIUM This cation proved to be fairly effective for development of a generator potential, although in TMA saline the latter is smaller than in Tris saline. In Fig. 10, only the dynamic (initial) phase of the generator potential was plotted. TMA did not affect the reproducibility of the responses in Tris or Na saline. The spike potential was abolished in TMA saline.

TETRAETHYLAMMONIUM On total replacement of sodium by TEA, the receptor cell failed to give a spike on antidromic and direct stimulation. The membrane potential did not change appreciably. Partial replacement led to a reduction of the amplitude of the generator potential with a burst discharge

of spikes on its initial peak. Total replacement further decreased the response stretch to a level similar to that obtained in choline saline. Recovery from TEA saline showed some peculiar results. When the cell was transferred from TEA saline to Tris saline, the stretch response sometimes did not reach the Tris level which had been attained previously in the same cell, although recovery from TEA saline to the normal saline appeared fairly complete. The possibility of interaction or competition between these cations for membrane sites has not been resolved.

AMMONIUM ION Ammonium ion in concentrations of 10–40 mmoles/liter was reported to have a depressing effect on toad's electroretinogram which was apparently irreversible (Furukawa and Hanawa, 1955). In the stretch receptor total replacement of sodium by NH_4 caused depolarization up to 30–40 mv.

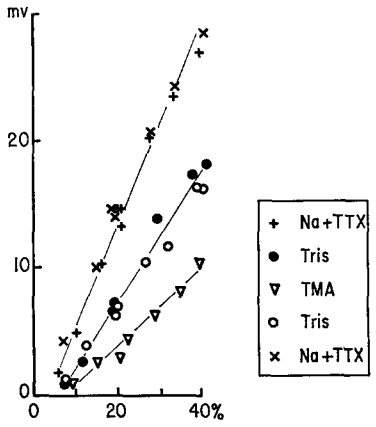

FIGURE 10. Stretch vs. generator potential relation in Na saline, Tris saline, and tetramethylammonium (TMA) saline. The resting potential in Na saline was 69 mv. The change of the resting potential in the test salines was about 1 mv or less. Only the initial peak amplitudes are plotted. Solutions were changed in the order shown in the box.

The antidromic spike was rapidly blocked. The large depolarization induced by this ion excludes a simple test of effectiveness of NH_4 for the generator potential, because of the technical difficulty in controlling the membrane potential by a current electrode.

GUANIDINIUM AND HYDRAZINIUM IONS The depolarization by these ions was smaller than that induced by NH_4, but still exceeded 20 mv. The axon spike appeared to be maintained and was evidenced in the soma by an electrotonic spread in most cases. Excitability of the giant axon of crayfish ventral cord is maintained in media in which Na was completely replaced by either guanidinium or hydrazinium ions (Yumoto and Ishima, 1966). An increase of Ca concentration up to 4–5 times control level appeared to antagonize the depolarizing effect of these ions. In hydrazinium saline with increased Ca, the amount as well as the rate of the depolarization was reduced, and under this condition the stretch response was found fairly large as compared with that in

Na saline with similarly increased Ca. However, the rate of the depolarization was still too rapid to permit quantitative analysis.

Current-Voltage Relations in Various Media

Current-voltage relations in various media relevant to the data in the preceding sections will be described briefly. Since the site responsible for the generator potential is supposed to be far from the cell soma, one only observes an electrotonic spread in the soma of the full potential change, along the cable-like structure of the dendrites. The observed potential, therefore, could be

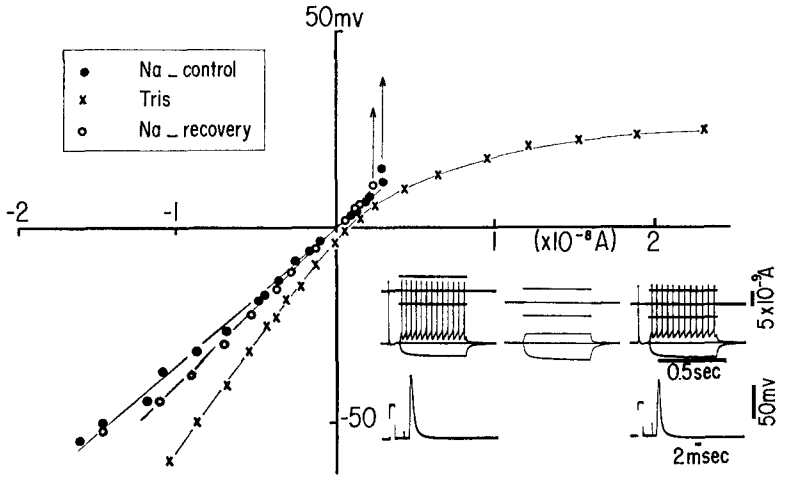

FIGURE 11. Current-voltage relations in Na saline and Tris saline. Note the resistance increase in Tris saline. Arrows show spike electrogenesis in control saline. Records in the insert (lower right) are for control, in Tris saline, and on return to Na saline, from left to right. In the upper row are shown the responses to current injection of 5×10^{-9} A, after antidromic spikes. The latter are seen on a faster sweep in the lower row. The origin of the curve in Tris saline is set at the level of the initial transient change of the membrane potential.

easily modified only if there were a change in the passive electrical properties of the cell when the outside medium is changed. To determine the cable properties in this case is almost impossible, because of the anatomical complexity of the cell. However, simple I-V relations in various media provide some estimates of the cable properties. One limitation in this approach would be that the comparison between various media should be made on one and the same cell, because of variability from cell to cell. Furthermore, it would be desirable that any change observed on changing media should be reversible.

In the standard solution, the I-V relation was generally linear for hyperpolarizations of 60–80 mv from the resting potential, where a deviation appeared indicating decrease in resistances that denotes hyperpolarizing activa-

tion (Grundfest, 1966). However, the ion species involved in the increased conductance was not determined in the present work. The critical depolarization ranged between 8 and 16 mv in the slowly adapting cell. In the depolarizing quadrant, in the presence of TTX a conductance increase appeared on depolarizing the cell over the critical depolarization level. In the normal

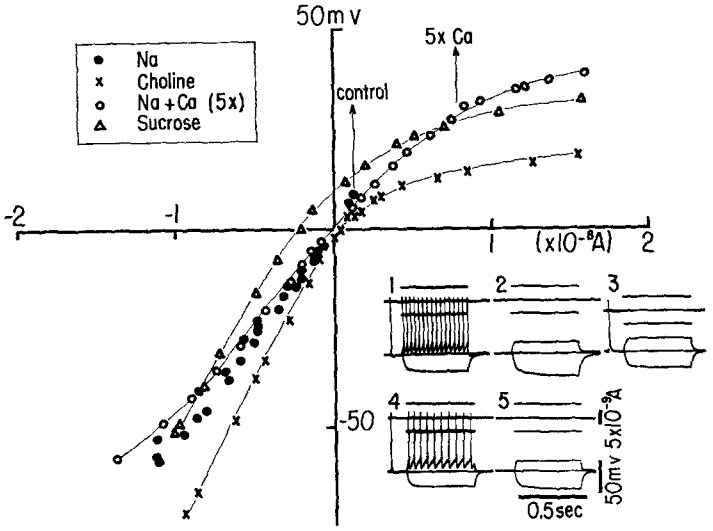

FIGURE 12. Current-voltage relations in various media. Measurements were made in the order shown in the box. After exposure to each of the test solutions the cell was returned to the control saline, all the latter measurements being indicated by filled circles. The critical firing level in the control saline (first arrow) was much lower than that in the high-Ca saline (second arrow). Spikes were never evoked in the choline and sucrose salines. The recording electrode was withdrawn from the cell before the exposure to sucrose saline, in order to check the membrane potential. The depolarization caused by the exposure to sucrose was not completely reversible.

Insert (lower right): Changes in membrane potential on applying depolarizing and hyperpolarizing currents of the same amplitude in the different media. An antidromic spike was evoked at the beginning of the record. 1 and 4, in Na saline; the spike frequency during the depolarizing current was lower in the later sequence. 2, in choline saline. 3, in high-Ca saline. 5, in sucrose saline. Note the larger hyperpolarizations in 2 and 5, and the absence of directly evoked spikes in 3, although the cell was capable of generating an antidromic spike and produced a train of spikes with larger depolarizing currents.

solution, it was usually masked by repetitive firing. The effective resistance of the slowly adapting cell was 2–8 MΩ, with a time constant of about 10 msec.

Tris saline and choline saline increased the effective resistance in the hyperpolarizing quadrant by about the same magnitude, 30–40% over the control (Figs. 11 and 12). The rectification was almost identical with that in the control. The I-V curve in sucrose saline (Fig. 12) was almost parallel to that in

Tris saline (Fig. 11) or in choline saline (Fig. 12) in spite of an irreversible depolarization which sometimes developed in this medium. Both Tris and choline appear to be as inert as sucrose with regard to the passive and electrically excitable properties of the cell membrane. The increase of the effective resistance, as well as the apparent absence of change in the resting potential aside from the initial transient, is at least qualitatively in agreement with the data given by Edwards et al. (1963).

Partial replacement of Na by TEA resulted in a prolongation of the spike, and in formation of a large plateau after an initial burst of a train of spikes on application of depolarizing currents (Washizu, 1965). After complete replacement of sodium by TEA, i.e. in TEA saline, however, no spike could be elicited on antidromic or direct stimulation. There was an increase of the effective resistance that was comparable to that in Tris saline. On application of depolarizing currents, the potential did not reach a steady level, but continued to increase during the application of current for as long as 500 msec, indicating that TEA had abolished the delayed conductance increase.

A fivefold increase in the calcium concentration caused little change in the slope in the hyperpolarizing quadrant, and almost no change in the resting potential (Fig. 12). However, the critical depolarization was raised to 30 or 35 mv. The spike train evoked by a suprathreshold current subsided rapidly, leaving a sequence of small oscillatory responses superimposed on the plateau. Rectification became less marked at lower depolarization, but developed when larger currents were applied. These data are also consistent with those reported by Edwards et al. (1963), who showed a decrease in firing frequency in fivefold Ca solution, for a given stretch or current pulse. Depression of rectification has also been observed in arthropod muscle fibers (Werman and Grundfest, 1961; Werman et al., 1961; Takeda, 1967).

Further increase of calcium, by replacing sodium, decreased and finally abolished the spike. Although the effective resistance seemed to increase, the validity of the measurements was lessened by frequent failure of recovery after high-Ca media.

DISCUSSION

The validity of the data regarding replacement of Na by other ions rests upon the accessibility to the substitutes of the receptor regions where the electrogenesis is produced. The experiments with Tris demonstrate, in agreement with Edwards et al. (1963), that there is no significant diffusion barrier at the crayfish stretch receptor like that which has been postulated to exist at the fine terminals of the muscle spindle (Katz, 1950; Ottoson, 1964) or the Pacinian corpuscle (Diamond et al., 1958). The absence of a barrier may perhaps be attributable to the open circulatory system of crayfish.

The generator electrogenesis does not appear to involve an increased conductance for Cl. Only indirect evidence was adduced by Edwards et al. (1963)

for the participation of Cl, and these workers ascribed only a minor role to the anion.

The present data indicate that the generator membrane of the crayfish receptor in its active state must be regarded as a cation-sensitive membrane. However, this membrane appears to be relatively nonselective for certain species of monovalent cations. It is noteworthy that most of the organic cations studied in the present work are completely inert for the spike electrogenesis. The divalent cation, Ca, does not appear to be capable of participating in the depolarizing electrogenesis of the receptive components, whereas large monovalent ions, such as Tris, TMA, or choline, can contribute to the generator depolarization. Calcium also does not contribute to the depolarizing electrogenesis of the excitatory synapses of crayfish muscle fibers (Ozeki and Grundfest, 1967). On the other hand, the acetylcholine-activated end plate of frog muscle becomes somewhat permeable to Ca (Takeuchi, 1963).

The basis of the selectivity of the generator membrane for monovalent cation species is unknown at present. The relative amplitudes of the generator potentials in the presence of the different monovalent cations, including Li (Obara and Grundfest, 1968), as Na substitutes can be presented as: Na $\doteqdot$ K > Li > Tris > TMA > choline or TEA. Although a further distinction in this series appears likely, for instance between NH_4, hydrazinium ion, and others, other complications induced by these cation species, which were described in the Results, preclude a simple comparison. The amplitude of the response of a cation-sensitive membrane depends on the activity of a given ion species in the bathing medium, and also on the mobility of the ion in the membrane. Assuming that there is no great difference among the activities of the Na substitutes in the test solutions, the above series of response amplitudes most probably reflects the mobility of these cations in the activated generator membrane. It is at least in keeping with the measurements of the relative conductance increase in normal and Tris salines (Fig. 8). More quantitative data, obtained by measuring the reversal level of the generator potential for each of the cation species (i.e. under zero current condition), would be desirable. The limitation and some of the complications involved in this approach were discussed briefly in the Results section.

In an attempt to obtain further information, conductivities of single Cl salt solutions of these cations were measured. Ample data are available on the limiting equivalent conductivity measurement of most of the cations which have been used here (cf. Robinson and Stokes, 1965). The conductivity of Tris solution was measured at pH 7.4. The relative order of the conductivities was not in keeping with the order of the response amplitudes as given above, the rank for the conductivities being NH_4, K, Na, TMA, Li, choline, TEA, Tris. The discrepancy may be due to difference in ion mobility in the aqueous solution and in the active membrane.

At first sight, the ionic contribution of Tris ion to bioelectrogenesis such as

that of the mechanosensory generator potential is surprising. In fact, Tris has been used as an inert Na substitute in connection with spike electrogenesis (cf. Lüttgau and Niedergerke, 1958), and its penetration into a cell has so far been shown only in red blood cells, probably in the un-ionized form (Omachi et al., 1961). The depolarizing electrically inexcitable response (EPSP) of crayfish muscle is abolished when Na is replaced by Tris (Ozeki and Grundfest, 1967). In *Limulus* the generator potential of photoreceptor is abolished in Tris solution, but only temporarily. After some 5–10 min in Tris, the cell again can respond to light but with a smaller generator potential (Millecchia et al., 1966; and personal communication). However, it might be pointed out that Tris is essentially a substituted ammonium ion, being an amine base, when it is ionized (Bates, 1961). Several other onium ions are also capable of substituting for Na in generator electrogenesis, as described in the Results.

Since Gomori (1946) introduced Tris as one of the versatile amine base buffer substances for use in the physiological pH range, a number of workers have reported on the physicochemical properties of Tris. Benesch and Benesch (1955) suggested that Tris has a symmetrical molecular structure with intramolecular hydrogen bonding. The minimum radius calculated on this molecular model is only slightly larger than that of TMA, although the validity of the model in an aqueous solution appears uncertain, as already suggested by the conductivity measurement.

It is interesting to note that in the acetylcholine-activated end plate of frog muscle, sodium can be replaced by ammonium (Furukawa et al., 1957), by methyl and ethyl derivatives of ammonium (Furukawa and Furukawa, 1959), by hydrazine (Koketsu and Nishi, 1959), or by other quaternary ammonium compounds including TMA (Nastuk, 1959). Thus, the chemically activated electrogenesis of the frog's end plate and the mechanically activated generator potential of the crayfish stretch receptor appear to have in common the property that the membrane in its active state becomes permeable to several large foreign cation species.

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