Interactions of Calcium with Sodium and Potassium in Membrane Potentials of the Lobster Giant Axon

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ABSTRACT Experiments were performed on the lobster giant axon to determine the relation between intracellular spike amplitude and external calcium ion concentration. Action potential decline in low external calcium is greatly accelerated by simultaneous removal of external sodium ion. Correlation of the time course of spike decline in low calcium–low sodium solution with the time courses of spike decline in low calcium alone and in low sodium alone indicates that the effect of simultaneous removal of both ions is significantly greater than the sum of the individual effects. For a given time of treatment, spike amplitude was a function of external calcium concentration. While spike height is proportional to the log of the external calcium concentration over the range 2.5 to 50 millimolar, the proportionality constant is dependent upon the sodium concentration. Under the conditions of low external sodium (50 per cent reduction) the slope of the linear relationship between the spike height and the log of the external calcium concentration is about 5 times greater than in normal external sodium. Decreasing external calcium concentration and simultaneously increasing external potassium concentration produce a greater spike reduction than the arithmetic sum of spike reductions in low calcium alone and in high potassium alone. It is suggested that calcium interacts strongly with sodium and potassium in the spike-generating mechanism. A theoretical basis for these results is discussed.

In the previous paper, evidence was presented to suggest that calcium ion exerts a complex effect on the spike process of a lobster giant axon (Dalton and Adelman (1960)). This effect was shown to be based on a relation somewhat different for the action of calcium ion on the action potential in terms of alterations in resting potential, than that existing for potassium ion. It has been shown recently in lobster limb axons (Adelman and Adams (1959))

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that zero external calcium drastically reduces the rates of spike rise and fall as well as reducing the peak spike amplitude. That calcium ion is capable of influencing sodium and potassium conductances in the squid giant axon has been shown by Frankenhaeuser and Hodgkin (1957) by means of voltage clamp techniques. These lines of evidence suggest that calcium ion might be playing a role in conjunction with sodium and potassium in the genesis of the action potential in invertebrate axons.

In order to shed further light on the role of calcium ion in the spike process of the lobster axon, experiments were performed in which the external calcium, sodium, and potassium ion concentrations were varied. Measurements of intracellularly recorded action potentials were made, and the spike amplitude was correlated with these varied ionic concentrations.

In terms of the initial hypothesis to be tested in these experiments it was expected that the predominant ion with which calcium would interact would be sodium, inasmuch as sodium has been considered (Hodgkin and Katz (1949); Hodgkin (1951)) as the major contributor to spike genesis.

Materials and Methods

The techniques of isolation, perfusion, and intracellular recording from the giant axons of the lobster circumesophageal connectives have been described previously (Dalton (1958); Dalton and Adelman (1960)). Axons were initially perfused with "normal" lobster solution which could then be exchanged for any desired test solution. The total osmolarity of most test solutions was kept constant by addition of either choline chloride or dextrose. In the cases in which the concentration of calcium ion was raised in excess of the normal value, the total osmolarity was not adjusted to normal by reducing the concentrations of other ions in the solution. It was found that lobster axons showed no changes in the measured values of spike amplitude and resting potential when perfused with equivalently high osmolarity solutions which were obtained by adding up to 75 mM dextrose to otherwise normal lobster solution.

RESULTS

Combined Effects of Calcium and Sodium on the Action Potential

Previous reports (Dalton (1958); Adelman and Adams (1959)) have indicated that the action potential amplitude of lobster axons continuously declines as a function of exposure time to external media deprived of calcium ions. However, the time course of spike decline in low external calcium has not been compared to that obtained upon exposure of such axons to low external sodium. Comparisons of the rates of decline of spike amplitude in low calcium solution and in low sodium solution were made and are shown in Fig. 1. The

0.1 × "normal" calcium concentration was 2.5 mM, while the 0.5 × "normal" sodium concentration was 233 mM. All other ions were kept at their normal concentration values. In Fig. 1 the spike amplitude in terms of per cent of the value obtained in normal lobster solution is plotted against the exposure time to the test solution. The mean spike amplitude values for seven axons are seen to decline continuously with time in these solutions. The vertical bars at the 14 minute values represent ±2 standard errors of the mean (S.E.M.). For comparison purposes the arithmetic sum of the mean values obained

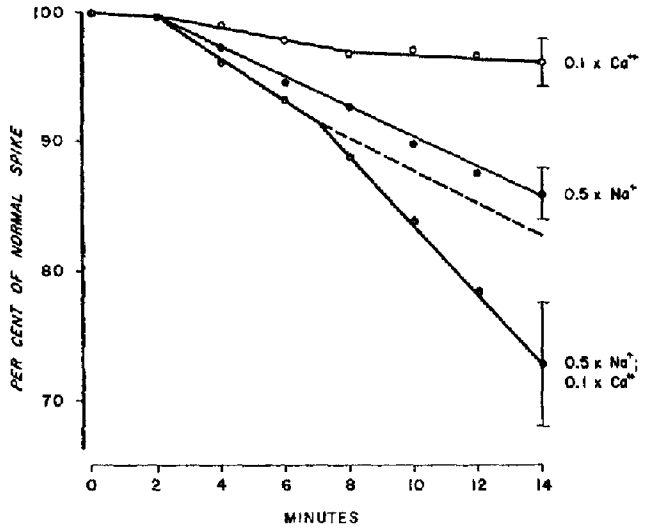

FIGURE 1. Time course of spike amplitude values upon exposure of axons to various test solutions, as indicated. Mean values were determined for seven axons in each test. For explanation, see text.

in the two test solutions (low calcium and low sodium) is represented by the dashed line.

Simultaneous reduction of external sodium and calcium resulted in a more rapid decline in spike amplitude (half-filled circles, Fig. 1) than anticipated on the basis of simple summation of the effects of individual reductions. The effect of low sodium and low calcium simultaneously on the action potential amplitude seems to suggest some sort of interaction between these ions in the spike process. It was thought that this interaction would be better visualized if the effect of a variety of calcium concentrations on spike amplitude were shown at two different sodium concentrations. In order for this to be done properly, the decline of spike amplitude and resting potential magnitude as a function of exposure time had to be considered. Up to 10 to 12 minutes' exposure to a low calcium medium the resting potential declines approximately exponentially with time; after 12 minutes' exposure this decline is

almost linear with time and it occurs at a reduced rate. Inasmuch as there was an indication of the establishment of a quasi-steady state after 12 minutes' exposure to low external calcium, 14 minutes' exposure was selected as a good time for comparison of the effects of various ionic concentrations on spike amplitude.

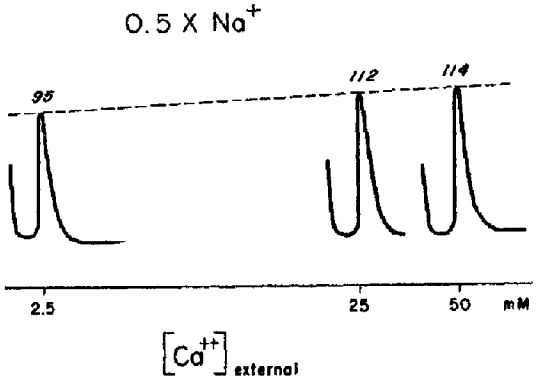

FIGURE 2. Typical action potential records obtained from an axon exposed to various concentrations of external calcium in half sodium solution. The numbers over the spikes give the magnitudes of the spikes in millivolts. The abscissa is logarithmic. See text.

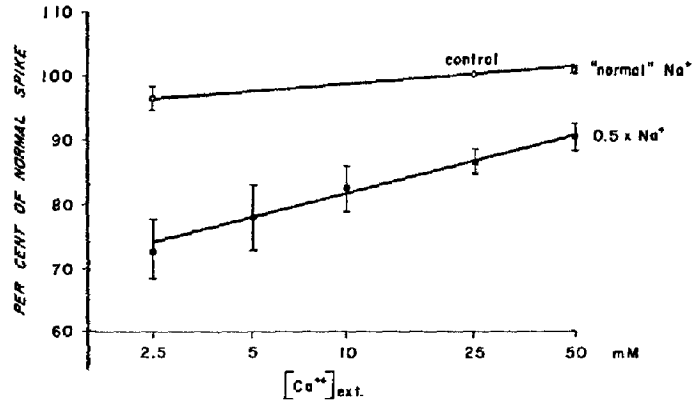

FIGURE 3. Mean spike amplitude values (per cent of normal) as a function of the external calcium ion concentration at two sodium concentrations. Abscissa is logarithmic. The vertical bars represent ±2 S.E.M.

The sort of data obtained under these conditions is shown for a typical axon in Fig. 2. The figure shows by means of action potential recordings the spike amplitude as a function of calcium concentration in low sodium solutions at 14 minutes' exposure time. The graph indicates that the spike amplitude is a linear function of the logarithm of external calcium concentration.

The mean effects of external calcium ion concentration on spike amplitude

at two different external sodium concentrations are given in Fig. 3. Again, all values represented in the figure were obtained at 14 minutes' exposure to the test solutions. The spike amplitude can be seen to be a function of the logarithm of the external calcium ion concentration both above and below normal concentrations. However, when the external calcium concentration is raised to values of 100 mM and above, spike amplitude values become erratic, and the linear relationship no longer holds. These values are not included

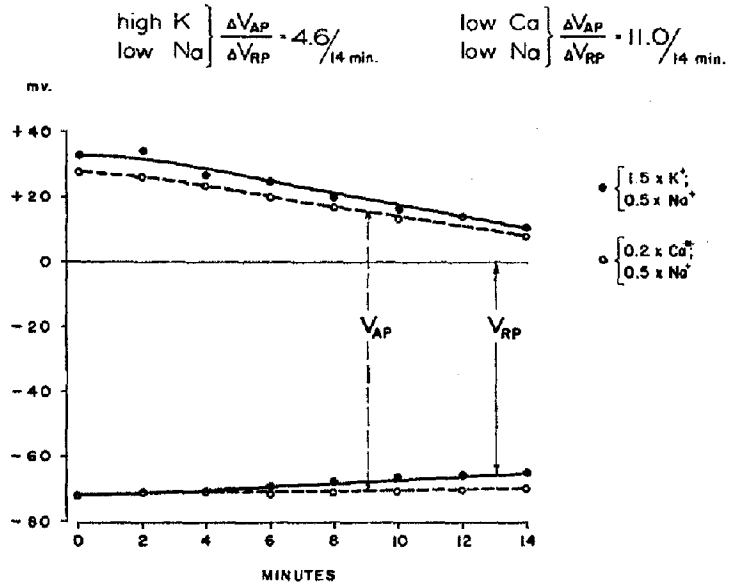

FIGURE 4. Typical transmembrane potentials plotted as a function of exposure time to two test solutions. Results are typical of five axons so treated.

among the data in Fig. 3, because of the possibility of effects produced by the hyperosmolarity of the solution.[1]

It should be emphasized that over the range 2.5 to 50 mM external calcium when the external sodium concentration is at its normal value of 465 mM, the relation between spike height and external calcium concentration has a different slope than the comparable relation obtained when the external sodium concentration is reduced to one-half. Indeed, the slope of the logarithmic relation for half-sodium is five times greater than in normal sodium. The marked increase in the effects produced by low external calcium when the external sodium is also low indicated that these two ions are involved in mutually dependent processes.

[1] There is a possibility of a direct effect of high external calcium on the nerve membrane, inasmuch as Lorente de Nó (1947) has demonstrated a breakdown of the myelin sheath of frog sciatic nerve fibers in isosmotic calcium.

It is conceivable that the effects of low sodium–low calcium mixtures on action potentials are produced by means of an alteration in resting potential. One could imagine that only the depolarization occurring in low calcium was altering the effects of sodium on the action potential. In other words, the relation between spike amplitude and sodium concentration may have a different slope when the resting potential is reduced than it has when the resting potential is at normal values. An attempt was made to test this possibility. Fig. 4 shows action potential (V_{AP}) and resting potential (V_{RP}) values as a function of time upon exposure of a typical axon to two different test solutions. One of these solutions contained low external sodium and high external potassium to produce a mild depolarization of the axon exposed to the effects of low external sodium. The effects so obtained were compared to those resulting from exposure to a solution containing low external calcium and low external sodium. It should be noted that even though the high external potassium produced a greater reduction in resting potential than the low external calcium, the alteration in action potential per unit change in resting potential in the low calcium was over twice that produced in high potassium. It is apparent that resting potential changes alone cannot account for the interactive effects of sodium and calcium on the action potential amplitude. This seems to lend further support for the contention that calcium is exerting a direct effect on the spike-producing mechanism, and that this effect is in some way mediated through sodium ion.

Combined Effects of Calcium and Potassium on the Action Potential

Recently, Frankenhaeuser and Hodgkin (1957) have shown for the squid giant axon that low external calcium concentration increases the rate of rise of potassium conductance for step depolarizations in the voltage clamp. They also showed that the potassium conductance occurs earlier than normal in low external calcium. If similar effects occur in the lobster giant axon, one might expect interaction between the effects of calcium and potassium on spike amplitude, even if there were no alterations in the sodium mechanism. The effects of low external calcium on the action potential might be explained solely on the basis of an earlier and more rapid potassium conductance, obliterating in part the voltage changes brought about by an otherwise normal sodium conductance. Since evidence has already been presented in this paper for an interaction between calcium and sodium, it is to be expected that lowering external calcium and raising external potassium simultaneously should produce greater alterations in spike amplitude than would either of these alterations alone.

Fig. 5, typical of results from five axons, shows such an interaction of cal-

cium and potassium on spike amplitude. As was true for simultaneous changes in sodium and calcium, it can readily be seen that reducing external calcium and raising external potassium simultaneously produce a greater reduction in spike amplitude than the arithmetic sum of the individual changes. To the right of the curves in Fig. 5 are given the percentage changes in action potential amplitude per millivolt change in resting potential over the 14 minute exposure period. Inasmuch as there is a disproportionate alteration in spike amplitude with respect to resting potential alteration, it would seem that again, this interaction is somewhat independent of changes in resting poten-

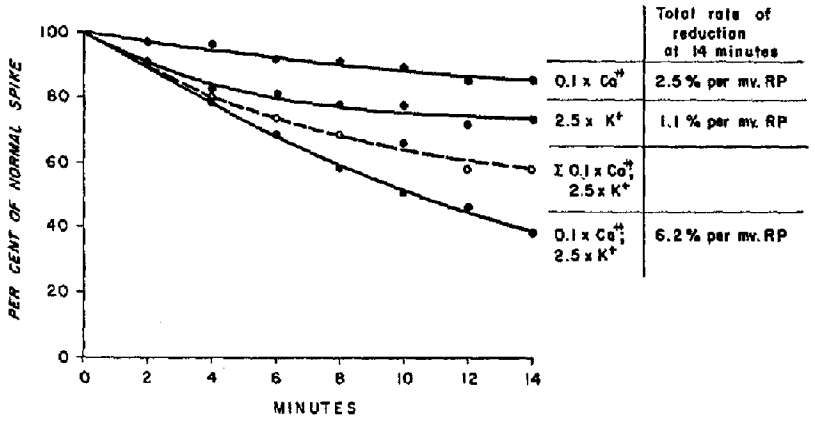

FIGURE 5. Time course of spike amplitude values (per cent of normal) upon exposure of an axon to various test solutions, as indicated. Results obtained are typical of five axons so treated.

tial. Such results are suggestive of an interaction between calcium and potassium directly on the spike-generating mechanism.

DISCUSSION

The results presented in this paper and in the preceding paper (Dalton and Adelman (1960)) suggest that external calcium plays an important role in the spike-generating mechanism of the lobster giant axon. Furthermore, this evidence supports the initial contention that such a role would be exerted in connection with the presupposed functions of sodium and potassium in the spike process. Inasmuch as there is ample evidence for a relation between the action of calcium ion on the action potential in terms of alterations in resting potential different from the relation existing for potassium ion, a more complex effect in the axon membrane must be ascribed to calcium than to potassium.

Cole (1949) has stated that the resistance of the squid axon membrane is proportional to the external calcium concentration. If such a relationship is also true for the lobster axon, one might explain resting potential changes with alterations in external calcium ion concentration on the basis of calcium ion having an influence on the general resting ionic permeability of the membrane. The simplest mechanism whereby calcium ion could influence resting membrane permeability is that proposed by Gordon and Welsh (1948). In this system calcium is envisioned as a "pore plug" limiting the movements of ions in membrane channels or pores and thereby contributing to the resting ionic gradients and in turn to the resting membrane potential. Whether such "pores" or "channels" exist cannot be determined at present, although some theoretical suggestions have been made recently (Mullins (1956)). These suggestions will be considered as a hypothetical basis for the action of calcium on the lobster axon.

In this connection it is interesting to note that an axon exposed to low external calcium continually loses membrane potential with time of exposure. Under these conditions no true equilibrium between external calcium ion concentration and resting membrane potential is reached in times comparable to the times required to reach an equilibrium with external potassium ion. Exposure to low external calcium ion may be envisioned as resulting in a continual slow loss of the potassium from the axon through these hypothetical channels. This would explain the continual decline in resting potential under such conditions. Brink (1954) has presented a compilation of the calcium-binding properties of proteins and other substances likely to be found in membranes. While such data in themselves tell nothing of the calcium-binding properties of such hypothetical membrane channel linings, it does suggest that such binding properties should be looked for in the nerve membrane.

With reference to the complex role of calcium in the genesis of the action potential, this role seems to be intimately connected with those ascribed to sodium and potassium. While direct evidence for calcium ion affecting sodium and potassium conductances has been presented (Frankenhaeuser and Hodgkin (1957); Shanes *et al.* (1959)) only for the squid giant axon, the evidence presented in this paper with respect to interaction of calcium with sodium and potassium effects on action potential adds further support for a similar effect in the lobster axon.

While there is ample evidence for calcium ion interaction with both sodium and potassium in the spike process, it is difficult to determine the exact nature of the interaction under normal conditions. However, there seem to be sufficient data now available on the effects of low external calcium on nerve function to make it possible to compare the logical consequences of an hypothesis similar to that proposed by Mullins (1956), with these data.

The hypothesis supposes that there are conductance channels to sodium

and potassium in the axon membrane. In the resting state these channels are closed by bound divalent calcium ions. The bound calcium must be temporarily removed in order for excitation to occur and for any rapid rise of sodium conductance to take place in the channels. Driving current through the membrane from an external source results in displacement at an external cathode of the calcium from its channel-occupying position. A certain minimal number of channels must be opened before a self-sustaining impulse may be initiated. Conductance channels are assumed to be like the macromolecular interspaces proposed by Mullins (1956). In this case calcium and sodium are assumed to compete for the same channels, inasmuch as their diameters will be nearly identical at all levels of hydration. The larger diameter hydrated potassium ion[2] is assumed to require a larger diameter channel than sodium or calcium. According to this hypothesis, while calcium and sodium compete for the same channel, the mobility of calcium in the channel is far less than that of sodium. This is assumed to be due to forces established between the divalent calcium ions and anionic sites on the membrane macromolecules. Mullins (1956) has assumed that, due to thermal agitation of the macromolecules, the size of the interspace channels varies. A channel in which calcium is bound may be considered as maintaining a relatively constant effective diameter. Removal of calcium from the channel momentarily leaves an opening of appropriate size for sodium entry. The monovalent nature of sodium is assumed to be less effective than calcium in maintaining an effective pore diameter less than that of the diameter of the hydrated potassium ion. The result of this situation is a rapid entry of sodium along its concentration gradient into the axon, followed in time by an enlargement of the effective pore diameter, producing a pore no longer favorable to sodium entry. Such a situation might come about through competition of internal potassium for these enlarged pores. While flow of ions in such a channel or pore can be either inward or outward, but not both at any one time, flow reversal must occur such that potassium ions move outwardly, inactivating further sodium entry. This series of events demands a statistical distribution in time in a given population of pores. The entry of sodium into such a population of pores produces the rising phase of the action potential. Repolarization is accomplished by the potassium movement in these channels (Hodgkin and Huxley (1952)). Return of the membrane potential from the peak of the spike toward the resting potential enhances the rebinding of calcium to the channel. As calcium is rebound inactivation is overcome by means of a return of the channel to a smaller effective pore diameter. Therefore, in order for

[2] Membrane channels are assumed to be circular in cross-section, with diameters about 4 A. This would mean that an effective ion diameter would be that corresponding to an ion with one or two hydration layers. For hydration layers less than three, sodium has a smaller diameter than potassium for a given number of hydration layers (Mullins, 1956).

inactivation of sodium to be removed, calcium must be present externally. If there is a slow loss of calcium from such channels, the resting inactivation should increase.

According to this theory, the hypothetical consequences of external calcium removal are: (1) low threshold to excitation inasmuch as the bound calcium may be removed from the channels more easily; (2) increased refractoriness due to sodium inactivation since a high proportion of channels are not available for rapid conductance changes; (3) decrease in resting potential with time and an increased resting and active potassium conductance since a high proportion of calcium-free channels implies that potassium will leak out of an axon at rest and will flow more readily during activation; and (4) these effects are completely reversible when calcium is restored to the membrane.

Many experimental facts are in complete accord with these postulates. Large diameter lobster axons exposed to low external calcium show a decreased threshold to excitation followed in time by an increase in accommodation (Adelman (1956)), a decreased resting potential (Dalton (1958); Dalton and Adelman (1960)), a decreased action potential amplitude partially independent of resting potential change (Dalton and Adelman (1960)), and a decrease in the rates of rise and fall of spike, as well as a marked increase in post-spike refractoriness (Adelman and Adams (1959)). In addition, evidence has been presented in this paper to indicate an interaction between calcium and sodium in the spike-generating mechanism of the lobster axon.

For the squid giant axon Frankenhaeuser and Hodgkin (1957) have reported increased sodium conductance in low external calcium. This was accomplished by overcoming the inactivating effects of low external calcium by hyperpolarization. An increased sodium conductance above normal was observed on subsequent depolarization. Associated with this increased sodium conductance was an increased potassium conductance. If one assumes that hyperpolarization drives calcium into pores, overcoming the loss due to diffusion in the face of the low concentration of external calcium, then the resting sodium inactivation is abolished. Subsequent depolarization can drive this calcium out of the pores more readily since this would be aided by the concentration gradient for calcium. According to our hypothesis, in the clamped depolarization there should be an increased sodium inactivation and an increased potassium conductance in time as the channels enlarge.

We would expect that these generalizations based on the membrane pore or channel hypothesis can be rationalized with the Hodgkin-Huxley theory (1952). While analog solutions of the Hodgkin-Huxley equations have been carried out only for alterations in external potassium (Dalton and Adelman (1960)), similar computations might be useful in deducing the effects of changes in external calcium on this system. Inasmuch as there is already a quantity of information available (Frankenhaeuser and Hodgkin (1957);

Shanes *et al.* (1959)) on the action of calcium on squid axon membrane parameters, such computations are planned for the near future. One way of approaching this problem stems from the pore hypothesis itself. Since our pore hypothesis suggests that low calcium (externally) implies a higher percentage of potassium-filled channels than normally, this would be equivalent to a change from the normal values for V_K and g_K, the potassium potential and the resting potassium conductance, respectively. Initial solutions are planned with this thought in mind.

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