EFFECTS OF CALCIUM LACK ON ACTION POTENTIAL OF MOTOR AXONS OF THE LOBSTER LIMB*

By W. J. ADELMAN, JR., AND J. ADAMS

(From the Department of Physiology, University of Buffalo School of Medicine, Buffalo)

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ABSTRACT

The effects of external calcium deprivation on certain characteristics of the action potential of the lobster motor axon have been studied. Upon exposure to calcium-free solution the spike amplitude is rapidly decreased within a few minutes and is followed by a slow linear decline. The rates of spike rise and fall are proportionally reduced more than the spike but follow similar time courses during calcium lack. Associated with these phenomena are the loss in the normal slow spike repolarization process, the development of a large and lengthy undershoot, and the appearance of a high degree of refractoriness. The mean increase in the refractory period is 525 per cent upon 10 minutes' exposure to calcium-free solution. These effects are completely reversible upon returning the axons to normal solution. These results are compared to similar effects of calcium deprivation on frog myelinated axons and squid and lobster giant axons recently observed by other workers.

Increased refractoriness has been suggested as a possible explanation for the cessation of spontaneous firing seen during calcium deprivation (Adelman, 1956). It is known that upon exposure to calcium-free *Homarus* solution, single lobster motor axons become spontaneously active, firing at frequencies as rapid as 100/sec. Within seconds, the discharge frequency declines, and eventually the spike discharge ceases altogether, in spite of local potential activity of high frequency and amplitude. At this time, stimulation with long duration constant currents can elicit single responses but not repetitive firing. The failure of the spike process, despite what seem to be favorable conditions for excitation, is assumed to be a result of the axon being in a state of general refractoriness.

That such a state could occur was indicated for the squid giant axon by Frankenhaeuser and Hodgkin (1957). Their work showed that in low or zero calcium a great tendency toward the refractory condition develops. Recently Dalton (1958) has found a tendency for spike "undershoot" (transient hyperpolarization following a spike) to develop in the "giant" axons of the lobster circumesophageal connectives upon exposure to low calcium. Such observations

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demonstrate that the effect of low calcium solutions may be either to induce oscillatory or refractory behavior, and indicate that the former develops into the latter during prolonged exposure to the low calcium solutions.

The experiments reported here were performed in order to inquire further into the relation of calcium ion to the spike process in single lobster motor axons. During exposure to zero calcium direct measurements of excitability following the propagated action potential were made and compared to various characteristics of the spike process itself.

Methods

Single motor axons (slow closers and openers) were prepared from the walking limbs of the lobster. Stimulation and recording techniques were essentially the same as those described in an earlier publication (Wright, Coleman, and Adelman, 1955). The method involves using an abrupt conductance change in the medium external to the axon to record a potential change which is similar to an internally recorded action potential, with the exception that it is somewhat attenuated in voltage. Advantages of this technique are that responses are initiated and recorded in physiological solution. Isolated axons may be used with no supporting neural tissue adjacent to the recording site, as is usually required for stabilizing small diameter axon preparations. The solution may be exchanged without disturbing the axon. The D. C. output amplifier previously described for mixing stimulating pulses (Adelman, 1956) was eliminated, by the use of a purely resistance-coupled device. In determining the refractory periods of the axons two independent short duration pulses were generated; the time interval between these pulses was measured by means of a megacycle time interval meter. In practice the first pulse initiated an action potential and the time interval to the second pulse was set and measured. Then the threshold intensity of the second pulse required to produce a second spike was determined. Spikes were recorded using the D. C. amplifiers of a DuMont 333 oscilloscope, or a Tektronix 535 oscilloscope.

Physiological lobster solution was that of W. Cole (1941) as modified by Dalton (1958) with the exception that sodium bicarbonate was used as a buffer. Calcium ion was replaced by substituting sodium, to maintain constant osmolarity. Similar precautions such as those taken by Frankenhaeuser (1957) were observed to insure "calcium-free" solutions.

RESULTS

Changes in the Action Potential in Calcium-Free Solution

One of the most dramatic effects obtained upon exposure of axons to solutions devoid of calcium ions is a reduction in the amplitude of the propagated monophasic action potential. Fig. 1 illustrates typical results obtained from a single motor axon. A typical spike, obtained in normal solution, is shown in record *A*. Highly characteristic of the normal spike is the existence of a delayed repolarization following the spike. The spike amplitude is 23 mv. in this record. Immediately after obtaining this record the external solution was exchanged for

one containing no calcium ions. Within 2 minutes the spike height was markedly reduced, as is illustrated in record *B*. At this time an undershoot or hyperpolarization develops which lasts for several milliseconds (Fig. 1 *B*). After 5 minutes' exposure to the calcium-free solution the recovery undershooting increased in magnitude, and became prolonged in duration (*C*). Associated with this change

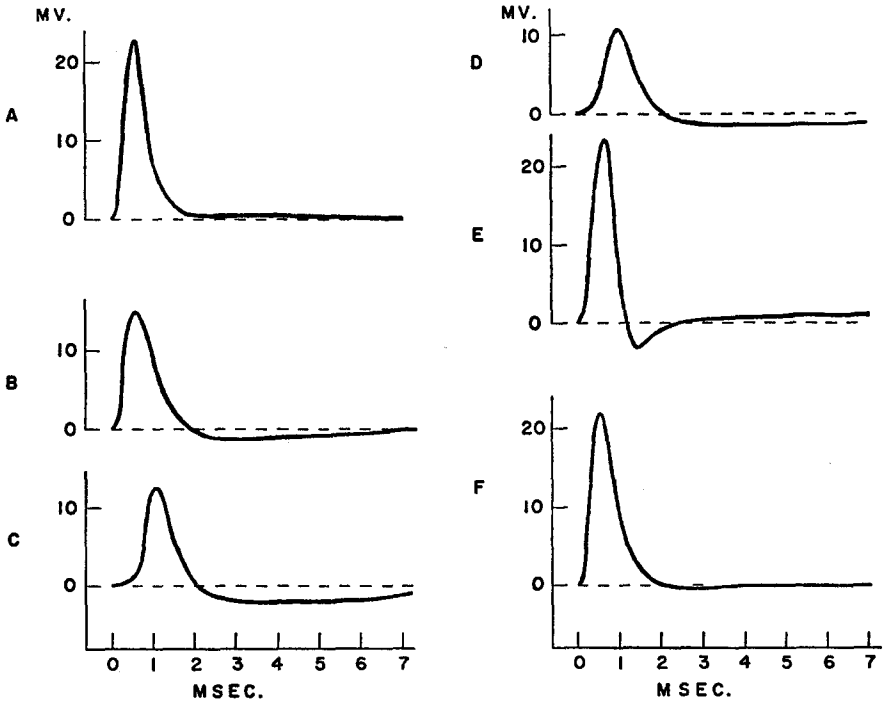

FIG. 1 Action potentials of a typical opener axon. *A*, normal solution initially. *B*, *C*, and *D*, calcium-free solution, 2, 5, and 14 minutes' exposure, respectively. *E* and *F*, 4 and 9 minutes after return to normal solution, respectively. Tracings of original recordings. See text.

was a slowing in the rate of rise and fall of the spike itself. Further exposure to calcium-free solution produced only a mild enhancement of these effects (*D*). The calcium-free solution was exchanged for normal solution after the axon had been exposed for 16 minutes. The spike amplitude promptly recovered, and within 4 minutes reached the initial value of 23 mv. However, undershooting still persisted, even though greatly shortened in duration (Fig. 1, *E*). In addition, this very transient trough of hyperpolarization was followed by a period of mild depolarization. Further exposure to normal solution resulted in the recovery of the spike to approximately normal characteristics (*F*). These results

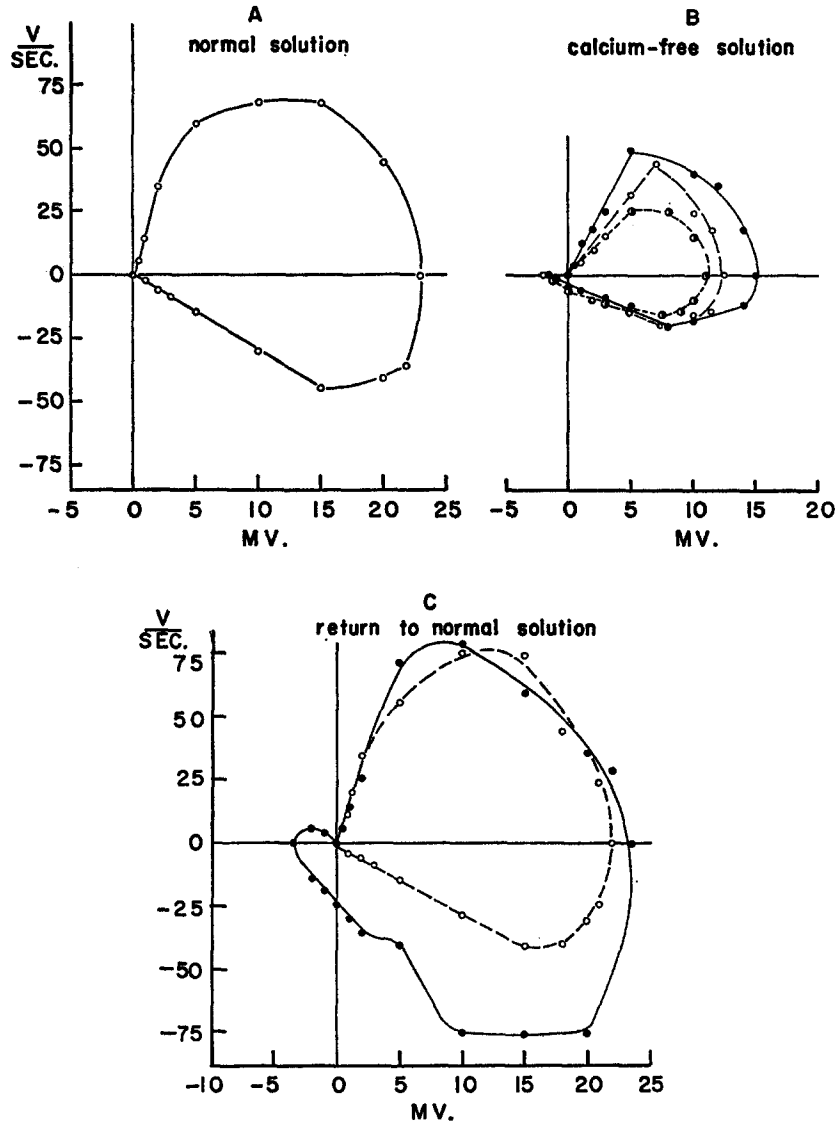

FIG. 2. Phase plane trajectories, dV/dt *vs.* V, of spikes in Fig. 1. *A*, normal solution initially. *B*, calcium-free solution, filled circles, 2 minutes' exposure; open circles, 5 minutes' exposure; half-filled circles, 14 minutes' exposure. *C*, filled circles, 4 minutes after return to normal solution; open circles, 9 minutes after return to normal solution. See text.

are typical of results obtained on ten different axons. In this particular axon (Fig. 1) the delayed repolarization following the spike was not recovered but in many other axons exposed to calcium-free media, complete recovery of this characteristic was accomplished in normal solution. Whenever axons are exposed to zero calcium for longer than 20 minutes blockade ensues and the axons become inexcitable.

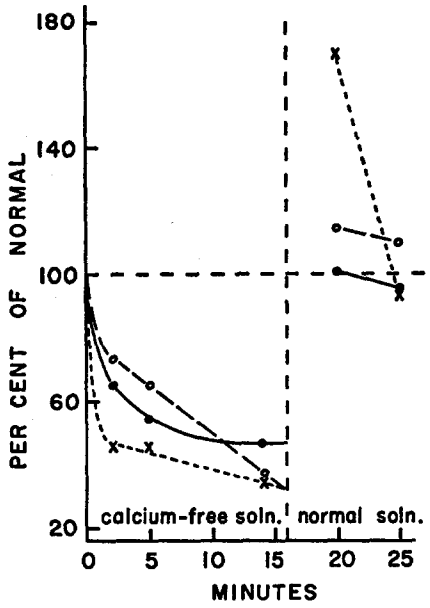

FIG. 3. Effect of calcium deprivation upon values of spike amplitude (closed circles) maximum rate of spike rise (open circles), and maximum rate of spike fall (crosses) in terms of per cent of initial values in normal solution.

While spike amplitude is the most obvious change in calcium-free solution, alterations in the rate of spike rise and fall are equally demonstrable. The use of the phase plane diagram (K. Cole, 1956) appears to be the best way of describing such data. Spikes such as those shown in Fig. 1 are differentiated and the closed trajectories of these derivatives (dV/dt) are plotted for each spike as a function of the recorded potential values (Fig. 2). In *A* may be seen the spike phase plane trajectory obtained in normal solution. The maximum rate of spike rise is reached at about 12 mv., with the whole rising phase of the spike being characterized by a somewhat symmetrical wavefront. The falling phase is most rapid as the potential decreases from 20 to 10 mv. Return to baseline characteristically shows the slow rates associated with delayed repolarization. The

entire trajectory sweeps out from the origin and smoothly returns to it with all voltage values remaining positive.

The spike phase plane trajectory is drastically altered in calcium-free solution. In Fig. 2 *B* this change is shown for 2, 5, and 14 minutes of calcium deprivation. A progressive decline is seen in the rate of spike rise and fall. After 14 minutes of exposure the maximum rate of rise is decreased to only one-third the normal value, whereas the spike amplitude is half the normal value. The undershoot, which is so characteristic of lobster axons in calcium-deficient solution, is clearly seen in the negative rates associated with the negative potential values to the left of the origin. In general, the entire spike process becomes more sluggish as the spike declines in low calcium.

The two phase plane trajectories in Fig. 2 *C* illustrate the recovery of the spike upon return to normal solution. After 4 minutes' exposure to normal solution the rates of rise of the spikes are approximately normal. However, the rapid fall of the voltage into the trough-like undershoot is clearly demonstrated. The maximum rate of repolarization is 75 v./sec., or almost double the value seen in the normal spike. The rapid repolarization and the trough-like undershoot completely disappear and the phase plane trajectory assumes normal proportions after 9 minutes in normal solution.

In Fig. 3 the variation in spike height is compared with the maximum rates of rise and fall of the spike upon exposure to calcium-free solution. All these parameters show a rapid reduction in the measured values within the first few minutes and then are followed by a slow linear decline. The rates of rise and fall are more affected than the spike magnitude, inasmuch as these values are down to one-third the normal value within 14 minutes, whereas the spike amplitude was reduced only to one-half its normal value.

Excitability Changes Following the Spike

The existence of the prolonged spike undershoot in the axons exposed to calcium-free solution suggested that there would be extreme changes in the refractory period of these axons. This suggestion was substantiated through direct measurements of the excitability cycle following a propagated spike. Fig. 4 represents such measurements obtained from a typical axon. On the ordinate is plotted the ratio of the threshold intensity (I_2) of a test shock to the threshold intensity (I_1) of another shock which precedes the test shock by some time interval, represented on the abscissa. When the I_2/I_1 ratio is 1, then the threshold is the same as that obtained with a single shock. The threshold curve obtained in normal solution shows a refractory period of 8 msec. (absolute plus relative). Very brief refractory periods are characteristic of axons that have spikes showing an abrupt return of the spike to the resting potential, with no subsequent potential oscillations. Even shorter duration refractory periods are

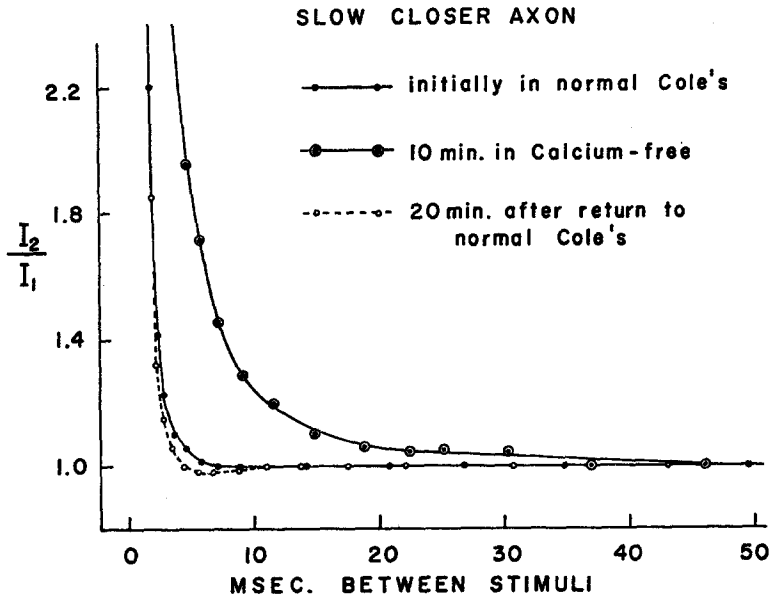

FIG. 4. Typical threshold changes following a propagated spike in normal and calcium-free solution. Axon normally having no supernormality. For detailed explanation see text.

TABLE I

Refractory Periods (Absolute + Relative)

Initially in normal solution	10 min. in Ca-free solution		10 min. recovery in normal solution
msec.	*msec.*	*per cent increase*	*msec.*
2.5	11.0	340	2.0
3.6	10.0	178	4.8
4.2	17.4	325	—
8.0	40.0	400	6.0
1.4*	14.0	900	2.1
1.7*	16.0	841	2.8
4.0	12.5	212	2.8
1.4*	7.4	428	2.3
12.5	42.0	236	6.4
4.7	70.0	1390	—
Means..... 4.4	24.0	525	3.7

* Axons showing pronounced supernormality in normal solution.

common in normal solution (see Table I). After 10 minutes' exposure to calcium-free solution, the refractory period value is five times the normal value. Twenty minutes after return to normal solution the threshold curve is essentially the same as that obtained initially.

Some axons show in normal solution the existence of a pronounced supernormal period. The post-spike threshold recovery curve of such an axon is illustrated in Fig. 5. In normal solution there is a pronounced supernormal

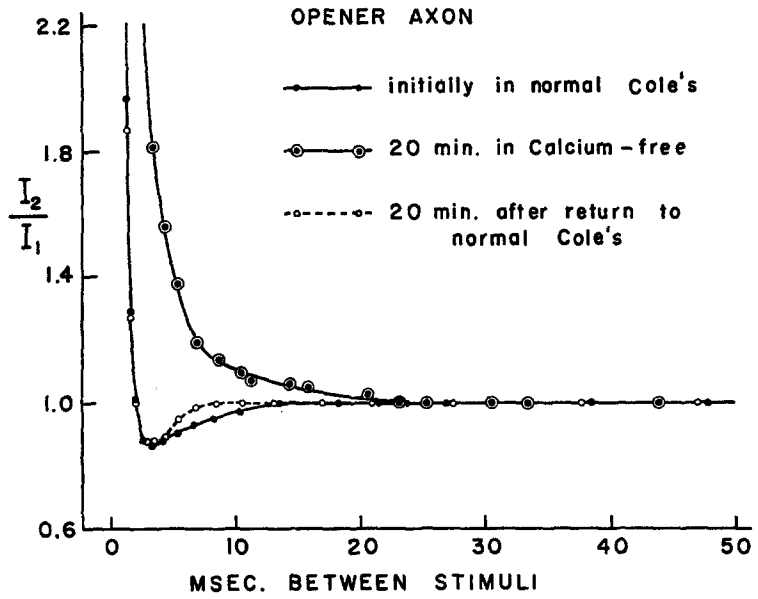

FIG. 5. Typical threshold changes following a propagated spike in normal and calcium-free solution. Axon normally possessing supernormality. See text and compare with Fig. 4.

period lasting for about 10 msec. This axon possessed a corresponding period of persistent depolarization following its spike. After 20 minutes' exposure to zero calcium, supernormality disappeared and a long lasting relative refractory period became apparent. This effect was completely reversible upon return of the axon to normal solution.

Table I summarizes the effects of calcium lack on the spike excitability recovery process. All axons so measured show large increases in the refractory period upon exposure to calcium-free solution. The mean increase, after 10 minutes' exposure, is 525 per cent. In all, it would seem that the spike process, once initiated in zero external calcium, imposes severe limitations on subsequent repeated spike initiation. Stimulation with long duration constant currents, at

the times when prolonged refractory periods were observed, showed that calcium-deficient axons are incapable of firing repetitive bursts even though they are capable of repetitive firing in normal solution.

DISCUSSION

The effects of external calcium deprivation on spike amplitude obtained in this study are in substantial agreement with those obtained by Dalton (1958) from the lobster circumesophageal giant axon. Inasmuch as spike amplitude was rapidly decreased and the axons eventually became inexcitable in zero external calcium, it is obvious that calcium is somehow necessary for spike development. While Frankenhaeuser (1957) was able to produce immediate blockage of frog myelinated axons in calcium-free solution, the lobster axon can be rendered immediately inexcitable only when decalcifying agents are applied along with calcium-free solution (Adelman, unpublished data). Frankenhaeuser (1957), comparing frog myelinated axon data to those obtained from cephalopod axons, suggested that, in the case of cephalopod axons, traces of calcium adhering to the axonal surface may prevent immediate blockade in "zero calcium" solution. The alternative to this suggestion is to conclude that "calcium in the external solution is not immediately necessary for impulse generation." However, it is conceivable that the frog nodal membrane is more exposed to ionic interchange than is the crustacean or cephalopod axonal membrane.

It seems unlikely that the complex effects of calcium deprivation on the action potential are brought about by alterations in resting membrane potential alone. Recently it has been demonstrated (Dalton, personal communication) that the crayfish giant axon undergoes a greater reduction in spike height per unit change in resting potential in low calcium than occurs with corresponding changes in resting potential brought about by exposure to high external potassium. If similar effects take place in the lobster limb axons, then alterations in membrane resting potential hardly seem sufficient to account for the even larger alterations in rate of spike rise and fall and the associated changes in post-spike excitability brought about by calcium deprivation.

The refractory period changes and the potential changes during spike repolarization induced by calcium lack might be interpreted as being due to an increased sodium inactivation and an increased potassium conductance respectively during the falling phase of the spike. If these assumptions are correct, then our work agrees with that of Frankenhaeuser and Hodgkin (1957). These workers were able to demonstrate in the squid giant axon, using the voltage clamp technique, that decreasing the external calcium concentration results in increased sodium inactivation and increased potassium conductance.

At the present time, it is tempting to speculate that calcium plays multiple roles in the membrane structure and its electrical and excitable properties (Brink, 1954). Work is in progress attempting to relate the effects of changes

in external calcium ion concentration with the effects of similar changes in other ionic constituents. It is hoped that this work will provide evidence for the multiple role of calcium in the nerve membrane.

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