

Dependence of Ionized and Total Ca in Squid Axons on Na_o-free or High-K_o Conditions

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ABSTRACT The level of intracellular Ca in squid axons (both ionized and total Ca) was studied as a function of the experimental variables [Na]_i, [Na]_o, pH_i, cyanide, and depolarization. Ionized Ca was measured by following the light emission of aequorin while total Ca was measured by the atomic absorption analysis of samples of axoplasm. Aequorin glow is known to be increased either by the application of Na_o-free solutions or by depolarization produced by external solutions containing greater than normal K concentrations. The present results show that if [Na]_i is low, the depolarization that is brought about by solutions with elevated [K] leads to a resting light emission that is decreased rather than increased, as is the case when [Na]_i is high. In axons where [Na]_i is varied, a comparison of the increments in light emission produced by the application first of Na-free and then of high-K solutions shows that they have an identical dependence on [Na]_i, with a half-activation of Ca entry produced by an [Na]_i of 25–30 mM. Changes in pH_i affect the aequorin signal produced by depolarization, with acidification reducing and alkalinization increasing the response. Cyanide did not greatly affect the size of the signal resulting from either Na_o removal or that from depolarization.

INTRODUCTION

Hodgkin and Keynes (1957) demonstrated that the depolarization of squid giant axons resulted in an increased influx of ⁴⁵Ca and they showed further that Ca entry could occur as a result of repetitive stimulation of the fiber. In a subsequent study (Baker et al., 1971), it was shown that the extra fluid of Ca with stimulation could be divided into a tetrodotoxin (TTX)-sensitive and a TTX-independent Ca entry with depolarization. A further elaboration of these two components of Ca entry with depolarization (Baker et al., 1973*a, b*) led to the conclusion that these studies with aequorin as an intracellular indicator of Ca necessarily required that some Ca entry be via Na channels, while the balance entered via a Ca channel that only very slowly inactivated. The entry observed was maximal at a membrane potential of ~0 and was very small at values of +40 mV or larger.

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A demonstration by Mullins and Brinley (1975) that Ca efflux from squid axons could be enhanced by passing electric currents that hyperpolarized the membrane suggested that the exchange of Ca_i for Na_o was electrogenic. Further studies (Mullins 1979, 1981) suggested that Na/Ca exchange, in addition to removing Ca from the fiber in exchange for Na, could introduce significant Ca into cardiac cells under appropriate conditions.

The experiments of Baker et al. (1973*b*) showed that applying high concentrations of K in seawater to axons injected with aequorin led to an increase in light emission that had both a phasic and a tonic component. Similar experiments by Mullins and Requena (1981), where the axons were tetanized in Li-containing seawater (a procedure that reduced Na_i to about half its normal value), led to axons that had only a very small response to depolarization. This response to depolarizing solutions could subsequently be enhanced ~20-fold or more simply by raising Na_i . It was suggested as a result of this study that the entire response of the axon to steady depolarization was Na/Ca exchange. A further study of this effect (Mullins et al., 1983), using arsenazo III as an intracellular Ca indicator and Na-sensitive electrodes to measure $[\text{Na}]_i$, showed that the relationship between the increase in Ca signal and $[\text{Na}]_i$ was such that the response was half-saturated at 25 mM and the relationship between $[\text{Na}]$ and response was very steep (Hill coefficient, 7).

Although these experiments clearly indicated the total sensitivity of the depolarizing response to Na_i , they did not rule out the possibility that a Ca channel was present in the squid axon that opened with depolarization and then inactivated so that the only way its contribution to Ca entry could be measured was by repetitive pulsing. Indeed, DiPolo et al. (1983) measured a current that flowed during the depolarization of squid axons when Na and K currents had been minimized. This current was enhanced by increases in Ca_o and inhibited by Cd^{2+} . Very recently, Mullins et al. (1985) have shown that in aequorin-injected axons subjected to voltage clamp and with Ca_o at physiological levels, a detectable entry of Ca with clamp pulses (in the presence of TTX) is only present if Na_o is decreased and Na_i is increased. More importantly, the response to voltage-clamp pulsing increases e-fold/22 mV of depolarization and the response to pulses showed no sign of saturation at membrane potentials close to E_{Ca} . This result strongly supports the notion that Na/Ca exchange is the major source of Ca influx during fast depolarization episodes in squid axons.

The purpose of the experiments reported here was to study the dependence of the levels of $[\text{Ca}]_i$ on Na_i or pH_i or cyanide (CN) during removal of external Na, a condition known to impair the operation of the Na/Ca exchange system in a forward-going direction, and to compare such dependence with that observed during K-induced depolarization. The results strengthen the notion that the entry of Ca observed during long depolarizations reflects mainly the operation of the Na/Ca exchanger and not that of a slow channel.

METHODS

Experimental Animals

The *Loligo pealei* used were collected and studied at the Marine Biological Laboratory in Woods Hole, MA, in the spring of 1982 and 1983, and the *Loligo plei* used were collected

in the Estacion de Investigaciones Marinas de Mochima, Edo Sucre, Venezuela, during the fall of 1982 and 1983.

Microinjection, Ca Measurements, and Microelectrodes

The experimental setup used has been previously described by Mullins and Requena (1981) as modified in Requena et al. (1985). The experiments were done at 16°C. Microelectrodes were manufactured as described in Mullins et al. (1983) and were from the same batch of ion-sensitive glass. The Ca and Na contents of axoplasm samples were measured as described in Requena et al. (1979) using atomic absorption and flame photometry. The analytical results were obtained with *L. plei* giant axons.

Solutions

The seawater used in these experiments had the following composition (mM): 440 NaCl, 10 KCl, 50 MgCl₂, 10 Tris buffer, pH 7.8, and 0.1 EDTA. Usually 10 mM CaCl₂ was added to the seawater. Variations in the composition of this solution were made by replacing all of the Na by K or Li or Tris to produce depolarizing or Na-free solutions. When higher concentrations of Ca were required, Mg or some Na was deleted from the solution in order to keep the osmolarity constant at 1,010 mosmol throughout. Phenol red was obtained from Sigma Chemical Co. (St. Louis, MO) and purified by several recrystallizations in Ca-free, double-distilled water during acid precipitation and solubilization by base. It was then passed twice through Chelex columns. Aequorin was kindly provided by Dr. John Blinks (Mayo Clinic, Rochester, MN) and used at a concentration of 12 mg/ml in 5 μM EGTA.

RESULTS

[Ca]_i in the Axon Periphery

Many of the experiments described here were done in axons injected with aequorin and phenol red in order to confine the light response to the peripheral part of the axoplasm (Mullins and Requena, 1979). It was thus of interest to know the level of ionized Ca in this region. For this purpose, the initial level of luminescence of an axon was compared with that observed under conditions in which the concentration of the intracellular ionized Ca was set by means of the Ca buffer EGTA. Fig. 1 shows that, in an axon, a roughly equimolar mixture of CaEGTA and EGTA produced twice as much light as the initial resting glow. The axon was bathed throughout in 10 mM Ca seawater. Thus, if the apparent dissociation constant of the CaEGTA complex under the conditions prevalent in the axoplasm is of the order of 150 nM Ca (DiPolo et al., 1976), it can be concluded that the level of ionized Ca in the subaxolemmal region is of the order of 75 nM. It should be recalled that at around pCa 7, the aequorin reaction with Ca, which leads to the production of light, shows a linear dependence on [Ca] (see Requena and Mullins, 1979, for a discussion of the data of Allen et al., 1977). This justifies the linear interpolation used above.

Decreased Ca Entry During K Depolarization

If [Na]_i is sufficiently low, then there is no enhancement of the aequorin light signal in response to a steady depolarization brought about by a high-K_o solution (Mullins and Requena, 1981; Mullins et al., 1983). This finding is the basis for the claim that Ca entry with steady depolarization is Na/Ca exchange. A new

finding in our present studies is that, in some axons, it is possible to observe a decrease in light emission during depolarization with high- K_o solutions.

An explanation for this effect is somewhat complicated and can best be understood by considering first the effect of Na_o -free solutions and, later, the effect of depolarization. If we consider an axon with a low Na_i and apply an Na_o -free solution, we expect that light emission from aequorin will be affected in the following ways: (a) Ca entry via Na/Ca will not be increased because $[Na]_i$ is too low; (b) Ca leaks by diffusion into the axon will not be changed; and (c) Ca efflux by Na/Ca exchange will be reduced. The expected result of this change is a rise in $[Ca]_i$ since influx remains unchanged while Ca efflux is reduced.

A somewhat different (and more complicated) situation arises if we depolarize the axon with a high- $[K]$ solution since (a) Ca entry via Na/Ca is not increased, owing to insufficient $[Na]_i$, and (b) Ca efflux has been decreased, both owing to the absence of Na in the external solution and because depolarization via Na/Ca



FIGURE 1. The effect on resting glow of equilibrating an axon with EGTA buffer containing ~ 4 mM total chelator. The axon was initially injected with aequorin and phenol red (220 mM K-salt) and it had a diameter of 0.600 mm. The membrane potential was -60 mV. The external solution was Na seawater with 10 mM Ca. The light trace collection was interrupted during the injection of the Ca buffer, but not so the time base in the record.

exchange decreased Ca efflux (Mullins and Brinley, 1975). Both of these changes would tend to increase the resting glow, as is the case for Na-free solutions. However, there are two other factors that are of substantial importance: (a) Ca entry via diffusive pathways is decreased since the electrical force driving Ca inward has been decreased by a reduction in E_m from -60 to $+5$ mV, and (b) depolarization reduces the number of open Na channels in the steady state according to the Hodgkin-Huxley analysis, and Ca entry that is TTX sensitive has been shown to be a substantial fraction of the resting Ca influx (DiPolo et al., 1982). Not considered here are the fast kinetics of the channel systems (for Na and K) since these inactivate on a time scale of milliseconds or hundreds of milliseconds, respectively. Hence, the response to steady depolarization of an axon with a normal $[Na]_i$ is not affected by Ca flow through these channels. We find that a decrease in aequorin light emission can occur with depolarization in the presence of TTX. Hence, it would appear that the influx of Ca via diffusive pathways is the principal source of resting Ca gain and this diffusive movement must be expected to be decreased by depolarization.

Examples of the decrease in light emission with K solutions are shown in Fig. 2. In the upper left, the transfer of the axon from Na-containing to Na-free

seawater led to an increased glow (because of a decrease in Ca efflux brought about by Na-free solutions), whereas the application of a high-K solution decreased the aequorin glow. In the upper right is a demonstration that TTX does not abolish the decrease in light emission with depolarization. The axon in the upper left was subsequently stimulated in Na seawater to increase $[Na]_i$, and a second test showed that the response changed from one where depolarization decreased light emission to one where depolarization increased light emission.

The possibility existed that this behavior of fresh axons was an artifact or simply a reflection of a slow rate of exchange between Na_i and Ca_o . An axon,

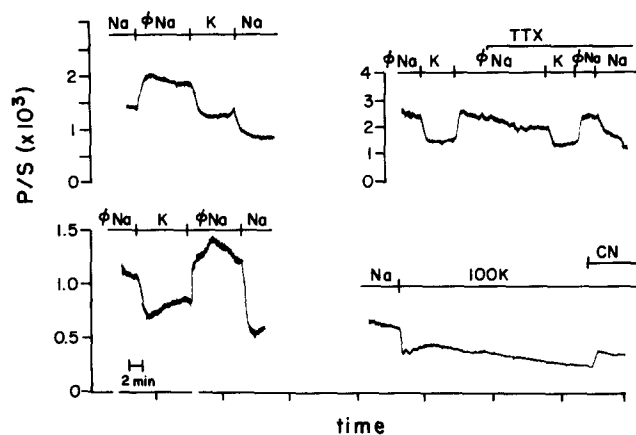


FIGURE 2. Composite of the time course of luminescence in four experiments showing that in each case, K depolarization resulted in a decrease of the Ca signal. All traces correspond to the beginning of the experiment. On the top left, an *L. pealei* axon (053182A) shows an increase in light upon application of Na-free Tris and a decrease upon depolarization. On the top right, the response to depolarization is not affected by TTX. On the lower left is a record from an *L. pealei* (R052782A) axon injected with aequorin and phenol red, and on the right the axon depolarized with 100 K seawater was a *L. plei* axon (R290382) injected with aequorin only. All experiments were at a Ca_o level of 3 mM. In the *L. pealei* axon, subsequent stimulation in Na seawater to raise the Na_i level above 35 mM resulted in the classical biphasic light increments with K depolarization. For more examples, see Figs. 3 and 7.

which produced a decrease in the light response with depolarization, was stimulated in low-Ca seawater in order to raise Na_i and tested again for its response to depolarization. This is shown in Fig. 3. The axon had an initial level of 12 mM $[Na]_i$ and, as can be seen in the top part of the figure, even at high- Ca_o levels, such as 50 and 112 mM, the response to a K depolarization was a decrease in the intensity of the light emitted by aequorin. The axon was then stimulated at 60/s until $[Na]_i$, as indicated by the Na electrode, was 42 mM. Then depolarizing tests at 10, 50, and 112 mM Ca_o resulted in increases in luminescence. The axon was kept in K-free Tris seawater when not being tested in order to prevent a decline in $[Na]_i$. Obviously, the reverse mode of operation of the Na/Ca exchange mechanism was activated by Na_i . In this particular axon, the magnitude

of the Ca signal owing to depolarization was studied as a function of Ca_o . It was found, in agreement with Mullins et al. (1983), that the effect was saturated at 3 mM Ca_o .

Ca Entry and $[Na]_i$

A series of experiments was undertaken to compare the quantitative relationship between the levels of $[Na]_i$ and the Ca entry observed during (a) the removal of Na_o , replaced by Tris, or (b) the removal of Na_o and the depolarization that

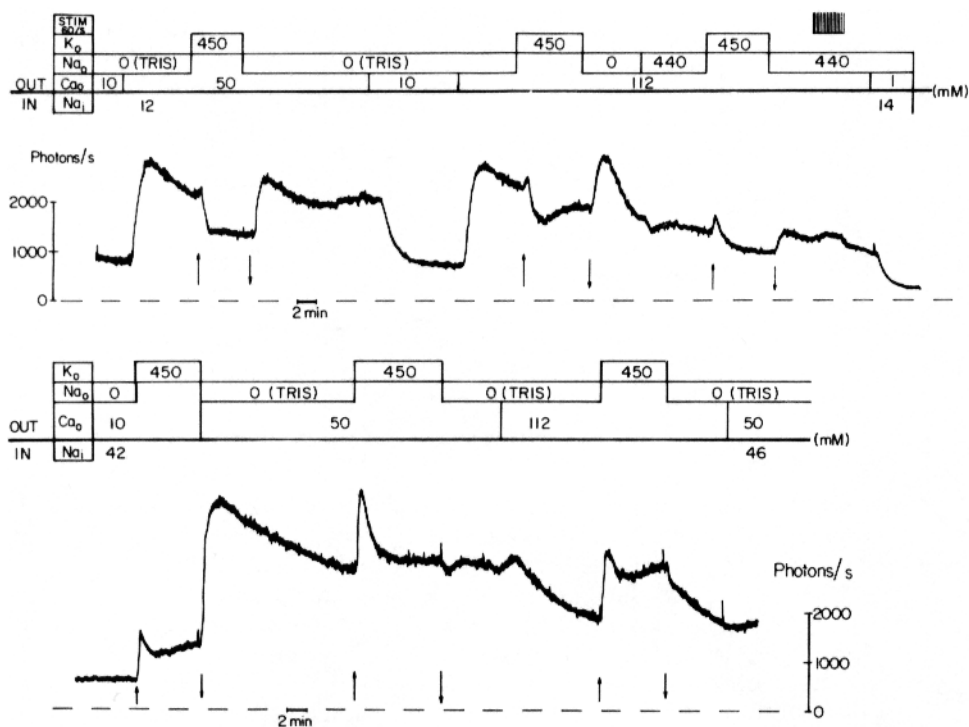


FIGURE 3. The time course of luminescence of an axon injected with aequorin and phenol red showing the effect of K depolarization in 450 mM K seawater with 50 or 112 mM Ca_o while $[Na]_i$ was 12 mM. Subsequently, the axon was stimulated at a frequency of 60 impulses/s for 17 min in Na (1 mM Ca) seawater to raise the level of Na_i to 41 mM. K depolarization tests were repeated at 10, 50, and 112 mM Ca_o . Axon diameter, 0.575 mm. The phenol red initial concentration was 220 mM K-salt. Membrane potential, -54 mV. The axon was impaled with an Na-sensitive microelectrode. RAEQPR052982A.

occurs when K is substituted for Tris. The method was to use an Na-sensitive microelectrode and progressively to raise the concentration of Na_i by brief 2-min periods of stimulation at 30 impulses/s. At each level of Na_i , the axon was tested for a Tris response for ~ 5 min and then for a K depolarization for another 5 min. These test conditions were followed by a rest period of at least 5 min, after which the stimulation episode was repeated and then followed with another rest period to allow for stabilization of the internal Na concentration. In Fig. 4,

aequorin responses for the two test conditions of Na-free and depolarized Na-free, in 10 mM Ca_o , are shown for various levels of Na_i concentration. It should be noticed that as long as Na_i was below 20 mM, there was no Ca signal associated with the removal of Na_o , whereas even at $Na_i = 12$ mM, there was a very small response to depolarization. Conversely, for a concentration level of $[Na]_i$ higher

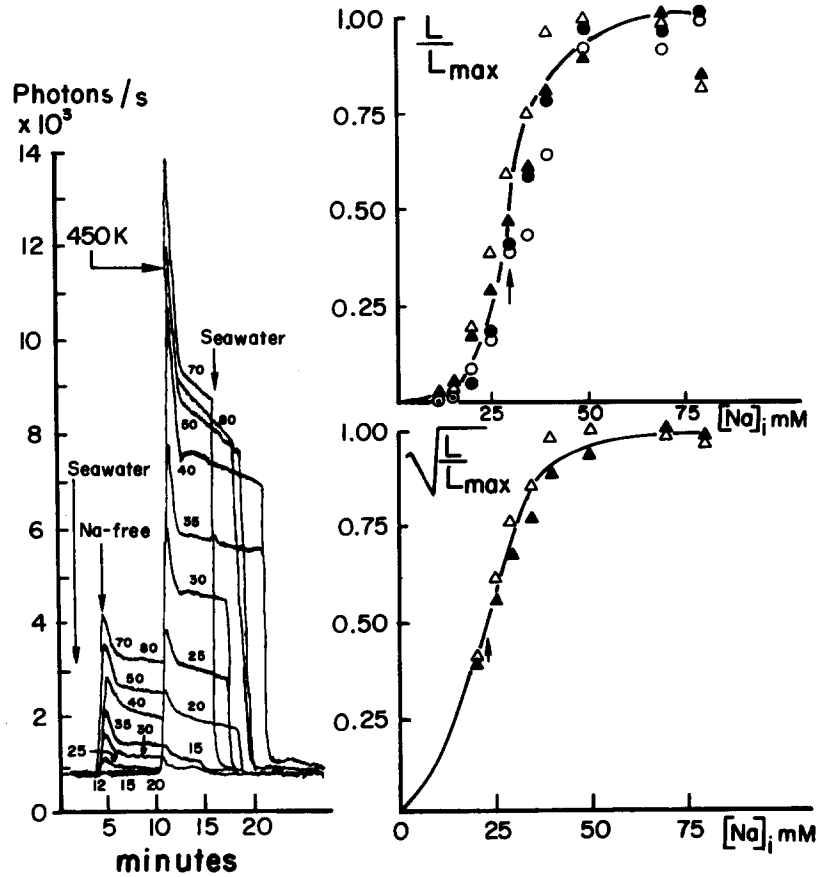


FIGURE 4. The dependence on Na_i of the magnitude of the Ca signal produced by the removal of Na_o , followed immediately by a K depolarization. The left panel shows the time course of records of aequorin signal superimposed for various Na_i levels, all in the same axon and for 10 mM Ca_o . Records were synchronized at the onset of depolarization. The right panel shows, as a function of Na_i , either the normalized (as a fraction of the maximum) magnitude of the increment in light (top part) or the normalized square root (as a fraction of the maximum) (bottom part) of the increment registered for the light signal. In the latter case, points were omitted for the low light emission range since this must be expected to be linear with $[Ca]$. The experimental points were: the peak of the rapid phasic response (▲) and the tonic or plateau (Δ) reached during a K depolarization and the peak (●) and the plateau (○) observed during the Tris (Na-free) episode. The axon was injected with aequorin only. Axon diameter, 0.600 mm. Membrane potential, -54 mV. 040683A.

than 50 mM, the aequorin-measured Ca signals from Na_o-free and K depolarization were all very similar. Between these concentration ranges, the Ca signals grew from virtually nil to several kilophotons per second.

The right part of Fig. 4 shows the normalized magnitude of the light increment (top part) and the normalized square root of this magnitude (bottom part), as a function of the internal Na concentration for the two test conditions: Na-free or Na-free and K-depolarized. The peak of the rapid phasic component and the plateau or tonic component of the Ca aequorin signal were measured. The experimental points from either component of the Ca signal owing to K depolarization fell on a single sigmoid curve, which can be described as highly cooperative, and had an apparent affinity constant of 30 mM Na_i for the linear aequorin-Ca reaction or 25 mM Na_i for the square law reaction. Similarly, for the components of the Ca signal owing to the replacement of Na_o by Tris, all measurements fell on the same sigmoid curve.

Three experiments similar to that of Fig. 4 were done on different axons; two of these were on axons injected with phenol red as well as aequorin. All results were in agreement in finding that a value of [Na_i] of 30 mM was necessary for the half-activation of either the peak aequorin light emission or its steady state value when K depolarizations were applied to the axon. A further finding of some interest was that as [Na_i] in these axons was increased by stimulation, the value of the resting glow increased. For the axon shown in Fig. 4, the initial aequorin luminescence was 750 photons/s at [Na_i] = 20 mM and it was 1,700 photons/s at [Na_i] = 80 mM. The data in Fig. 4 all have initial resting glows normalized to a value of 900/s in order to make it possible to compare responses to Na-free and high-K solutions.

Ca Entry and pH_i

Mullins et al. (1983) studied in some detail the effect of pH_i on Ca entry with depolarization and concluded that entry is strongly inhibited by a decrease in pH_i. We have extended these measurements by comparing the aequorin Ca signal resulting from the replacement of Na_o by Tris with depolarization in high-K seawater. These tests were done as a function of various levels of pH_i monitored by a glass pH microelectrode positioned inside an axon. The intracellular pH was varied with increasing [NH₄⁺] from 2 to 10 mM applied in seawater (Boron and De Weer, 1976). At each stable pH_i, the axon was tested first for Na removal (replacement with Tris), followed by a K depolarization. At least 10–15 min was permitted to elapse between depolarizing episodes. Once a complete pH_i excursion was done, the axon was stimulated at 90 impulses/s for 10 min. The protocol was then repeated. The first pH_i excursion was, presumably, at a normal level of Na_i, whereas the second one was at a higher concentration of Na_i since this was elevated during the period of stimulation.

The left part of Fig. 5 shows (as an example and from the experiment just described) a Ca signal at the onset of the experiment, caused by an Na-free episode followed by a K depolarization. It corresponds to a normal [Na_i] (trace at pH 7.16). It is five times smaller than the one toward the end of the experiment (trace at pH 7.10). Notice that both tests were done at virtually the same pH. In the right part of the figure, the magnitude of the aequorin light emission is

plotted, as a function of the internal pH, for the two levels of Na_i and for the three experimental parameters measured: the peak of the rapid phasic component, the tonic or plateau of the response during a K depolarization, and the plateau recorded during the Tris episode.

At levels of Na_i that are physiological, there is relatively little sensitivity of Ca entry to changes in pH_i , whether the entry of Ca is brought about by Na_o -free solutions or by K depolarization. This may reflect the fact that when the reverse Na/Ca is relatively small, Ca entry via other modes may be dominant. Our results show that, at elevated $[\text{Na}]_i$, Ca entry with depolarization is greatly enhanced by

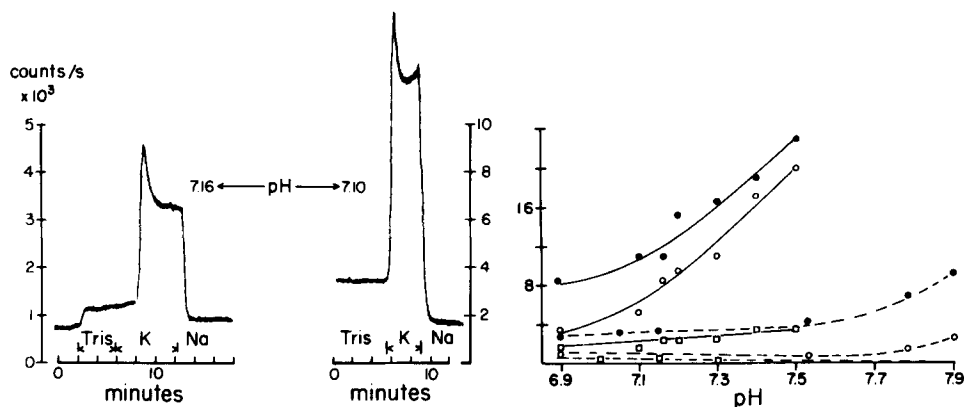


FIGURE 5. The effect of pH_i on the magnitude of the Ca signal of the removal of Na_o followed by K depolarization. The axon was injected with aequorin only, impaled with a pH-sensitive microelectrode, and exposed throughout to 10 mM Ca_o . In the left part, two selected traces are shown corresponding to Ca signals caused by the substitution of Tris for Na_o , followed by a K depolarization before (trace at pH 7.16) and after stimulation (trace at pH 7.10), a procedure used to elevate Na_i above the physiological level. Note the different ordinates for the two traces. In the right part of the figure, the magnitude of the aequorin signal is plotted as a function of the pH_i and for the two levels of Na_i , elevated (solid lines) and physiological (broken lines). The experimental points are: the peak of the rapid phasic response (●), the plateau (○) during a K depolarization, and the tonic level (□) during the Tris episode. Initial pH_i , 7.39; membrane voltage, -59 mV; diameter, 0.650 mm. 050683B.

low $[\text{H}]_i$, whereas the axon shows a Ca entry with Na-free conditions that is quite insensitive to changes in $[\text{H}]_i$. This finding of an identical sensitivity of both treatments to $[\text{Na}]_i$ and a differing sensitivity to $[\text{H}]_i$ suggests that H^+ may act only on the part of the Na/Ca exchange cycle that is controlled by membrane potential.

Finally, in contrast to the results of Baker and Honerjager (1978), but in agreement with other studies (Lea and Ashley, 1978; Mullins and Requena, 1979; Mullins et al., 1983), acidification of the intracellular media increases the level of ionized Ca; at physiological concentrations of Na_i . Measurements of resting glow for the axon of Fig. 5 (low Na_i) showed that the glow of aequorin at pH 6.90 was of the order of 1,200 photons/s, whereas at pH 7.05 it was 900

photons/s, and at pH 7.15 it was 700 photons/s. From pH 7.50 through pH 7.90, the luminescence was steady at 600 photons/s. Contrarily, at an elevated level of Na_i , a dependence of the resting of ionized Ca on acidification was not found: from pH 7.05 through pH 7.92, the Ca signal was steady at 1,700 photons/s.

Effect of CN on $[Ca]_i$

In view of the fact that the response of $[Ca]_i$ to a given value of Ca influx depends on how much of the Ca is buffered, it seemed important for us to make additional measurements of the effects of CN on the Na-free and high-K effects. It has been known since the original experiments of Brinley et al. (1978) that Ca uptake by mitochondria does not take place at physiological values of $[Ca]_i$, but that after a significant elevation of internal Ca, these organelles provide a powerful buffering system for Ca. An additional observation made by Mullins and Requena (1979) was that Ca entry into phenol red-injected axons with stimulation often was not visible except in the presence of CN. Taken together, these observations suggested that peripheral mitochondria might be functional even though they might not contribute much to the buffering of Ca entry in regions of the axoplasm where $[Ca]_i$ was low.

Fig. 6 shows an experiment in which an axon was depolarized with high-K solution and subsequently treated with Na-free solution. CN was then added and the conditions applied above were repeated. Finally, the CN was removed and the original responses to both depolarization and to Na-free solutions were obtained. It is clear from this experiment that CN has, at best, a modest effect on the response of the aequorin reaction to the experimental conditions imposed.

If one uses a phenol red-injected axon with a low $[Na]_i$, then one would expect there to be a decrease in light emission upon depolarization and, indeed, as Fig. 7 shows, this was found to be the case. Under these conditions, CN again had a very modest effect in modifying the response of the aequorin to a Ca concentration change.

Finally, it was thought useful to repeat the original experiments of Mullins and Requena (1981) but on phenol red-injected axons in the presence and absence of CN. These involved depolarizing a freshly isolated axon (with an Na electrode inside) and noting the response of the aequorin signal. After this, the axon was stimulated in Li seawater and Na_i was observed to decline. A second test depolarization was then made. The stimulation was repeated but in Na-containing seawater until $[Na]_i$ rose to high levels and a final test depolarization was again made. In Fig. 8, the first two panels correspond, in an axon injected with aequorin and phenol red, to the response to test K depolarizations in the absence and presence of CN. The signals are only slightly different. The axon was then stimulated in Li to reduce the level of Na_i and the light signal associated with a third K depolarization is shown to be very much smaller than the initial control one. A subsequent K depolarization test in the presence of CN resulted in an enhanced signal. The magnitude of this enhanced signal, however, was still smaller than that observed for any of the initial control depolarizations. After stimulation in Na, to raise Na_i , the record finishes with a K depolarization. This last Ca signal is comparable in magnitude to that observed at the onset of the

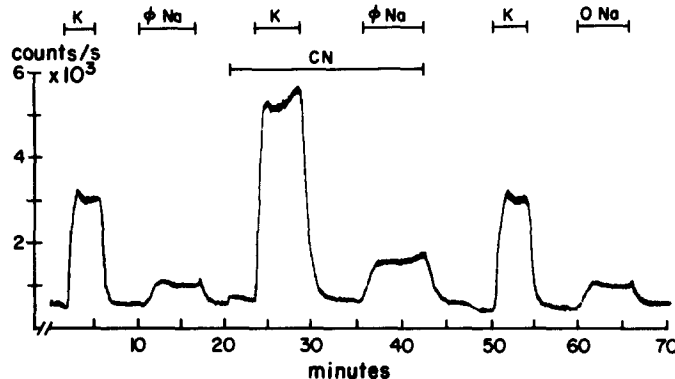


FIGURE 6. The effect of CN on the magnitude of the Ca aequorin signal induced by Na removal and K depolarization. The luminescence time course of an axon injected only with aequorin is shown when tested in the presence and absence of 2 mM CN for Na-free (Tris) and K seawater, both containing 10 mM Ca_o . Axon diameter, 0.600 mm. Membrane voltage, -55 mV. R051882C.

experiment. Once more, it has been shown that CN does not greatly affect the magnitude of the Ca aequorin signals.

Analytical Ca Studies

In order to follow up the experiments outlined above, the total Ca content of intact axons was determined after a period of exposure of 2 h to seawater

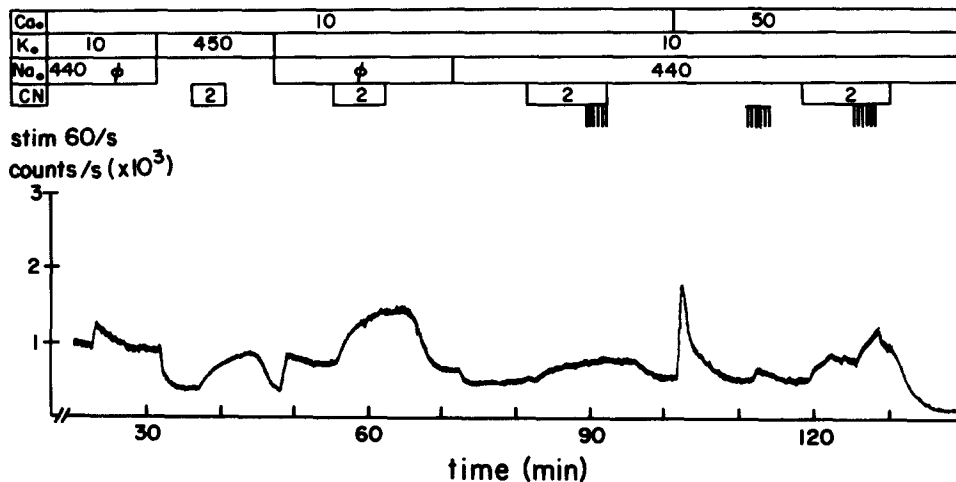


FIGURE 7. The effect of CN on the negative response in luminescence induced by a K depolarization. An axon injected with aequorin and phenol red (220 mM K-salt) gave a reduction in Ca signal when exposed to 450 mM K, even when treated with 2 mM CN. Subsequently, a CN pulse in the middle of the Na-free condition revealed the magnitude of the Ca buffering under metabolic control. Finally, a light signal caused by stimulation (period 60 impulses/s) is clearly seen when high Ca_o (50 mM) and CN were combined. Membrane voltage, -58 mV. Axon diameter, 0.600 mm. The initial Na_i level read by a microelectrode was 18 mM. R053082A.

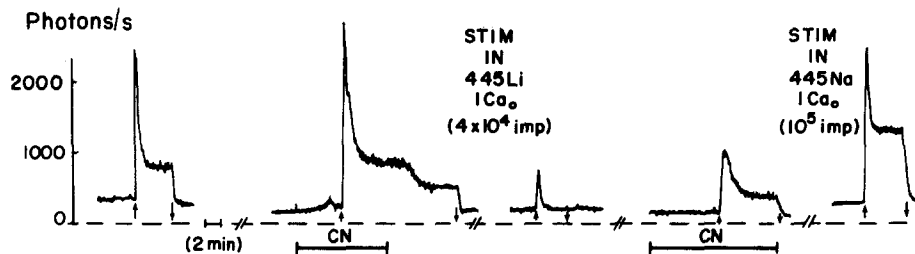


FIGURE 8. The effect of CN on the magnitude of the Ca aequorin signal produced by K depolarization under conditions of high and low Na_i. An axon with an Na-sensing electrode positioned inside, which read 19 mM Na, was injected with aequorin and phenol red (220 mM K-salt) and exposed to 450 mM K, 10 mM Ca seawater in the absence and presence of CN. Subsequently, it was stimulated in 450 mM Li (1 mM Ca) seawater for 40,000 impulses at 60 impulses/s and the Na_i level fell below 15 mM. K depolarization tests were then repeated. Finally, the axon was stimulated in Na (1 mM Ca) seawater for 100,000 impulses until the Na_i concentration read by the microelectrode was above 110 mM Na. Under these conditions, the axon was K depolarized again. Initial membrane voltage, -55 mV. Axon diameter, 0.600 mm. RAEQPR060182A.

containing either Na, Tris, or K, and for two initial conditions of internal Na, elevated or at normal physiological levels. Axons were quickly dissected from live animals at the seashore and lightly cleaned before being placed, at room temperature, in seawater either with stimulation at 60 impulses/s for a total of 50,000 impulses in 0-Ca, full-Na seawater or in the same solution without stimulation. These procedures were applied so that in one group of axons, the [Na]_i was increased by ~50 mM (Caldwell-Violich and Requena, 1979), whereas the other group maintained a physiological level of [Na]_i (a mean value of 23 mM). Next, axons were soaked for 2 h in 10 mM Ca seawater containing either 440 mM Na, 450 mM Tris (0 Na), or 450 mM K, all of them with 100 nM TTX and 10⁻⁴ M ouabain. The drugs were used to block, as much as possible, Na and Ca movement through mechanisms unrelated to Na/Ca exchange. The axons were then rinsed twice in isotonic sucrose with 10 mM EGTA and blotted and the axoplasm was extruded, weighed, and stored in plastic tubing for later atomic absorption analysis for Ca and flame analysis for Na. In Table I, the analytical

TABLE I
Axoplasmic Concentrations of Ca and Na After 2 h Exposure to Solutions
Containing 10 mM Ca, 50 mM Mg, and 450 mM of the Cation Indicated as a
Function of Two Initial Levels of Internal Na

Initial [Na] _i level	Ca (total)			Final [Na] _i		
	Na	Tris	K	Na	Tris	K
mM	μM	μM	μM	mM	mM	mM
Normal (20's)	149±67 n = 6	304±58 n = 9	336±56 n = 6	38±4	15±3	21±3
Elevated (70's)	153±61 n = 7	887±153 n = 8	1,172±151 n = 8	71±5	26±5	25±4

* Mean ± SEM (of n samples). n for Ca = n for Na.

results are summarized for the two intracellular cations of interest, Ca and Na. It can be seen that in the group with a physiological level of $[Na]_i$, exposure to either Tris or K only doubled the Ca content as compared with the level of Ca detected in the group soaked in Na seawater. The Na_i level was ~20–30 mM for all of these axons.

For axons with an initial high level of Na_i , which was of the order of 70 mM, the Ca content of the group stored in K-rich seawater was eightfold larger, whereas for those stored in Tris, the Ca content was sixfold greater than that of the control axons stored in Na seawater. It should be noted that at the end of the 2-h soak, the level of $[Na]_i$ in the Na-free treated axons was close to normal. Obviously, the load of internal Na slowly dissipated during the soak period through a possible Na gradient-dependent Mg transport. Contrarily, in the set of axons soaked in Na-containing seawater, the level of $[Na]_i$ at the end of the soak period was 71 mM.

These results leave no doubt that in axons with physiological levels of Na_i , there is a much diminished reverse Na/Ca exchange; this is significantly enhanced by raising $[Na]_i$, as originally noted by Baker et al. (1969).

DISCUSSION

Ionized [Ca]_i Visible in Phenol Red–Aequorin Axons

The value of ionized $[Ca]_i$ measured in a phenol red axon (75 nM) can be compared with that measured in the center of an axon with aequorin confined to a dialysis capillary that was 20 nM (DiPolo et al., 1976) and with a value of 50 nM as measured with arsenazo III, where the sampling was an average of $[Ca]_i$ everywhere in the axoplasm. These measurements are reasonably concordant and are understandable on the basis that $[Ca]_i$ would be highest in the axon periphery and lowest at the core and have some mean value as measured with arsenazo III. Note that a gradient in $[Ca]$ in the axoplasm would be expected if $[Ca]_o$ was 10 mM, as it was in this case.

A Decline in Aequorin Glow with Depolarization

In explaining the decline of aequorin glow with depolarization, as found with some axons in this study, note should be taken of the findings that depolarization activates Na/Ca exchange to move Ca inward and that channels of a variety of sorts are also opened. On the other hand, the electrical gradient driving Ca^{2+} inward is diminished by depolarization. If Na/Ca exchange and channel movements of Ca are negligible, then the expected result of depolarization is a decline in Ca entry (reflected in the aequorin measurements as a decline in aequorin glow). In every axon where there was a decline in aequorin glow with depolarization, this response could be reversed by the simple expedient of raising $[Na]_i$ so that an exchange of Ca_o for Na_i was activated. It might be thought that since E_{Ca} is of the order of +140 mV and the change in membrane potential with K depolarization is from -60 to +5 mV, the purely linear change in driving force on Ca^{2+} would be from 200 to 135 mV. However, it is quite unlikely that passive Ca currents are purely ohmic. All measurements suggest that a constant field equation is the more likely expression for electrical driving force. A solution of

this equation shows that in going from -60 to $+5$ mV, there is of the order of a 10-fold decrease in the electrical force driving Ca inward. There is a very much smaller change in driving force as one approaches the reversal potential for Ca^{2+} .

It is useful to list the mechanisms described in the literature that pass Ca^{2+} upon depolarization and then to draw conclusions about such mechanisms. There is a component of Ca entry via Na channels (Baker et al., 1971; DiPolo et al., 1982); this is readily blocked by TTX. A second mode of entry is via Na/Ca exchange; this is readily blocked by making $[\text{Na}]_i$ low (for a review, see Requena, 1983). A third sort of entry that needs to be considered is the possibility that Ca entry takes place via K channels, and a final route is via nonspecific leakage pathways. As far as K channels are concerned, these are known to be inactivated over the time scale we have under consideration. Our finding that TTX does not alter the decrease in aequorin glow with depolarization could again be because, although TTX cuts off Ca influx (DiPolo, 1979), it also raises membrane potential and this could produce an enhanced Ca efflux. Hence, there would be a decline in glow. Depolarization (of a steady sort, lasting several minutes) will act in complex ways on these various mechanisms: for Na channels, at a membrane potential of -60 mV, some of these are open in the steady state according to the usual Hodgkin-Huxley analysis. A very much smaller fraction of Na channels will be open in the steady state at a membrane potential of zero according to this same analysis. Hence, the expected effect of depolarization on Ca entry via Na channels is that entry will be reduced by depolarization. Upon removal of Na_o , Na/Ca exchange will only pass Ca inward if Na_i is above a level of ~ 15 mM, while, simultaneously, Ca efflux by Na/Ca exchange will be strongly inhibited. Since the aequorin light signal depends upon the differences between all the mechanisms bringing Ca into the fiber and those extruding it (as well as the interaction of the net Ca flux with intracellular buffers (Brinley et al., 1977)), it is not obvious what aequorin light emission is to be expected.

Our measurements do suggest that when the inward movement of Ca by Na/Ca exchange is blocked (low $[\text{Na}]_i$), any channel mechanism that might be activated by depolarization is quantitatively much smaller than the diffusive leak of Ca into the fiber, since aequorin light declines.

Comparing Na_o -free and High- K_o Conditions

Baker and DiPolo (1984) have suggested that the results of Mullins and Requena (1981), showing that Na_i changes could either enhance or abolish the response to depolarization, could be explained by assuming that changes in Na_i would change Ca buffering. There seems to be genuine agreement that Ca entry in response to Na-free conditions measures Na/Ca exchange, although it is argued that K depolarization measures an additional contribution to Ca entry from some channel-mediated process.

Our experiments show that both Na-free responses and K depolarizing responses, when plotted as a function of $[\text{Na}]_i$, fall on the same curve, which makes it difficult to assume that there are two different mechanisms contributing to the response. As to the possible effect of changes in $[\text{Na}]_i$ in changing Ca buffering, it is important to point out that there is no change in the Ca entry induced by

repetitive stimulation (a Ca entry mainly via Na channels) whether $[Na]_i$ is low or high (Mullins and Requena, 1981), whereas the response to steady depolarization is changed by at least 20-fold.

CN Effects

Experiments with CN were undertaken to examine the sensitivity of an axon to Ca entry with and without mitochondrial Ca buffering. Previous experience (Requena et al., 1977) had shown that, in fresh axons, mitochondria had little Ca to release when poisoned, which implies that they were not highly functional at levels of $[Ca]_i$ that occur in axoplasm. On the other hand, the experiments of Mullins and Requena (1979), which showed that in phenol red-injected axons, some entry of Ca was only visible on the record if CN was present, made it desirable for us to look further into this effect. Our results show that there is at best a three- to fourfold enhancement of the signal generated by membrane depolarization and, hence, that mitochondria are not major sources of Ca buffering. Recently (Requena et al., 1985), it has proven possible to inhibit nonmitochondrial buffering in squid axons and to produce, as a result of this inhibition, very large Ca signals, which again suggests that mitochondria are not the major buffer at physiological levels of $[Ca]_i$. Some caution should be exercised here because in axons isolated for long periods of time, the nonmitochondrial buffering appears to decline markedly (the "sensitive" axons of Baker et al., 1971). Under such conditions, mitochondria buffering could be very large.

$[H^+]_i$ Effects

A study of the effect of pH_i on Ca entry was undertaken to see whether there was a difference between the response to Ca entry via Na-free and K depolarizing conditions. The main finding was that with high $[Na]_i$, the response with depolarization is greatly enhanced by making the axoplasm alkaline, whereas the response to Na-free conditions was less dependent on pH_i . This may indicate that changes in $[H]_i$ affect a part of the Na/Ca exchange cycle that is potential dependent, but not just the total reverse cycle.

Analytical Ca

Analytical studies of total Ca content of axons avoid the problems of ionized vs. buffered Ca since both are measured. The results obtained persuasively indicate that if the initial $[Na]_i$ is <20 mM, there is little net Ca gain even in Na-free or high-K solutions. A modest Ca gain in 2 h was deliberately induced since seawater was 10 mM Ca rather than 3 mM, where Ca gains have been shown not to occur. By contrast, if one starts with $[Na]_i$ at ~ 70 mM, there is no difference in analytical Ca if seawater contains Na as compared with the case where $[Na]_i$ is low. This suggests that the Na electrochemical gradient is of a size adequate to maintain Ca_i at the same level as when Na_i is normal.

With a normal Na_i , treating an axon with Tris rather than Na seawater can be expected to reduce Ca efflux (Ca influx will be little affected because of the low Na_i). One also notes that the final values for $[Na]_i$ increase somewhat in Na seawater but remain at close to control values (20 mM) in Tris or K solutions.

This result is expected if the Na pump is inhibited by ouabain since losses of Na by diffusion cannot be expected to be >0.3 mmol/h, whereas Na influx is 100 times this value.

For an $[Na]_i$ of 70 mM, a Tris seawater leads to axons with substantial Ca gains both because Ca efflux is inhibited in such solutions and because Ca influx is greatly enhanced. A similar, larger Ca gain is obtained in elevated K solutions. There is a large (45 mM) decline in $[Na]_i$ in Na-free solutions. As noted above, this is much too large to be the result of passive diffusion and it suggests that the Na extrusion has been the result of an exchange of a cation other than Ca (possibly an Mg/Na exchange).

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