The "Late" Ca Channel in Squid Axons

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ABSTRACT Squid giant axons were injected with aequorin and then treated with seawater containing 50 mM Ca and 100-465 mM K⁺. Measurements of light production suggested a phasic entry of Ca as well as an enhanced steady-state aequorin glow. After a test K⁺ depolarization, the aequorin-injected axon was stimulated for 30 min in Li seawater that was Ca-free, a procedure known to reduce [Na]ᵢ to about one-half the normal concentration. Reapplication of the elevated K⁺ test solution now showed that the Ca entry was virtually abolished by this stimulation in Li. A subsequent stimulation of the axon in Na seawater for 30 min resulted in recovery of the response to depolarization by high K⁺ noted in a normal fresh axon. In axons first tested for a high K⁺ response and then stimulated in Na seawater for 30 min (where [Na]ᵢ increases ~30%), there was approximately eightfold enhancement in this response to a test depolarization. Axons depolarized with 465 mM K seawater in the absence of external Ca for several minutes were still capable of producing a large phasic entry of Ca when [Ca]₀ was made 50 mM, which suggests that it is Ca entry itself rather than membrane depolarization that produces inactivation. Responses to stimulation at 60 pulses/s in Na seawater containing 50 mM Ca are at best only 5% of those measured with high K solutions. The response to repetitive stimulation is not measurable if [Ca]₀ is made 1 mM, whereas the response to steady depolarization is scarcely affected.

INTRODUCTION

It has been known since the pioneering study of Hodgkin and Keynes (1957) that squid giant axons incur an increased influx of Ca either when they are repetitively stimulated or when they are subjected to a steady depolarization by increasing the K concentration in seawater. A further study of this Ca entry using aequorin rather than ⁴⁶Ca was made by Baker et al. (1971), who showed that Ca entry could be divided into a tetrodotoxin (TTX)-sensitive fraction and a TTX-independent fraction. A subsequent examination of Ca entry occasioned by depolarization was made by Baker et al. (1973a and b), who concluded that the TTX-insensitive Ca entry with depolarization was effected via a "late" Ca channel that opened with depolarization and subsequently inactivated.

A study using aequorin confined to a dialysis capillary in squid axons
(Requena et al., 1977) showed that steady depolarization with 100 mM K seawater led to a continuous Ca entry and the [Ca]i reached a maximum after ~1 h.

More recently, Keynes et al. (1979) have shown that in voltage-clamped squid axons internally dialyzed with CsF and bathed in Tris Cl (with TTX) or in this medium plus 66 mM Ca, there is an inward current during repolarization that must represent Ca moving through K channels. Ca movement through K channels was further confirmed by the demonstration by Inoue (1980) that an action potential can be produced in TTX-poisoned squid axons containing 30 mM NaF inside and 100 mM Ca plus TTX outside.

It has also been shown that Ca efflux that is dependent on Na0 is sensitive to depolarization and that this movement is inhibited by a reduction in membrane potential (Mullins and Brinley, 1975), whereas Baker and McNaughton (1976) show that Na efflux coupled to Ca entry is enhanced by depolarization. Thus, one concludes that depolarization is likely to enhance Ca entry via the Na/Ca exchange mechanism running backward.

A summary of the observed effects of depolarization on Ca entry would therefore be that Ca entry can be expected to be enhanced by its moving through (a) Na channels, (b) K channels, (c) "late" Ca channels, and (d) Na/Ca exchange. The purpose of the experiments reported here is to measure the Ca entry occurring via the various pathways that have been proposed. The results show that for steady depolarizations, Ca entry via Na/Ca exchange is by far the most important mechanism. For action potentials at a frequency of 50–60 pulses/s, it is likely that Ca entry via Na and K channels is more important than entry via Na/Ca exchange unless [Na]i is made artificially high.

**METHODS**

**Experimental Animals**

The squid used were collected and studied at the Marine Biological Laboratory in Woods Hole, Mass. from April to June, 1980.

**Aequorin**

This material was a gift from Dr. O. Shimomura and was prepared as previously described (DiPolo et al., 1976). The solution used was substantially more dilute than that used previously; this dilution was deliberate because with photon counting there was ample sensitivity. For the experiments described here, we estimate that the resting glow actually measured was 1% of that previously measured with a concentrated aequorin solution. This value was selected to give a reasonable photon count rate as described below. The handling of aequorin and its microinjection were as described in Mullins and Requena (1979).

**Microinjection and Experimental Chamber**

A microinjector described previously was modified so that it operated horizontally rather than vertically. Microinjection could thus take place in a dialysis-type chamber with a light pipe array as previously described by Requena et al. (1977). Glass capillaries used for the microinjection of aequorin were stored in 10 mM K2EGTA,
pH 7, and were extensively rinsed just before use with 1 μM K2EGTA. The usual length of injection was 15 mm and a 1-μl Hamilton syringe (Hamilton Co., Reno, Nev.) was normally used.

**Light Measurement**

The chamber used in these aequorin light measurements was the same as that used in Mullins and Requena (1979). The outputs from the two optical light guides were fed into a housing (model 3262/F-AD4; Pacific Photometric Instruments, AD4, Emeryville, Calif.) that contained a specially selected photomultiplier tube for photon counting (model 9542A; EMI Gencom Inc., Plainview, N. Y.). Photon counting was carried out on a Spex DPC2 Digital Photometer (Spex Industries, Inc., Metuchen, N. J.), which provided a high voltage supply (-900 V), a discriminator network for pulse height selection, a counting and integrating network with digital display of the count, and a variety of subtraction networks that allowed one to subtract one full scale on the analog output, a feature called “wrap-around” on the instrument. This was convenient for large signals and the instrument also had the capability of integrating and counting over a wide variety of preset times. The usual integration time was 10 s. The counts at the resting glow level were ~300 counts/s. The response of the system was limited with respect to time by the fact that the chart recorder used to follow the rise in aequorin glow had a response of 0.5 s for a full scale, and in any event, responses were probably more limited by the rate at which solutions could be changed in the bath than by any other parameter.

**External Solutions**

Seawater was driven through the slot of the experimental chamber at a flow rate of 4 ml/min, and because the volume of the slot was ~0.1 ml, this gave a change of solution of ~0.6 of the slot volume per second. Effective changes in the solution as judged by loss of excitability in Na-free solutions was ~5–10 s and there is every reason to believe that high potassium solutions used in these studies were also effective in that period of time. The basic seawater used had the following composition: Na 465 mM, Ca 50 mM, TES 10 mM (pH 7.8), and EGTA 0.1 mM. Solutions with 100, 200, or 465 mM K were obtained by replacing Na with an equivalent amount of K. Solutions that were Ca-free had 50 mM Mg as a replacement for this ion, as did solutions with 1 mM Ca. Choline or Li seawater was made by replacing all Na by choline or Li. All solutions were adjusted to 1,000 ± 10 mosmol using a Wescor Dewpoint osmometer (Wescor Inc., Logan, Utah) and to pH 7.8 ± 0.05 with a pH meter. Temperature was maintained at 15°C.

**Changing [Na]i**

An important requirement for the experiment was to be able either to increase or decrease [Na]i. Sophisticated techniques of internal perfusion (Baker et al., 1962) or of internal dialysis (Brinley and Mullins, 1967) are available but have the disadvantage of having to control [Ca]i with EGTA buffers, and these have been shown (Baker and McNaughton, 1976) to affect the coupling of Ca entry to Na exit via Na/Ca exchange. Because it is this process that we wished to study, simpler techniques for Na change were used. The first (Frumento and Mullins, 1964) involves the stimulation of squid axons in Li seawater. The idea is that Li enters during the depolarizing phase of the action potential as Na emerges. The study cited above gave analytical values for [Na]i, [K]i, and [Li]i in axoplasm as shown in Table I, line 1. These measurements can be compared with those of Baker et al. (1969), who stimulated *Loligo* axons both in Na-containing and Li seawater with the results as shown in Table I, lines 4 and 5.
Another method used to reduce $\text{Na}_i$ (Frumento and Mullins, 1964) was to soak axons in Na-free seawater for 6 h; analysis then showed a $\text{Na}_i$ of 35 mM as shown in line 2.

Measurements similar to those detailed above also have been made by Caldwell de Violich and Requena (1977) on the tropical squid $\text{Doryteuthis}$ when subjected to stimulation in Li or Na seawater.

Another method of getting at changes in $\text{Na}_i$ produced by stimulation (Keynes, 1951 and personal communication) is by measurement of Na influx and efflux; a substantial amount of such data leads to the conclusion that at $20^\circ\text{C}$ Na influx is 10.3 pmol/cm$^2$-impulse, whereas efflux is 6.6 pmol/cm$^2$-impulse, for a net flux of 3.7 pmol/cm$^2$-impulse. For 30 min of stimulation at 60 pulse/s, this is a net Na gain of 32 mM (Keynes, 1951). The problem of a value for $\text{Na}_i$ in axons is considered by Mullins (1979) with a best estimate of 80 mM for an axon that has been cleaned, mounted in an injection chamber and injected with some substance, and analyzed at 3,600 s (Table I, line 3).

A summary of the $\text{Na}_i$ to be expected under the various experimental conditions used is that fresh axons ($\text{Na}_i = 80$ mM) undergo a halving, roughly, of this value of $\text{Na}_i$ (to 36 mM) during Li stimulation; upon restimulation in Na seawater they recover $\text{Na}_i$ at a rate of 3.7 pmol/cm$^2$-impulse or a gain of 32 mM to a value of 68 mM. Axons that were stimulated in Na seawater without a previous stimulation in Li seawater might be expected to gain 50 mM Na if the frequency were 100 pulse/s for 1 h (line 5), but we used 60 pulse/s for 1/2 h so that the expected gain is $(50 + 2) \times 0.6$ or 15 mmol/kg Na gain or a value of 95 mM Na, whereas on the basis of a net gain of Na (3.7 pmol/cm$^2$-impulse), the final $\text{Na}_i$ would be $(80 + 32) = 112$ mM. We have taken an average value of 104 mM for the likely increase in $\text{Na}_i$.

Another detailed study of $\text{Na}_i$ and $\text{K}_i$ in squid axons is that of Hinke (1961). He found an analytical $\text{Na}_i$ of 91.5 mM for extruded axoplasm and an activity of Na of 41.8 mM, which suggests that part of the $\text{Na}_i$ is bound. It should be noted that the values in Table I are all analytical and may need to be divided by 2 to yield Na$^+$ activities. The net gain of Na with stimulation as measured by Na-sensitive electrodes was 3.8 pmol/cm$^2$-impulse, which is in close agreement with the value cited above as measured with isotopes.

### Table I

| Line | Treatment | Seawater | Frequency | Time | $\text{Na}_i$ | $\text{K}_i$ | $\text{Li}_i$ | Reference |
|------|-----------|----------|-----------|------|-------------|-------------|-------------|-----------|
| 1.   | Stimulation | Li       | 60        | 1,800 | 36          | 158         | 117         | Frumento and Mullins (1964) |
| 2.   | Unstimulated | Na-free  | 0         | 21,600 | 35          | --          | --          | Frumento and Mullins (1964) |
| 3.   | Unstimulated | Na       | 0         | 3,600  | 80          | --          | --          | Mullins (1979) |
| 4.   | Stimulation | Li       | 100       | 3,600  | 30          | --          | --          | Baker et al., Table 10 (1969) |
| 5.   | Stimulation | Na       | 100       | 3,600  | 130         | --          | --          | Baker et al., Table 10 (1969) |
Membrane Potential vs. [K]$_o$

Because the measurements made required long periods of repetitive stimulation, the additional complication of making membrane potential measurements on the same axon was not attempted. Control measurements on other axons for [K]$_o$ were 100 mM ($-20$ mV), 200 ($-11$ mV), and 465 (+5 mV).

RESULTS

Ca Entry upon Depolarization

When a squid giant axon kept in 50 mM Ca, 465 mM Na seawater is exposed to 50 mM Ca, 465 mM K seawater, the results, as described by Baker et al. (1973 b), is a phasic change in aequorin light emission, as shown in the left-hand trace of Fig. 1. The restoration of the membrane potential by making the [K]$_0$ then leads to a reduction of light emission. This sort of record is the basis for a claim that there is a "late" Ca channel that opens with depolarization and then inactivates as depolarization is maintained.

If, however, the axon shown in Fig. 1 is placed in Li seawater containing 50 mM Mg so that Ca loading is not brought about, and the axon is stimulated at 50 pulses/s for 2,700 s, a treatment that has been shown to reduce [Na]$_i$ to one-half of its initial value (see Methods), then a test with 465 mM K, 50 mM Ca (choline) seawater yields the aequorin record shown in the middle trace of Fig. 1. Clearly the response has been reduced to $\sim1/15$ of its control value as a result of stimulation, which, in addition to reducing [Na]$_i$, also increases [Li]$_i$ and decreases [K]. The effect on [Ca]$_i$ appears to be minimal because throughout the stimulation, the aequorin glow was less than three times its initial value in Na seawater. A final treatment of this axon was to repeat the stimulation episode in Na seawater instead of Li seawater. Measurement of Na fluxes with isotopes suggest that this treatment increases [Na]$_i$ by $\sim30$ mM. Thus, the axon now has a value of [Na]$_i$ roughly equivalent to that at the start of the experiment. A 465-mM K test treatment now yields a response that is at least qualitatively similar to that observed initially.

These results can be summarized by saying that the increase in Ca$_i$ of a squid giant axon in response to steady depolarization brought about by high [K]$_o$ appears to depend critically on the internal Na concentration because a halving of this [Na]$_i$ reduces the aequorin light from Ca entry about 15-fold.

At the same time, one can see from Fig. 1 that the response of the axon to a test stimulation (60 impulses/s for 30 s) is (a) small compared with steady depolarization and (b) unaffected by a reduction in [Na]$_i$ (compare the left-hand and center responses).

Another test was to keep axons in one case for 4 h and in another for 6 h in Na-free, Ca-free (50 mM Mg) choline seawater after they had been tested for a response to 465 K, 50 Ca seawater. Because the time constant by which [Na]$_i$ changes is $\sim300$ min,$^1$ this treatment should have reduced [Na]$_i$ substantially (Table I, line 2), even though no stimulation was applied. Test responses

$^1$ Because Na efflux into choline seawater at 15°C is $\sim50 \times 10^{-12}$ mol/cm$^2$·s, S/V for a 500-$\mu$m axon is 80 cm$^{-1}$ and [Na]$_i$ is 80 nM, we have (80 $\mu$mol Na/cm$^3$) = $(50 \times 10^{-12}$ mol/cm$^2$ × 80 cm$^{-1}$) or a time constant of 333 min. The rate constant for Na efflux is concentration independent (Brinley and Mullins, 1968).
FIGURE 1. This record of light emission vs. time compares the response of an axon stimulated at 60 pulses/s for 30 s in 50 mM Ca (Na) seawater with the aequorin response to a steady depolarization from 465 mM K, 50 mM Ca seawater. The left trace is for a normal, freshly injected axon, whereas the center trace is the response after the axon has had [Na], lowered by stimulation in Li seawater. The responses to 60 pulses/s stimulation vs. steady depolarization differ by 17-fold in an axon with a normal [Na], whereas the responses to 60 pulses/s and to steady depolarization are comparable after the decrease in [Na]. Time on the abscissa is measured from the time of aequorin injection.

of such soaked axons showed responses very similar to but smaller than the response after the Li+ stimulation shown in Fig. 1.

Response of Axons with High [Na]:
To judge the physiological importance of [Na], in regulating Ca entry after depolarization, a different sort of experiment was carried out, as shown in Fig.
2. Here an axon was maintained in 465 mM Na, 50 mM Ca seawater and tested for a response to 200 mM K (Na) seawater. The Na in seawater was retained so that foreign cations such as choline were not introduced, and the response observed in the left-hand trace of Fig. 2 shows that the usual sort of light response was obtained. The response is much smaller than in Fig. 1, which agrees with our finding that the response to depolarization is always

![Figure 2](image-url)

**Figure 2.** An axon in 465 Na, 50 Ca seawater was transferred to 200 K, 265 Na, 50 Ca, as indicated on the left-hand trace. The ordinate is aequorin light and the abscissa is time. After the test depolarization, the axon was stimulated in Ca-free (Mg) seawater for 1,750 s and the depolarizing test was reapplied with the result shown on the right.
larger in 465 mM K seawater than in 200 mM K (Na) seawater. This axon was now stimulated in Ca-free, Na seawater for a shorter time than the axon in Fig. 1. The seawater was Ca-free so that the stimulation would result in no Ca loading of the fiber, and it contained Na so that the expected result of the stimulation was a roughly 1.3-fold increase of [Na]i (Table I). A subsequent test of the axon with 200 mM K (Na), 50 Ca seawater is shown in the right-hand trace of Fig. 2 as an eightfold increase in the light emission of the fiber in response to an identical test pulse of a 200-mM K solution. Two conclusions from this sort of experiment are: (a) that an increase in [Na]i brought about by a stimulation in Na seawater is capable of increasing the response of the axon to depolarization, and (b) that since Requena et al. (1977) have shown that [Na]o = 180 mM is capable of maintaining a normal [Ca]i, the change in E m alone produced by 200 mM K (265 mM Na) is capable of reversing the Na/Ca exchange. Taken together, Figs. 1 and 2 suggest that Ca entry induced by K depolarization is a steep function of [Na]i. Note that in Fig. 2 both the peak phasic Ca entry and the level of the plateau were enhanced by an increase in [Na]i.

The main purpose of this investigation was to see whether the depolarization response could be reduced by reducing Na. Because, as noted earlier, there might be some reservation about the use of Li for this purpose, two control experiments were done using only (a) a test response in 465 mM K and in 200 mM K seawater, followed by (b) a 30-min stimulation episode in Na seawater (a treatment that increases Na). Table II shows the way the axons responded first to control depolarizations and then after stimulation for 30 min in 1 Ca (50 Mg) Na seawater to test depolarizations where [K]o = 200-465 mM.

In both axons, there was an approximately 4.5-fold increase in the response to a test 200-mM K pulse after stimulation in Na seawater, whereas one axon (270580) gave a large response to 465 mM K before Na loading and this response did not increase much after stimulation, which suggests that perhaps the processes governing Na/Ca exchange had become saturated.

Ca Entry vs. [K]o

The results of a series of test depolarizations brought about by increasing [K]o from 10 to 465 mM are shown in Fig. 3 A for a single axon. Depolarization appears to result in a peak increase in light emission of about fivefold for this

| Axon       | Freshly injected with aequorin | After stimulation in Na seawater: 60 pulses/s for 30 m |
|------------|-------------------------------|----------------------------------------------------------|
|            | [K]o = 465                   | [K]o = 200                                                |
| 270580     | 18.0                         | 22.0                                                     |
| 230580B    | 1.9                          | 13.7                                                     |
| 230580     | 1.0                          | 7.7                                                      |
axon (i.e., one freshly injected with aequorin and labeled "normal"). The subsequent stimulation of such an axon in Li seawater at 60 pulses/s for 30 min leads to a substantially decreased sensitivity of the aequorin response of the axon to depolarization (curve labeled "after Li stimulation"), whereas a second stimulation of the axon in Na seawater leads to a curve that is similar to the normal curve (labeled "after Na stimulation"). These responses suggest that the peak Ca entry is a function of [Na] because a decrease of this ion in axoplasm is the principal effect of stimulation in Li seawater. Similarly, a recovery of the "normal" response to K depolarization after subsequent

**Figure 3.** (A) This is a plot of light emission beyond the steady resting glow measured at \([K]_o = 10 \text{ mM}\) when the \([K]_o\) indicated is applied to the axon. Values measured in a fresh axon 10–20 min after aequorin injection are shown as solid circles and labeled "normal." The axon was then stimulated in Li for 30 min at 60 pulses/s and tested again for response to increments in \([K]_o\) (open circles). Finally, the axon was stimulated in Na for 30 min at 60 pulses/s and tested once more for response (open triangles). (B) Responses to \([K]_o\) change for all axons studied are shown as increase in photon count over baseline (oblique lines) divided by increase in light for stimulation at 60 pulses/s for 60 s. The response is thus compared with a known Ca entry that has been isotopically measured. Solid points are freshly aequorin-injected axons; open points represent the response after stimulation in Li seawater. Points of the same shape represent a particular axon. (C) Response as in A for axons studied when treated with various \([K]_o\) for fresh, Li-stimulated, and Li-stimulated followed by Na-stimulated axons.

stimulation in Na seawater suggests that [Na] is an important parameter in fixing the peak Ca entry with depolarization.

The responses to depolarization as a function of \([K]_o\) in seawater for all axons tested are shown in Fig. 3 B. We have taken the increment in photon count from the resting level to the peak of the \([K]_o\) transient and divided this by the increment in photon count when the axon was stimulated at 60 pulses/s for 60 s, because during steady depolarization, the reduction in photon count with a reduction in [Na] is substantial but this procedure scarcely affects the
response to repetitive stimulation. This is a method of standardizing responses to a known value for the Ca influx. Isotope measurements have shown that Ca influx is 1 fmol/cm²·impulse mM [Ca]o; thus, for our test stimulations in 50 mM Cao at a frequency of 60 pulses/s, the influx is 3 pmol/cm²·s. A test Ko depolarization that is 10 times the response to stimulation would therefore indicate an influx of 30 pmol/cm²·s if both responses took place over the same time scale and if the aequorin response were linear with [Ca]. Finally, Fig. 3C shows the responses of all axons tested to both a lowering of Na by stimulation in Li seawater, followed by a return of Na by stimulation in Na seawater. Here the mean peak response of a fresh axon was 6 × 10³ photons/s to 465 mM [K]o and the mean background from which these measurements was made equaled 0.3 × 10³ photons/s, so that light emission in a fresh axon was 6 + 0.3 or 18 times background. Upon Li stimulation this response was reduced to 2 times background. In every axon examined, a reduction in [Na] produced a substantial (greater than fivefold) reduction in light emission upon depolarization. Further stimulation of the axons in Na seawater to increase [Na] toward initial values leads to a recovery of 80 ± 14% of the initial response to stimulation.

Inactivation of Ca Entry

Baker et al. (1973b) observed that if a depolarization brought about either by KCl or voltage clamp were closely followed by a second depolarization, then the second gave a much smaller Ca entry as judged by aequorin light emission. This finding was assumed to indicate an inactivation of the "late" Ca channel as a result of depolarization. Reasons have been given above for supposing that the "late" Ca channel is in fact an aspect of Na/Ca exchange rather than a separate channel, so that "inactivation" in the sense used for the h parameter in the Hodgkin-Huxley analysis may need an alternative explanation. We have not examined in any systematic way the time dependence of recovery of the response to steady depolarization. We have, however, not imposed such depolarizations closer in time than 10 min.

Recently, it has been clearly shown (Tilston, 1979) that it is Ca entry itself that produces inactivation of Ca currents in molluscan neurons; one might therefore expect that Ca entry in squid axons might also be sensitive to the quantity of this ion upon entry rather than to the change in membrane potential. Indeed, one can expect that Ca entry via Na/Ca exchange will be more sensitive to changes in [Ca] than Ca entry via channels because for a given change in [Ca] (and hence of $E_{\text{Ca}}$), the change in driving force on Ca is greater for Na/Ca exchange. For a membrane potential of 0, $E_{\text{Ca}} = 145$ mV, $E_{\text{Na}} = 60$ mV, and a change of $E_{\text{Ca}}$ of 25 mV as a result of Ca entry, we have for channels a driving force ($E - E_{\text{Ca}}$) of 145 mV, and its decrease to 120 after Ca entry. For Na/Ca exchange, with a 4 Na/Ca coupling, the driving force is $(2E_{\text{Na}} - E_{\text{Ca}})$ (Mullins, 1976) and this changes from −25 to 0 as a result of the same Ca entry.

Another feature of the aequorin response is its nonlinearity of light output with [Ca] when this is in the range of $10^{-6}$–$10^{-5}$ M. There are good reasons for supposing that the aequorin signal upon depolarization may result from
Ca entry into the most peripheral 1% of the axoplasm. Because the resting glow from this element of volume is negligible compared with the total resting glow, there could be a large increase in $[\text{Ca}]_i$ in this region with only a negligible increase in total resting glow. Additionally, in some unpublished measurements (Tiffert, Vassort, Whittembury, Brinley, and Mullins, unpublished observations) using arsenazo III as a Ca indicator, it is clear that the response to depolarization is a sustained Ca entry rather than a phasic response.

Because Na/Ca exchange is implicated in Ca entry, it is necessary to consider whether, during a depolarization, Ca entry involves an increase in $[\text{Ca}]_i$ or a decrease in $[\text{Na}]_i$. In view of the fact that $[\text{Na}]_i$ is tens of millimolar, a change in this variable seems unlikely. The experiments so far reported support the notion that upon depolarization of the membrane, Ca entry is controlled by some power function of $[\text{Na}]_i$. If $[\text{Na}]_i$ could be shown to change sensibly with depolarization, it might be possible to explain inactivation of Ca entry in this way. Repetitive stimulation of a squid axon at 100 pulse/s leads to a net entry of Na of 300 pmol/cm$^2$·s. If such an entry changed $[\text{Na}]_i$ just inside the membrane, one might expect to find differences in action potentials recorded at 1 pulse/s and 10 pulses/s because $E_{\text{Na}}$ would be expected to be different in the two cases. Common experience shows, however, that there is no difference in action potential shapes. Similarly, if an axon is depolarized with high $[\text{K}]_o$, the Na pump is stimulated by the chemical action of K on the Na/K transport, but the efflux of Na is constant over time of the order of minutes, which suggests that $[\text{Na}]_i$ is constant. It is also possible to observe inactivation of Ca entry with depolarization whether this is produced by elevated $[\text{K}]_o$ or by electrical depolarization (Baker et al., 1973 b), with the former procedure expected to decrease and the latter expected to increase $[\text{Na}]_i$. An additional argument against the existence of a compartment for $[\text{Na}]_i$ that might be affected by depolarization is that the response to depolarization is always larger if the axon is kept in choline seawater before depolarization rather than in Na seawater, and one would think that choline seawater would deplete any such internal compartment of Na.

Finally, one might consider what the expected changes might be if there were Ca channels and changes in $[\text{Na}]_i$ had some direct effect on them. It has been shown (Reuter, 1973) that Na moves through the Ca channels of cardiac cells and it is argued that the reversal potential of $I_{\text{Ca}}$ is in fact the potential where inward current carried by Ca and Na is balanced by an outward current composed mainly of Na. Thus, a decrease in $[\text{Na}]_i$ to low values ought to promote Ca entry because this change would favor a larger inward current at membrane potentials of $\approx 0$. This expectation is exactly the opposite of experimental findings and, hence, leads to the conclusion that a coupled Na/Ca mechanism is the most likely explanation of our results.

Our experimental approach to the study of inactivation has been first to produce a very small Ca entry by using 100 mM K for depolarization and reducing the usual 50 mM Ca in our seawater to 1 mM. The expectation was that this small Ca entry would not inactivate during the depolarization pulse since it corresponded to the expected level of aequorin glow during the
plateau. It can be seen from the experiment shown in Fig. 4 A that the glow rose smoothly to the plateau and was at the same level some 30 min later. The application of 50 mM Ca (while continuing the depolarization produced by 100 mM K) gave the usual phasic response even though there was no change in membrane potential; hence, the decline in light level must be due to factors other than a change in membrane potential. Note that it is possible to recover

![Graph showing the effects of calcium and potassium on glow levels.](Image of graph)

**Figure 4.** (A) An axon in 1 mM Ca (choline) seawater was treated with 100 mM K, as shown by the arrow. It was subsequently changed to 50 mM Ca, then back to 1 mM Ca, and then to 365 Na, 1 Ca seawater. (B) An axon in 465 choline, 50 mM Mg, Ca-free seawater (50 mM Mg) was transferred to 465 mM K (50 Mg) Ca-free, and 3.5 min later the solution was changed to 465 mM K, 50 mM Ca. This led to a very large Ca entry and a spontaneous return of light to base-line values. The transient at 10-12 × 10^3 on the ordinate is an artifact of the automatic range-changing system.

the initial level of resting glow not by repolarizing, but by replacing [choline]o by [Na]o. This record was made with the axon in Na-free solutions for 30-min, so only a trivial decrease in [Na] and in sensitivity to depolarization is to be expected.

A second sort of experiment is shown in Fig. 4 B. Here an axon in choline, Ca-free seawater (50 mM Mg) was changed to 465 mM K, Ca-free (50 mM Mg)
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Mg) seawater for 3.5 min. If membrane potential controlled inactivation, this time was long enough for the abolition of Ca entry. A change to 50 mM Ca, 465 mM K seawater produced a much larger than normal Ca entry rather than a lesser entry, which suggests that inactivation is not membrane potential dependent. Some small change in the membrane field may have occurred because we changed from 50 mM Mg, 465 K solution to 50 mM Ca, 465 K, and Ca is known to adsorb to the membrane and increase membrane field. This change must be expected to be small and moreover would be in the direction of increasing membrane potential, thus reducing Ca entry. There is also the fact that Mg is an inhibitor of Ca entry (Baker et al., 1973a and b); again an effect that would make Ca entry less than expected.

One concludes, therefore, that Ca entry itself is responsible for the inactivation that is observed both during a depolarizing pulse and after repolarization. The long recovery time (minutes) is compatible with time necessary to pump Ca from the fiber.

Effects of Stimulation

An important observation (Hodgkin and Keynes, 1957) was that repetitive stimulation of squid axons led to an increased Ca entry as judged by ⁴⁰Ca entry. Subsequent measurements in aequorin-injected axons (Baker et al., 1971) showed that this Ca entry could be divided into two parts: a TTX-sensitive entry and one that was insensitive to this drug. Such findings led to the idea that there was a Ca entry that took place through Na channels and another part of Ca entry that was via a “late” Ca channel. Such a conclusion was essentially confirmed by measurements on perfused squid axons by Rojas and Taylor (1975) and by Meves and Vogel (1973), who were able to measure a TTX-sensitive current of Ca that passed through the Na channels. It is important to emphasize that although inward currents carried by Ca have been measured in molluscan neurons, barnacle muscle fibers, and cardiac cells (Geduldig and Junge, 1968; Hagiwara and Naka, 1964; Reuter, 1973), it has proved impossible to measure a TTX-insensitive inward current in squid axons that could be ascribed to Ca. In fact, Rojas and Taylor (1973), Meves and Vogel (1973), Meves (1975), and Baker and Glitsch (1975) all comment that such an inward current for both Mg and/or Ca ought to have been measurable, and their failure to find it suggested that the divalent cation entry might be brought about by some electroneutral ion exchange. An alternative explanation is that the outward current expected from Na/Ca exchange escaped notice. Thus, the “late” Ca channel must be expected to be an entity quite different from the Ca channels that have been studied in the preparations cited above.

Movements of Ca via Na channels are difficult to compare quantitatively with the Ca entry that results from a steady depolarization. Therefore, in each measurement of the Ca entry in response to depolarization from elevated [K]o, we have compared this with a measurement of the response to stimulation at 60 pulses/s for 30 s as a calibration of Ca entry via transient vs. steady depolarization.
A typical record is shown in Fig. 1 where a fresh axon (with [Na]$_i$ presumably equal to ~80 mM) was subjected to stimulation in Li seawater (with a reduction in [Na]$_i$ to about one-half). In each case, however, a response of the axon to a stimulation in Na seawater (in a normal axon in 465 Na; in the Li axon to a 265 Na seawater) is compared and the conclusion is clear that although the response to steady depolarization is reduced about 15-fold, the response to stimulation at 60 pulses/s for 30 s is virtually unaffected. An important conclusion from this finding is that a reduction in [Na]$_i$ cannot have changed the buffering of Ca in axoplasm because if this were so, then Ca entry via Na channels and via Na/Ca exchange would appear to have been equally affected.

Baker et al. (1971) noted that the usual aequorin response to stimulation was an increase in light emission that rose to a steady level with a time constant of 10–15 s and that the amplitude of such a light level was proportional to the frequency of stimulation. Exceptionally, there were axons that not only produced much larger responses to stimulation but the response was now proportional not to the frequency of stimulation but to a power function of frequency that could be as much as 2.4. Our conclusion is that such a finding is difficult to explain on a Ca channel basis but is understandable if Ca entry by Na/Ca exchange depends in a complex way on the time integral of the membrane potential beyond the carrier reversal potential.

Our original plan was to use the $^{45}$Ca-measured Ca influx per impulse as compared with the aequorin-measured increment in light per impulse to calibrate the aequorin responses to steady depolarization. This procedure appears, in fact, to be a valid one if [Na]$_i$ is kept normal or less than normal, as the results in Fig. 5 (filled symbols) show. Here we compare the response of normal freshly injected axons to a 60-pulse/s test stimulation in 50 Ca (Na) seawater (as increase in peak light output) with the same axon after a 30-min stimulation in Li seawater, followed by a second test stimulation in 50 Ca (Na) seawater. The responses to stimulation are similar within the inevitable scatter in the data. Subsequent stimulation of these axons in Na seawater followed by a third test stimulation again produced little change in the response.

If, however, an axon is given a test stimulation and then stimulated in Na seawater for 30 min, one obtains results for two axons that are shown in Fig. 5 (open symbols). Here there is an approximately threefold increase in the response to the test stimulation (there is a 4.5-fold increase in the response to steady depolarization) (Table II), which suggests that as [Na]$_i$ is raised beyond normal levels, a greater fraction of the response to tetanic stimulation becomes dependent on Na/Ca exchange. It is useful to recall that although the depolarization occurring during an action potential is larger than that produced by 465 mM K$_o$, the potential change during an action potential at 60 pulses/s lasts only 5% of the time. If the quantity of Ca contributed by excitable channels and that contributed by Na/Ca exchange during steady depolarization were equal, then stimulation at the rate used would add only 5% to the response (i.e., it would not be measurable). A doubling of Na$_i$ and
the assumption that Ca entry is proportional to \(([Na]^i)^4\) would mean a 16-fold increase in Ca supplied by Na/Ca exchange so that the response would now be 1.9 times the control.

To further document the difference in the behavior of Ca entry with repetitive stimulation vs. steady depolarization when \([Na]_i\) is normal, the experiment shown in Fig. 6 was carried out. Here stimulation at 60 pulses/s for 60 s is compared in 50 mM Ca (Na) seawater with the aequorin response to steady depolarization produced by 100 mM K. A change to 1 mM Ca, 50 mM Mg led to the disappearance of a response to stimulation (Ca entry would be expected to be 1/50 of that at 50 mM) but to little effect on the response.
to steady depolarization. The rise time of the light emission is clearly slower but the amplitude is roughly the same, which suggests that Na/Ca exchange is different from a channel-mediated process in that it may be close to saturation at [Ca]o in the low millimolar range.

**DISCUSSION**

Our measurements have shown that Ca entry brought about by depolarization is a function of [Na]i and we have used this as a definition of Na/Ca exchange. This conclusion follows from the assumptions that if Ca is at a saturating level and Na/Ca exchange involves the simultaneous translocation of Ca in one direction and Na in the other, then the rate will be Na dependent. Furthermore, if 4 Na are required per Ca translocation, the flux will be dependent not only on membrane potential but also on the 4th power of [Na]i for values of Na well below the $K_m$. Values in the literature suggest a $K_m$ of $\sim 50$ mM, or something near the value found in fresh squid axons, so that in going from 80 mM Na in fresh axons to 36 mM Na in Li-stimulated axons, we might expect a 2-fold or 16-fold change in Ca entry. Our average change is closer to 10-fold, but in the absence of exact analytical figures for each axon, the findings can be understood on the basis of Ca entry as a power function of Na. If [Na]i is raised fourfold (something that can occur during a long experiment), then one expects a 256-fold increase in Ca entry via Na/Ca exchange. Such large sensitivities have been described by Baker et al. (1971) in responses to repetitive stimulation and by Baker et al. (1973a and b) in responses to steady depolarization in axons that had been isolated for many hours and, hence, had presumably gained substantial Na. These papers provide a complete voltage-clamp study of Na/Ca exchange. It is important to note that the resting glow in such Na-loaded axons is normal (it is controlled
by Ca buffering), whereas the Ca entry produced by depolarization cannot be controlled by buffers instantaneously.

The entry of Ca in response to steady depolarization appears to inactivate, and recovery from this inactivation has a time constant of the order of minutes. We have shown that a maintained depolarization does not inactivate Ca entry if no Ca is included in the seawater; hence, this finding suggests that it is the entry of Ca that produces its own inactivation. A previous study of the thermodynamics of Na/Ca exchange (Mullins, 1976) showed that if the membrane potential were made zero, 

\[
[Ca_o] \frac{[Na_o]^4}{[Na_i]^4} = [Ca_i] \frac{[Na_o]^4}{[Na_i]^4}
\]

would be the relationship at equilibrium. With \([Na_o] = 400\), \([Na_i] = 80\), and \([Ca_o] = 50\) mM, the value for \([Ca_i] is 80 \mu M\). This value is one at which there would be no further net Ca flux. If one also assumes that a rise of \([Ca_i]\) stimulates buffers to initiate Ca accumulation, then the infinite-time \([Ca_i]\) will be one in which the net flux of Ca across the axoplasmic membrane just equals the net flux into the buffer systems. A termination of Ca influx by repolarizing the surface membrane will initiate Ca extrusion. There will therefore be a transient rise in \([Ca_i]\) upon depolarization as the system attempts to reach equilibrium (80 \mu M), followed by a steady state where buffer Ca uptake matches Ca entry. Refractoriness will be observed in the system because repolarization will not reduce \([Ca_i]\) to resting levels at once but rather, \([Ca_i]\) will be maintained at higher than normal levels until the Ca load has been discharged.

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