

The Influence of Chemical Agents on the Level of Ionized $[Ca^{2+}]$ in Squid Axons

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ABSTRACT Squid giant axons injected with either aequorin or arsenazo III and bathed in 3 mM Ca (Na) seawater were transferred to 3 mM Ca (K) seawater and the response of the aequorin light or the change in the absorbance of arsenazo III was followed. These experimental conditions were chosen because they measure the change in the rate of Na/Ca exchange in introducing Ca into the axon upon depolarization; $[Ca]_o$ is too low to effect a channel-based system of Ca entry. This procedure was applied to axons treated with a variety of compounds that have been implicated as inhibitors of Na/Ca exchange. The result obtained was that the substances tested could be placed in three groups. (a) Substances that were without effect on Ca entry effected by Na/Ca exchange were: D600 at 10–100 μ M, nitrendipine at 1–5 μ M, Ba^{2+} and Mg^{2+} at concentrations of 10–50 mM, lidocaine at 0.1–10 mM, cyanide at 2 mM, adriamycin at a concentration of 3 μ M, chloradenosine at 35 μ M, 2,4-diaminopyridine at 1 mM, Cs^+ at 45–90 mM, and tetrodotoxin at 10^{-7} . (b) Substances that had a significant inhibitory effect on Na/Ca exchange were: Mn^{2+} , Cd^{2+} , and La^{3+} at 1–50 mM, and quinidine at 50 μ M. (c) There were also blocking agents and biochemical inhibitors whose action appeared to be the inhibition of nonmitochondrial Ca buffering in axoplasm rather than an inhibition of Na/Ca exchange. These were the general anesthetic 1-octanol at 0.1 mM and 1 mM orthovanadate plus apyrase.

INTRODUCTION

The purpose of experiments reported here was to examine the sensitivity of the various systems that allow Ca entry with depolarization, as well as those buffering Ca internally in squid giant axons, to a variety of chemical agents that have been reported to influence Ca movement in cells.

Since in many excitable cells there are at least two voltage-sensitive mechanisms that can allow Ca entry, the Ca channel and Na/Ca exchange, it is important to distinguish between these two entities in drawing conclusions about the effects

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of drugs on Ca movement. In addition, it is also known that Ca can enter via Na channels (a tetrodotoxin [TTX]-sensitive Ca entry; Baker et al., 1971) and it seems possible that Ca is also capable of entering via K channels (Keynes et al., 1979). Therefore, it becomes a matter of some experimental difficulty to sort out the various mechanisms that allow Ca entry. An additional complication is that if Ca entry is measured with substances such as aequorin or arsenazo III in the presence of a chemical agent, one must be certain that the agent applied does not interact with the Ca indicator (aequorin or arsenazo III) and further that the chemical agent does not affect intracellular Ca buffering or the release of Ca from stores, as any or all of these actions could be confused with an altered Ca entry with depolarization.

We have been able to distinguish between Ca entry via Ca channels and Na/Ca exchange by the relatively simple expedient of defining the Ca entry with depolarization that occurs in the absence of $[Na]_i$ as a Ca entry that might be via a channel mechanism (Ca, Na, K), while the entry of Ca that is a function of $[Na]_i$ is defined as Na/Ca exchange (Mullins and Requena, 1981). The practical consequence of this definition is that virtually all the measurable Ca entry with steady depolarization at $[Ca]_o = 3$ mM is Na/Ca exchange.

Our experimental findings are that the Ca channel blockers of an organic chemical sort (D600, nitrendipine) are virtually without effect on Ca entry with depolarization in squid axons. Similarly, Na channel blockers (lidocaine and TTX) are also without effect on Ca entry with steady depolarization.

An organic molecule that appears to have some inhibitory action on Na/Ca exchange is quinidine (Parker, 1978); interestingly, while it primarily affects the Ca entry with depolarization, it also appears to have an effect on intracellular Ca buffering.

The divalent heavy metal cation Mn^{2+} is known to be an inhibitor of Na/Ca exchange (Blaustein, 1977) and, indeed, we have found it to be an effective inhibitor of Ca entry. On the other hand, the divalent cation Ba^{2+} , which moves through Ca channels more readily than Ca, has little effect on the Na/Ca exchange process; the divalent cation Mg^{2+} is also without effect.

It has been known since the study of Blaustein and Hodgkin (1969) that metabolic inhibition with cyanide (CN^-) increases the $[Ca^{2+}]_i$ of the axoplasm, presumably by releasing stored Ca from the mitochondria. This conclusion leads to the notion that a change in $[Ca^{2+}]_i$ in the axoplasm can affect the extent of Ca extrusion as a result of metabolic inhibition. At the same time, the studies of Brinley et al. (1977) have shown that there are at least two mechanisms that buffer Ca^{2+} in cells and that one of these (the X buffer) is effective at very low internal $[Ca^{2+}]_i$, while the other mechanism (mitochondrial buffering) is sensitive either to electron transport poisons such as CN^- or to substances such as carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP) that are H^+ ionophores and collapse the proton gradient across the mitochondrial membrane.

We have noted that a substance tested as a general anesthetic, 1-octanol, has the property of effectively modifying the buffering properties of what has been previously described as the X buffer by inhibiting whatever process it has for the uptake of Ca.

Finally, both Na/Ca exchange and substrate-driven Ca pumps appear to be

affected by adenosine triphosphate (ATP) (DiPolo, 1976). We have used the hydrolytic enzyme apyrase to reduce ATP to low levels and orthovanadate to prevent the phosphorylations that presumably underlie the phosphorylating processes; this sort of treatment leads to a loss of Ca buffering, which in some respects resembles that induced by the treatment of axons with octanol.

METHODS

Axons

The experiments were performed at the Marine Biological Laboratory, Woods Hole, MA, during the months of May and June, 1983. Axons were dissected from freshly caught living squid and were cleaned and mounted in a dialysis-type chamber on glass end cannulas as described by Requena et al. (1977).

Microinjection

A horizontal microinjector was used to introduce aequorin, arsenazo III, or other substances that are not permeable. The usual injection distance was 18 mm and the axon was tested for excitability during and after the microinjection process. Total aequorin counts injected were $1-3 \times 10^9$ as measured by treating the axon with 10 mM CaCl_2 solution (in distilled water) at the end of an experiment. Since resting glow is $\sim 10^3$ counts/s, aequorin in the axon was being consumed at 10^{-6} /s.

Arsenazo III was injected such that the final concentration in the axoplasm was ~ 10 μM ; apyrase (Sigma Chemical Co., St. Louis, MO) was injected to produce a final concentration of 1 U/mg axoplasm. Vanadate was another substance introduced by injection and its final concentration in axoplasm was calculated to be 1 mM.

Light Measurement and Absorbance

Light measurement was as described by Mullins and Requena (1981), except that the photomultiplier was cooled to -20°C by a thermoelectric cooling device. The resulting background count of the tube was reduced severalfold to 15 counts/s. Spectrophotometric measurements of arsenazo III were as described by Brinley et al. (1977).

Solutions

The seawater used in these experiments had the following composition (mM): 440 NaCl, 10 KCl, 10 CaCl_2 , 50 MgCl_2 , 10 TES pH buffer, pH 7.8, and 0.1 EDTA. Variations in the composition of this solution were made by replacing all the Na^+ by K^+ or by Tris^+ to produce depolarizing or Na-free solutions. Inhibitors used in millimolar concentrations (MnCl_2 and CsCl) were added to seawater and an equivalent concentration of Mg^{2+} was subtracted. Drugs in the micromolar concentration range were added to seawater with no change in the resulting seawater. Internal injection solutions were either aequorin (200 μM), made by adding 1 mg of salt-free aequorin (kindly supplied by Dr. John Blinks), to ~ 200 μl of highly purified, quartz-distilled water or to the same volume of 1 μM EGTA solution. Other internal solutions were apyrase, orthovanadate, or arsenazo III. The last two substances were made up in 330 mM KTES buffer, pH 7.3. Water-insoluble drugs were dissolved in dimethylsulfoxide (DMSO) or ethanol at a concentration such that a 1:1,000 dilution would give the required concentration in seawater.

Na Electrodes

In a number of the axons studied, a glass Na-sensitive electrode was introduced into the axon. The details of the fabrication of such electrodes and the circuits used for measuring

are as described by Mullins et al. (1983). The electrodes were essential in differentiating between axons that were low in Na_i (<15 mM) or high in Na_i (>20 mM). These changes in $[\text{Na}]_i$ have previously been shown (Mullins and Requena, 1981) to be the dominant factor in controlling the response to depolarization that is Na/Ca exchange. The Na-sensitive electrodes were calibrated at several Na concentrations in the range of 10–50 mM and the results are reported as $[\text{Na}]_i$ rather than as Na activity. The notation $[\text{Na}]_i$ therefore means internal Na concentration and Na_i means internal Na (in contrast to external Na).

RESULTS

Ca Channel Blocking Agents

An original suggestion regarding Ca entry with depolarization in squid axons was that it took place in part via Na channels and in part via Ca channels that provided a TTX-insensitive pathway for Ca entry (Baker et al., 1971). It was, therefore, important for us to show that in spite of the claims that D600 inhibited Ca entry with depolarization, this finding could not be observed if the Ca entry in squid axons was confined to Na/Ca exchange. Some confusion arises because to observe a Ca entry that is sensitive to Ca channel blocking agents, it was necessary for Baker et al. (1973a) to work at rather high $[\text{Ca}^{2+}]_o$ (112 mM), while Mullins et al. (1983) have shown that the Na/Ca exchange process is effectively saturated at a concentration of ~3 mM. Obviously, a large $[\text{Ca}^{2+}]_o$ leads to entry of Ca that is via processes other than the exchange reaction.

We have tried D600 in seawater (at a concentration of 10–100 μM) on the response of a squid giant axon to depolarization produced by changing the external solution from a 10 mM K (3 mM Ca) seawater to a 450 mM K (3 mM Ca) solution. The axon had been injected with arsenazo III and the response of the optical signal was measured with a spectrophotometer. The result is shown in Fig. 1, in which it can be noted that arsenazo III has little or no effect on the Ca entry with depolarization. A similar experiment in an aequorin-injected axon bathed in 10 mM Ca^{2+} with 100 μM D600 showed no effect on Ca entry with depolarization.

In Table I we list a number of substances that have been described in the literature as either Ca channel blocking substances (D600, nitrendipine) or inhibitors of Na/Ca exchange (adriamycin, quinidine, Mn^{2+}). The two organic Ca channel inhibitors were without effect at doses much larger than those used to block Ca channels in other preparations and there were only modest (12–30%) decreases in the response to depolarization at very high dose levels. Quinidine and Mn^{2+} had a substantial inhibitory action on Ca entry with depolarization and were studied in more detail using aequorin-injected squid axons (see below).

Divalent Cations

It has been known since the study of van Breemen and De Weer (1970) that La^{3+} has a strong inhibitory effect on Ca efflux from squid giant axons; subsequent studies have shown that divalent cations such as Mn^{2+} have an inhibitory action on Ca entry through Ca channels. Baker et al. (1973a) showed that in

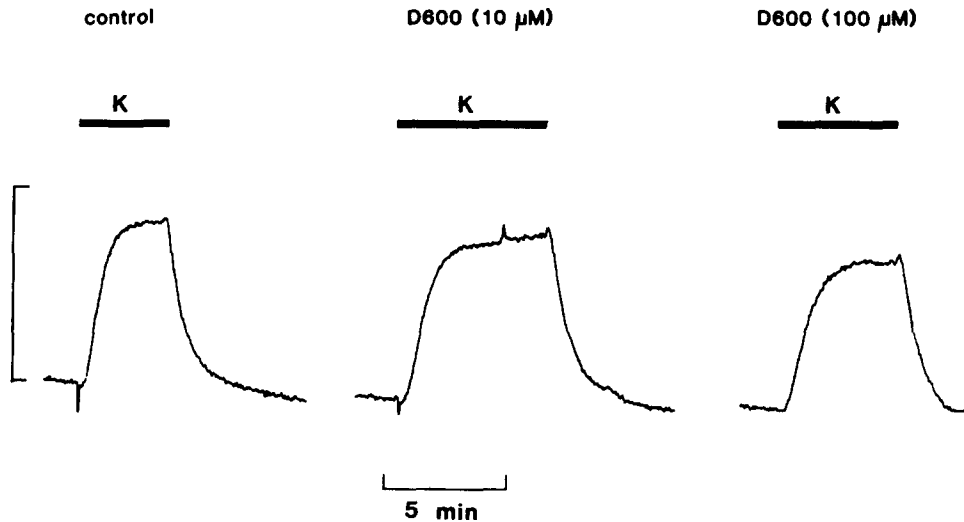


FIGURE 1. An arsenazo III-injected axon was depolarized by changing its external solution from a 10 mM K (3 Ca) solution to a 450 mM K (3 Ca) solution. The resulting absorbance change is shown on the left. Following a return to seawater, D600 at concentrations of 10 and 100 μM was applied and the depolarization was repeated. The results are shown on the center and right panels. The vertical bar is 0.05 in absorbance.

aequorin-injected squid axons, this ion inhibited the response to depolarization; they suggested that this was because it interfered with Ca entry via a Ca channel. Mullins and Requena (1981) demonstrated that Ca entry with depolarization as measured with aequorin depended on Na_i , and Mullins et al. (1983) showed that there was an identical dependence on Na_i in arsenazo-injected axons. Thus, it appeared that the action of Mn^{2+} was more than that of simply interfering with Na/Ca exchange. To further examine this effect, we depolarized squid giant axons in seawater containing either 10 or 50 mM Ca^{2+} , and as $[\text{Mn}^{2+}]$ was varied, we followed the response of aequorin luminescence. A plot of our experimental results is shown in Fig. 2. Note that with the higher Ca concentration, the inhibitory action of Mn^{2+} is somewhat overcome, so that a 50 mM Mn^{2+}

TABLE I
Effects of Various Substances on the Arsenazo III Response to Depolarization by Na-free (K) Solutions (3 mM Ca)

Drug	Concentration	Description
D600	10 μM	No effect ($n = 3$)
D600	100 μM	12% and 30% decrease ($n = 2$)
Nitrendipine	1 μM	No effect ($n = 2$)
Nitrendipine	5 μM	20% decrease ($n = 1$)
Adriamycin	3 μM	No effect ($n = 2$)
Quinidine	200 μM	Mean decrease of 80% ($n = 3$)
Mn^{2+}	10 mM	Mean decrease of 80–90% ($n = 3$)

concentration is required to produce 50% inhibition of the response. On the contrary, with 10 mM Ca^{2+} , 5–7 mM Mn^{2+} is required to produce 50% inhibition. Since the $[\text{Ca}^{2+}]$ required in seawater to half-saturate the Na/Ca exchange is of the order of 0.5 mM (Mullins et al., 1983), the present findings suggest that a simple competition between Mn^{2+} and Ca^{2+} for a site may account for the results.

A second divalent cation that was tested was Ba^{2+} . Here the idea was to evaluate the extent to which Ba^{2+} might compete with Ca^{2+} for the exchanger site, and to note whether the shape of the aequorin light response with depolarization might be changed because Ba^{2+} acted as a poison for K^+ channels. Neither

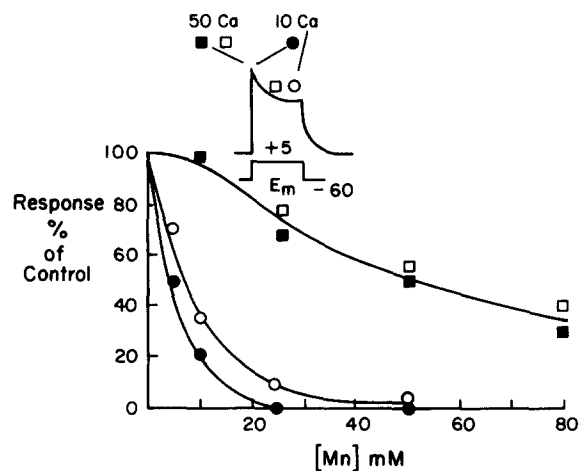


FIGURE 2. This shows the effects of increasing the Mn^{2+} concentration in seawater from 0 to 80 mM. Mg_o^{2+} was 100 mM minus $[\text{Mn}^{2+}]$. Two $[\text{Ca}^{2+}]_o$ were used, 10 and 50 mM, and a membrane potential change from -60 to $+5$ mV, using 450 mM $[\text{K}]_o$, was employed. The ordinate is the light emission from aequorin, and the abscissa is $[\text{Mn}^{2+}]$ in seawater. Note that half-maximal suppression of the Ca entry with depolarization was at a $[\text{Mn}^{2+}]$ of ~ 5 –7 mM when $[\text{Ca}^{2+}]_o$ was 10 mM, but raising $[\text{Ca}^{2+}]_o$ to 50 mM changed the level of this suppression to ~ 50 mM.

of these effects was prominent, as the results of Fig. 3 show. The amplitude of the response was slightly decreased in the presence of 20 mM Ba^{2+} , but there was essentially no change in shape. A complication in this record is that Ba^{2+} itself could be expected to enter the axon and it is known that Ba^{2+} has some ability to activate aequorin. This activating effect has, however, been shown by Blinks et al. (1977) to require a $[\text{Ba}^{2+}]$ more than three orders of magnitude greater than that for $[\text{Ca}^{2+}]$. An interesting point is that Ba^{2+} often passes the Ca channel more easily than Ca^{2+} ; thus, it would appear that Ba^{2+} has little effect on Na/Ca exchange.

A third divalent cation related to Ca^{2+} that we studied was Mg^{2+} . The lower part of Fig. 3 shows an experiment in which Mg^{2+} was removed from seawater and the response to depolarization was measured. There is clearly no difference between this record and that of a control containing 50 mM Mg^{2+} . We concluded that Mg^{2+} does not interact with the Na/Ca exchange system. This observation

is also supported by observations of Na/Ca exchange using isotopes where Mg^{2+} was substantially without effect. On the other hand, Baker et al. (1971, 1973b) observed substantial inhibitions of Ca^{2+} entry with depolarization when $[Ca^{2+}]_o$ was 112 mM and Mg^{2+} was present.

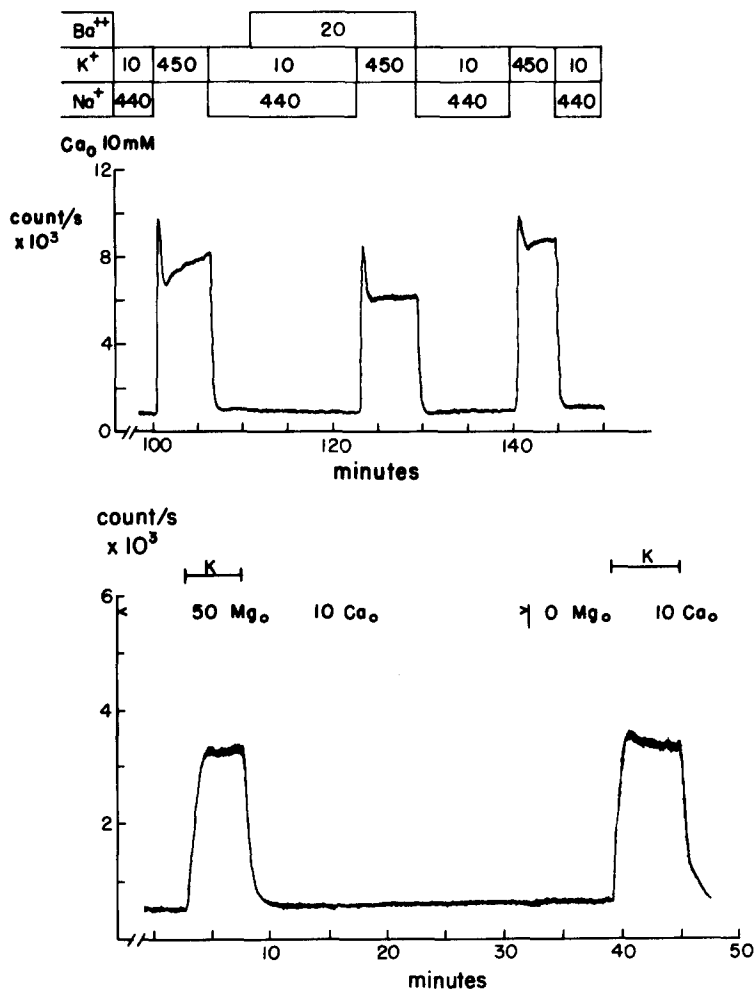


FIGURE 3. This plot (upper panel) shows the effect of applying 20 mM Ba^{2+} to seawater containing 10 mM Ca^{2+} and 50 mM Mg^{2+} when depolarizing concentrations of K^+ were added to the seawater. Note that although Ba^{2+} does influence the aequorin reaction, the response of aequorin to this ion is three orders of magnitude smaller than that for Ca^{2+} so that Ba^{2+} entry could not influence the light emission from Ca^{2+} entry with depolarization. The record shows essentially that Ba^{2+} is without effect on Ca^{2+} entry with depolarization. In the lower panel, a 450 mM K depolarization in 10 mM Ca^{2+} , 50 mM Mg^{2+} seawater is compared with a similar depolarization in 10 mM Ca^{2+} seawater that was Mg^{2+} -free (80 mM Tris Cl replaced Mg^{2+}).

Octanol

It seemed useful to examine the effects of substances that are classified as generalized blocking agents (ethers, halogenated paraffins, higher alcohols), and octyl alcohol (octanol) was selected as a representative substance. It was necessary first to test this substance on the aequorin reaction *in vitro* to be sure that there was no direct effect on the Ca-aequorin reaction, since Baker and Shapira (1980) had reported a modest enhancement of light emission when substances such as urethane were added to the *in vitro* aequorin-Ca reaction. The question of direct

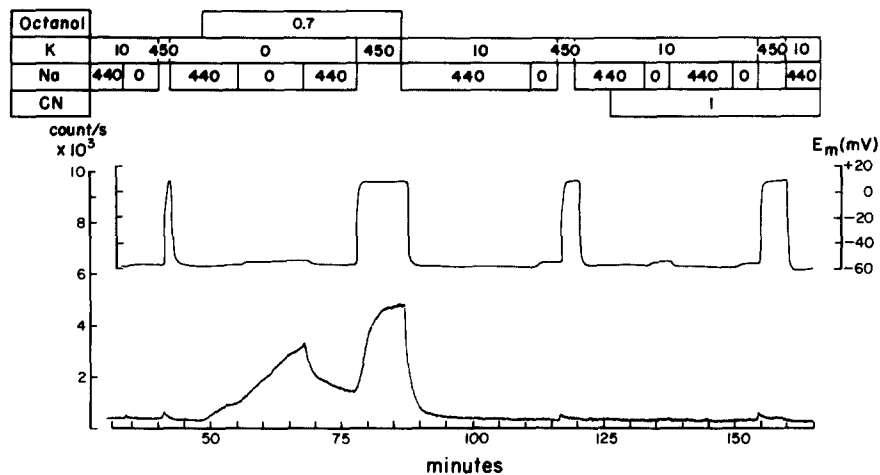


FIGURE 4. Response of aequorin to depolarization in a freshly isolated axon where internal $[Na^+]$ was between 12 and 15 mM. Note that a depolarization applied at ~32 min gave a very small increase in aequorin glow, but when this was repeated in the presence of 0.7 mM octanol, there was a very large response. In between, simply applying octanol led to an increase in aequorin glow and the application of Na-free solutions further increased this response. The removal of octanol led to a return to the initial response to depolarization. This behavior was not a result of some inhibitory action on mitochondrial buffering, as shown by applying 1 mM CN^- in seawater in the last part of the record. The response of aequorin to depolarization remained at control levels.

effects of anesthetic agents on aequorin has been examined in detail by Blinks et al. (1982) and their finding is that there is little such effect. Our findings *in vitro* showed that there was no effect of octanol on the aequorin light reaction if precautions were taken to ensure the absence of Ca contamination by the use of high concentrations of CaEGTA/EGTA buffers.

In a freshly isolated aequorin-injected squid axon (with a low $[Na]_i$) (Mullins and Requena, 1981), there is at best a very small increase in aequorin glow upon K depolarization. This finding is confirmed by the measurement at minute 33 in Fig. 4, where K^+ depolarization yielded a barely measurable response. Just before the high- K^+ step, the axon was in Tris seawater, which had no effect on the resting glow. After the K depolarization, the axon was returned to Na seawater;

this was followed by treatment with Na seawater that contained octyl alcohol at 0.7 mM. This change led to an increase in resting glow of approximately fourfold. A change to Na-free seawater also containing octyl alcohol then led to a large increase in glow. This increase was reversed by a return to Na seawater and the application of a high-K⁺ solution led to a light emission that was ~17 times resting glow. Finally, the octyl alcohol was removed and the axon was retested with a high-K⁺ solution; the response was virtually identical to the initial depolarization.

One possible explanation for the effect of octyl alcohol was that it was inhibiting the mitochondrial sequestration of Ca; a test of this is shown on the right-hand side of Fig. 4. Here, 1 mM CN was applied and then Na-free and high-K solutions were again applied; the response was identical to that of control. We conclude that for the low concentrations of Ca that occur upon depolarization in low [Na]_i axons, the mitochondria are not involved in Ca buffering, except possibly in the very periphery of the axon.

The remaining possibilities are that octyl alcohol affects the Na/Ca exchange mechanism such that it causes it to operate at a higher velocity, or that octyl alcohol inhibits nonmitochondrial buffering. One can decide between these possibilities by noting that in every instance where octyl alcohol is applied in the presence of normal external Na and a normal membrane potential, there is a rise in aequorin glow. Since under these experimental conditions there should be a net efflux of Ca via Na/Ca exchange (most of Ca influx at the resting potential has been shown to be TTX sensitive; DiPolo et al., 1982), it would appear that octyl alcohol affects a nonmitochondrial buffer.

To examine further the actions of octanol in affecting [Ca²⁺]_i, it was necessary to work at a higher [Na]_i so that larger entries of Ca would be brought about both by Na-free conditions and by depolarization. Accordingly, axons were first examined for a small response to high-K solutions and a corresponding enhancement with octanol and were then stimulated for 10⁵ impulses and again depolarized in high-K solutions. After this control, the axon was treated with varying concentrations of octanol and the steps outlined above were repeated. The results are shown in Fig. 5. It can be seen that in the absence of octanol, the Na-free conditions produced an easily measured increase in aequorin glow and depolarization produced an increase of 4,000 counts/s in glow compared with <200 counts/s in a low-Na axon. This response to depolarization was enhanced by the lowest dose of octanol tested (0.15 mM) and grew to more than fivefold the control value at 0.75 mM. Proportionally, the octanol effect is much greater when [Na]_i is low, which suggests that when the aequorin count is low, there is little or no mitochondrial contribution to Ca buffering. By contrast, since the aequorin count in axons with an enhanced Na_i is 10 times greater, mitochondrial buffering may be important.

In another axon treated with octanol, the increase in aequorin glow was measured when [Na⁺]_i was high under the following conditions: (a) when octanol was applied in Na seawater, (b) under Na-free conditions, and (c) under high-K solution conditions (i.e., with depolarization). The results from this study are plotted in Fig. 6, in which it can be seen that all three conditions yield higher

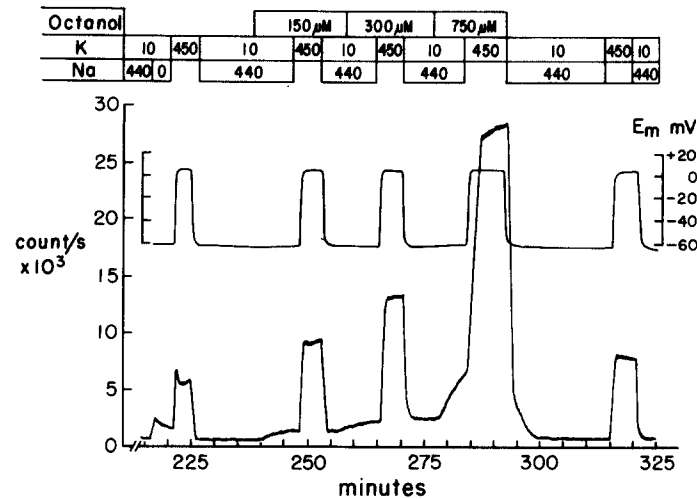


FIGURE 5. Response of an axon to depolarization when $[Na^+]_i$ was of the order of 20 mM, as measured with a Na electrode; the measurement was of aequorin light emission. Note that there is also an increased aequorin glow when octanol at concentrations of 0.15–0.75 mM was applied. The record shows that the control response is regained when octanol is removed from the axon.

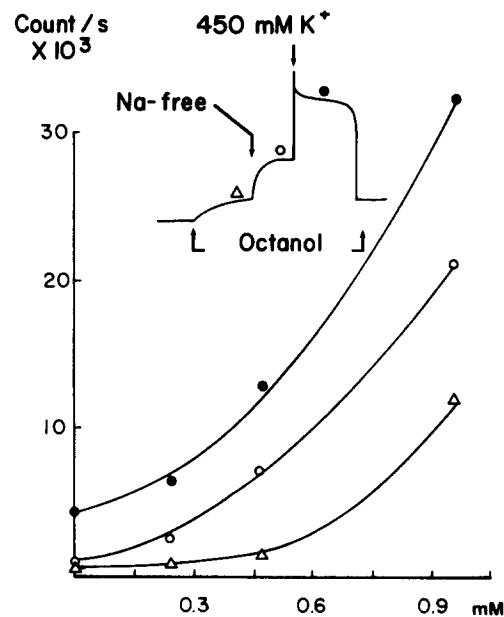


FIGURE 6. In another axon, a plot is made of the response of the light emission from aequorin in the presence of varying concentrations of octanol during resting glow conditions, during Na-free and during K-rich applications. The axon had a $[Na^+]_i$ of 26 mM, as measured with an electrode, and thus was Na-loaded.

aequorin glows as the octanol dose is increased. Concentrations above 0.93 mM were not used, because the membrane is liable to undergo an irreversible increase in Ca permeability at higher concentrations. At low octanol concentrations (0.5 mM), conduction is not blocked in squid axons, but the Ca signal from aequorin is enhanced. This result suggests that octanol may be useful as a tool to obtain an increased sensitivity to the measurement of Ca entry with depolarization.

The Action of Quinidine

A report that quinidine is an inhibitor of the Ca-induced increase in K permeability (Armando-Hardy et al., 1975) has led to several additional studies suggesting that this substance affects the binding of Ca²⁺ to a receptor that activates K permeability (Atwater et al., 1979; Fishman and Spector, 1981) and to a study by Parker (1978) showing that Ca influx in dog red cells is almost completely inhibited by doses of quinidine of the order of 50 μM.

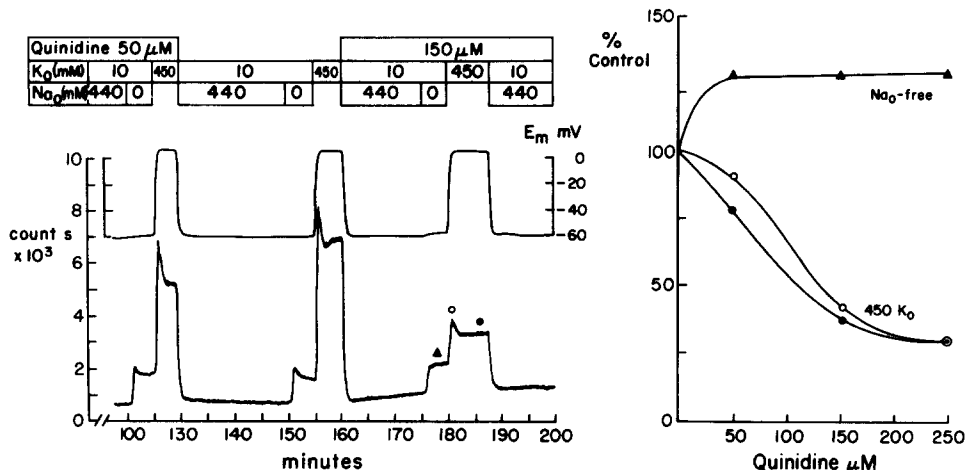


FIGURE 7. The response of aequorin glow to Na-free and K-rich solutions is shown for an axon that was initially treated with 50 μM quinidine. This treatment was followed by a control response in the absence of quinidine. Finally, two further concentrations of quinidine were applied (only the response to 150 μM is shown). At the right, the curve shows a dose-response set of curves for aequorin glow with depolarization and under Na_o-free conditions.

Since this Ca influx in dog red cells depends on [Na⁺]_i, it is considered to be a form of Na/Ca exchange; hence, it was of interest to examine the sensitivity of the squid axon response to depolarization as affected by quinidine.

Fig. 7 shows an experiment in which an axon was treated first with a Na-free solution and then with 450 mM K solution. This procedure was repeated for several concentrations of quinidine and the results are plotted in the right-hand part of Fig. 7. It is clear that quinidine has a differential effect in enhancing the response to Na-free solutions while reducing the response to depolarization. Since it is thought that the entry of Ca on applying Na-free solutions is always smaller than the response to depolarization because the presence of a membrane

potential represents a larger barrier to Na/Ca exchange than does the release of carrier sites occupied by external Na, the results with quinidine suggest that it acts on a different part of the carrier cycle where the rate-limiting step is sensitive to membrane potential.

Other Inhibitors

While it has been known since the study of Baker et al. (1973*b*) that TTX does not affect the response of an aequorin-injected axon to steady depolarization, it is not so clear that Ca may not pass through K channels during steady depolarization. Substances such as TEA⁺ are not of much help, since in squid axons they only work on the inside of the axon to block outward K⁺ movement and Ca²⁺ movement must be expected to be inward through K⁺ channels. We have therefore tried Ba²⁺ (mentioned above), Cs⁺, and 2,4-diaminopyridine as inhibitors of ion movement in K channels. None was effective in reducing the response

TABLE II
*Effect of Various Substances on the Aequorin Response to Depolarization
by 450 mM K Solutions (10 mM Ca²⁺)*

Axon	Drug	Concentration	Description
030682A	Cs ⁺	45 mM	No effect (<i>n</i> = 2)
		90 mM	No effect (<i>n</i> = 1)
140583A	2,4-diaminopyridine	1 mM	No effect (<i>n</i> = 1)
150583A	D600	100 μM	No effect (<i>n</i> = 2)
100583B	D600	100 μM	No effect (<i>n</i> = 1)
030583A	Chloradenosine	35 μM	No effect (<i>n</i> = 2)
010683A	Lidocaine	0.1 mM	No effect (<i>n</i> = 1)
		10 mM	30% increase in aequorin signal (<i>n</i> = 2)
030584	Cd ²⁺	5 mM	30% inhibition (<i>n</i> = 2)
051482A	La ³⁺	2 mM	90% inhibition (<i>n</i> = 1)

of axons to steady depolarization, as the results in Table II show. Two axons injected with aequorin were treated with D600 and depolarized. In agreement with the results with arsenazo III (presented earlier), there was no effect of this inhibitor.

Chloradenosine, at a concentration of 10 μM, has been shown to be a potent inhibitor of a class of Ca channels (Henon and McAfee, 1983); it was tested and found to be without effect on Na/Ca exchange. The local anesthetic lidocaine was also tested since it has been shown to be a Na channel blocker and an inhibitor of intracellular Ca release from the sarcoplasmic reticulum. It was essentially without effect. Two additional ions tested were Cd²⁺ and La³⁺, since they are often used as Ca channel inhibitors; in the doses employed, the former reduced the response to depolarization by 30% and the latter by 90%.

Vanadate and Apyrase

30 min after a squid axon is injected with apyrase, its level of intracellular ATP falls to values in the tens of micromolar. Although such concentrations may

activate Ca extrusion pumps and/or Ca buffering entities in axoplasm, the addition of orthovanadate, which has been shown by DiPolo et al. (1979) to inhibit Ca extrusion from axons in the absence of external Na, must be expected to inhibit Ca efflux almost totally. Hence, we have employed a mixed injection of orthovanadate to give a final concentration of 1 mM and apyrase to give a final concentration of 1 U/mg axoplasm. Such axons have a resting glow that is perhaps 15-fold greater than unpoisoned axons, but they do have a stable resting glow in the presence of external Ca and Na. This situation can be contrasted

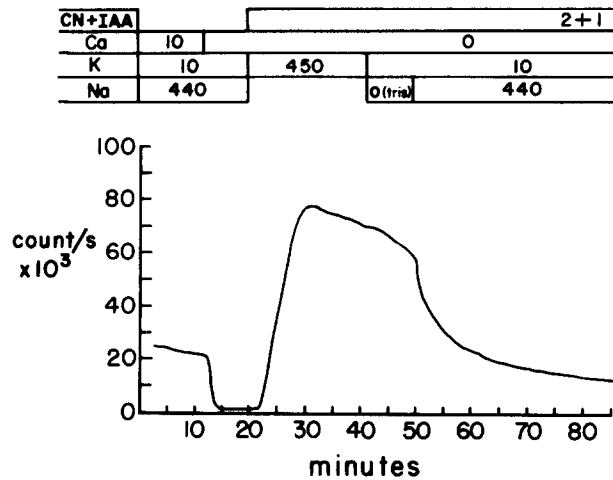


FIGURE 8. A freshly isolated axon was injected with vanadate, apyrase, and aequorin. Vanadate, 1 mM final concentration; apyrase, 1 U/mg. The resting level of glow in 10 mM Ca seawater was ~15-fold greater than that in axons without the inhibitor and apyrase. Removal of external Ca^{2+} results in a resting glow that is virtually normal, which suggests that Ca^{2+} extrusion is possible, while ATP-requiring systems ought to be fully poisoned. The application of CN^- leads to a release of stored Ca^{2+} from the mitochondria and to a stabilization of the aequorin glow at a high level. External Na^+ brings down this glow, presumably by its ability to extrude Ca^{2+} in an ATP-independent manner.

with apyrase-injected axons in which the aequorin was confined to a dialysis capillary on the axis of the axon, and in these axons there is no difference in luminescence between control and apyrase-injected axons.

If external Ca^{2+} is removed, then, as the record in Fig. 8 shows, aequorin light emission falls over a time course of 2 min to a very low level. In an unpoisoned axon, this time course of fall would be tens of minutes. The axon, in effect, behaves as if (a) buffering by nonmitochondrial buffers had been greatly decreased, and (b) a Ca extrusion system were fully functional. The expected effect of a buffering decrease would be that more Ca^{2+} would be free in axoplasm and less would be stored, thus allowing the rapid fall of Ca_i when Ca_o is made zero. Next, the axon is both depolarized and treated with CN. Then depolarization will not lead to a Ca^{2+} entry since Ca_o is zero, but CN^- will release stored Ca^{2+} from the mitochondria at once, since ATP levels in the axoplasm in the presence

of apyrase are such that they cannot support Ca sequestration when electron transport is blocked (Requena et al., 1977). Repolarization of this axon in Tris⁺ solution does not affect the time course of aequorin light, but a replacement of Tris with Na in the external solution brings $[Ca^{2+}]_i$ down to low levels.

DISCUSSION

One of the purposes of this study was to compare the results of Ca^{2+} entry with steady depolarization when $[Ca^{2+}]_o$ was low (3–10 mM) with the previous results of Baker et al. (1971, 1973*a, b*). The absence of an effect of Mg^{2+} or of organic Ca^{2+} channel blockers such as D600 on Ca^{2+} entry with steady depolarization when $[Ca^{2+}]_o$ is low emphasizes that the Ca^{2+} entries presently being measured and those in the references cited above are two different processes. Moreover, our measurements have been made with two different intracellular indicators of $[Ca^{2+}]_i$, aequorin and arsenazo III, and both show a lack of effect of organic Ca^{2+} channel blocking agents on Ca^{2+} entry with depolarization.

TABLE III
Agents Affecting Ca^{2+} Entry at Rest and During Depolarization

Agent	Resting Ca^{2+} influx	Ca entry with depolarization	
		Late Ca channel	Na/Ca exchange
TTX	Blocks 70%*	No effect [‡]	No effect
TEA ⁺		No effect [‡]	No effect
Mg ²⁺	No effect [†]	Blocks [‡]	No effect
Mn ²⁺		Blocks [‡]	Blocks
Co ²⁺		Blocks [‡]	—
Cd ²⁺		Blocks [†]	Blocks
La ³⁺		Blocks [‡]	Blocks
D600	Blocks 50%*	Blocks [‡]	No effect

* DiPolo et al. (1982).

‡ Baker et al. (1971).

‡ Baker et al. (1973*a*).

† Brinley et al. (1977).

† DiPolo et al. (1983).

Results in the last column are from this study.

Heavy metal divalent cations such as Mn^{2+} have traditionally been used as Ca channel blocking agents, and indeed they have this effect. They do block, at virtually the same dose, the Na/Ca exchange system, as the present study shows. Hence, they are not useful in separating Ca entry via Na/Ca exchange from Ca entry via channels.

Measurements have been made of isotopic Ca influx at the resting membrane potential and of the influence of a variety of chemical agents on this influx (DiPolo et al., 1982). In particular, it has been shown that ~70% of this resting influx is cut off by the application of TTX to seawater and only 20% at most is Ca influx that is dependent on $[Na]_i$. The application of the Ca channel blocking drug D600 has only a very small effect on Ca entry since this is mostly via Na channels. On the other hand, if $[Na]_i$ is made 90 mM (a value many times that of normal), Ca influx is elevated and D600 has a much greater effect. We have

no explanation for this finding. If Ca can pass K channels, it is possible that the effects observed are an indirect effect of changes in K permeability. A comparison of the blocking agents discussed above is made in Table III for both the resting Ca influx and the two sorts of Ca entry with depolarization—the late Ca channel and Na/Ca exchange. It is useful to note that among these substances, only Mg²⁺ and D600 clearly differentiate between channels and Na/Ca.

The substance quinidine is interesting in that it does produce an inhibition of Ca entry with depolarization; however, this inhibition is only partial.

Octyl alcohol has long been used as an agent to block conduction in nerve and cause general anesthesia in a variety of systems. The mechanism for this sort of action has usually been thought to be via its effect on the time constants of the Na channel (Haydon and Urban, 1983). Recently, however, a number of reports (Wojtczak, 1984; White et al., 1984) have indicated that octyl alcohol produces uncoupling in cardiac cells. This effect is indicative of a rise in the myoplasmic Ca concentration, precisely the effect that octyl alcohol produces in squid axoplasm. The alteration of buffering that this substance produces may be an important aid in understanding Ca entry phenomena. Octyl alcohol also has been shown to have an action on Na channels incorporated into a planar bilayer (Urban et al., 1984). Here the finding is that octanol increases the fraction of time that a Na channel remains closed.

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