Effects of Internal Divalent Cations on Voltage-Clamped Squid Axons

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ABSTRACT We have studied the effects of internally applied divalent cations on the ionic currents of voltage-clamped squid giant axons. Internal concentrations of calcium up to 10 mM have little, if any, effect on the time-course, voltage dependence, or magnitude of the ionic currents. This is inconsistent with the notion that an increase in the internal calcium concentration produced by an inward calcium movement with the action potential triggers sodium inactivation or potassium activation. Low internal zinc concentrations (∼1 mM) selectively and reversibly slow the kinetics of the potassium current and reduce peak sodium current by about 40% with little effect on the voltage dependence of the ionic currents. Higher concentrations (∼10 mM) produce a considerable (ca. 90%) nonspecific reversible reduction of the ionic currents. Large hyperpolarizing conditioning pulses reduce the zinc effect. Internal zinc also reversibly depolarizes the axon by 20–30 mV. The effects of internal cobalt, cadmium, and nickel are qualitatively similar to those of zinc: only calcium among the cations tested is without effect.

INTRODUCTION

Divalent cations in the external media of nerve cells play a unique role: small concentrations are necessary for, but large amounts reduce, excitability. Very little is known about their effects within these cells, however. Grundfest et al. (1954) and Hodgkin and Keynes (1956) microinjected divalent cations into squid giant axons and found that the large concentrations injected (∼0.25 M for an eventual axoplasmic concentration of 10–25 mM) produced a depolarization of the resting membrane potential as well as conduction block. Meech (1972) using Aplysia neurons, and Krnjević and Lisiewicz (1972) using cat motor neurons concluded that increased internal Ca++ produced an increase in potassium conductance. The latter investigators suggested that the entry...
of Ca++ during the early part of the action potential (Hodgkin and Keynes, 1957; Baker and Hodgkin, 1971) may activate potassium conductance.

To investigate this possibility, we perfused squid giant axons with solutions containing Ca++ and measured the ionic currents under voltage clamp. We found that as much as 10 mM of internal Ca had little, if any effect on the magnitude, voltage dependence, or time constants of the ionic currents. It is therefore unlikely that the potassium conductance is activated by the very small change in [Ca++]i, which results from the influx of Ca++ during an action potential (Hodgkin and Keynes, 1957; Baker and Hodgkin, 1971).

In order to determine if calcium was alone in its lack of effect, we perfused axons with solutions containing Zn++, Co++, Cd++, and Ni++. Unlike Ca, these other cations produced marked effects on the magnitude and time-course of the ionic currents. Our results suggest that the divalent cations other than Ca alter the ionic channel gating structure, probably as a result of their binding to the protein constituents of the ionic channels.

METHODS

The experiments reported here were performed on segments of isolated giant axons of the squid, *Loligo pealei*, obtained at the Marine Biological Laboratory, Woods Hole, Mass. The average diameter of 51 axons was 392 μm, with a range of 270-500 μm. The average initial resting potential of the axons was −60 mV.

The voltage clamp circuit was essentially that of Moore (1971) modified to include series resistance compensation. Compensation for a series resistance of 4–6 Ω·cm² was usually applied.

The single cannula perfusion technique described by Fishman (1970) was used, employing the enzyme papain (at a concentration of 1 mg/ml in KF) to facilitate removal of the axoplasm. The chamber was similar to that described by Armstrong, et al. (1973) except that air gaps were used to electrically isolate the ends of the axon from the voltage-controlled region. Temperature measurement and control was also similar to that of Armstrong et al. (1973).

Electrodes

The external electrode was a sea-water bridge connected to the external voltage preamplifier via a Ag/AgCl wire. The internal electrode was of the “piggy-back” arrangement (Chandler and Meves, 1965) with current delivered via a 50 μm platinum-black Pt-Ir (10%) wire. The voltage electrode was a glass pipette 50–75 μm at the tip, containing a solution of 0.56 M KCl; a Ag/AgCl wire connected the KCl solution to the internal voltage preamplifier. A 25-μm bright Pt-Ir (10%) wire inserted into the shank of the pipette was used to reduce the high frequency impedance.

At the start of each experiment, the internal electrode was placed in the external solution and any DC bias was removed with the DC-offset control of the internal voltage preamplifier. No correction was made for junction potentials.

Solutions

The axons were bathed in an artificial sea water (ASW) of the following composition (mM): Na+ 440, Ca++ 10, Mg++ 50, Cl− 560. The internal perfusion fluid was usually
(mM): K+ 275, Na+ 44, anion -319. Isethionate, glutamate, or Cl were the anions used for the calcium experiments and F was used for the rest of the experiments. Sucrose was used to make the internal solutions isotonic with the ASW. Sucrose was removed to maintain isotonicity when divalent cations were added. Both internal and external solutions were buffered with 1 mM HEPES (N-2-Hydroxyethylpiperazine-
N'-2-ethanesulfonic acid) to a pH of about 7.4.

It is clearly important to know the activity of the various divalent cations used in this study. We were unable to find any published data on the degree to which Ca may complex with isethionate; we therefore set out to determine how much complexation occurs. To measure divalent cation activity in the presence of various concentrations of Cl (control), isethionate and glutamate and at the pH of our solutions, we used an Orion model 92-32 divalent cation electrode (Orion Research, Inc., Cambridge, Mass.) and a double junction reference electrode with an Orion model 801 digital pH meter. An apparent stability constant ($K_1$) of 4.1 for the calcium monoglutaamate complex at the ionic strength and pH of our solutions was calculated. This is different than the values reported in Sillen (1964). However, the apparent $K_1$ should be rather dependent on pH and ionic strength and the conditions used in the measurement of the reported values are not the same as those in our solutions. Therefore, from this $K_1$ we find approximately 60% of the Ca is complexed at the concentration of glutamate employed. Isethionate also forms a complex with Ca, but to a much smaller degree, and is negligible for our purposes.

While ZnF$_2$ is soluble, Zn does form a monofluoride complex with a stability constant ($K_1$) of 5.01 (Sillen, 1964). Ni complexes with fluoride but to a small degree ($K_1 = 0.22$), and there is no evidence for any monofluoride complexes of Co or Cd (Sillen, 1964). Using the above stability constants, and an activity coefficient of 0.30 for the divalent cations at this ionic strength (see Shatkay, 1963), we determined the activities shown in Table I. The concentrations of divalent cations referred to hereafter are total concentrations; Table I should therefore be consulted when activities are required.

To accelerate recovery after exposure to the divalent cations, EGTA was often used before and after exposure. Control experiments showed that EGTA itself had a negligible effect on all the properties of the ionic currents of interest.

**Conventions and Definitions**

The membrane potential, $V$, is the potential inside the axon minus the potential outside. Therefore, a depolarization of the axon causes $V$ to become more positive. The movement of positive ions outward through the membrane constitutes positive current and in all figures is in the upward direction.

The early or transient current (corrected for leak) is carried predominantly by Na ions and will be called $I_{Na}$. Similarly, the late or steady-state current (corrected for leak) is predominantly K ions and will be called $I_K$.

The term sodium conductance ($g_{Na}$) is used as defined by Hodgkin and Huxley (1952):

$$g_{Na} = I_{Na}/(V - V_{Na}),$$

where $V_{Na}$ is the sodium reversal potential. Potassium conductance ($g_K$) here is the
maximum slope conductance as defined by

$$g_K = \frac{dI_K}{dV}.$$  

This definition is more useful than the chord conductance since we did not measure the potassium reversal potential.

**RESULTS**

Internal Calcium Does Not Affect Ionic Currents

Study of the effects of internal calcium is complicated by the fact that CaF$_2$ is only very slightly soluble and internally perfused axons do not survive well unless the perfusion fluid contains a high fluoride concentration. Tasaki et al. (1965) examined the ability of internal anions to maintain excitability; they found the order of preference to be: F$^-$ > HPO$_4^{2-}$ > glutamate > aspartate > SO$_4^{2-}$ > acetate > Cl$. Adelman et al. (1966) found that the leakage current was abnormally increased when F$^-$ is replaced by Cl$^-$, and this is probably the reason that excitability is not well maintained in the absence of F$^-$. Our internal Ca experiments were performed using glutamate, isethionate, and Cl$^-$ as internal anions. We also found that leakage current increased rapidly in the absence of F$^-$ and that $I_{Na}$ and $I_K$ decayed rapidly in magnitude. The experiments were therefore performed rapidly with a minimum exposure to each solution, to minimize the effects referred to above. We often measured perfusion flow rates using the dye phenol red as a marker and exchanged the internal volume 10–20 times before making a measurement.
The results of perfusing an axon with 1 mM Ca in a glutamate solution are shown in Fig. 1. Neither $I_{\text{Na}}$ nor $I_{\text{K}}$ were appreciably affected by internal Ca: the small reduction observed is probably due to deterioration of the axon in the absence of $F^-$. There was no change in the apparent sodium equilibrium potential, nor in the voltage dependence of $I_{\text{Na}}$. Data on $I_{\text{Na}}$ from similar experiments are summarized in Table II. Internal Ca concentrations as large as 10 mM had no effects on $I_{\text{Na}}$ significantly different than the zero calcium control experiments. In two experiments (axons C-28, C-29) the order of perfusion was reversed: the axon was first exposed to 1 mM Ca and then to zero Ca. In one case (C-28) there was a slight increase in $I_{\text{Na}}$ when the 1 mM Ca solution was replaced by a zero calcium solution, in the other (C-29) there was a small decrease. Thus, the order of exposure to calcium-containing solution introduces no appreciable differences in the conclusion that internal calcium has little or no effect on $I_{\text{Na}}$. 

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**Figure 1**. Current-voltage relations in a squid axon perfused with a potassium glutamate solution containing zero Ca (open symbols) and one containing 1 mM Ca (filled symbols). The lines in all figures except 4 and 6 are merely for visual reference.

**Figure 2**. Sodium current-voltage relation in an axon perfused with a potassium fluoride solution containing zero Zn (filled circles), a solution with 1 mM Zn (filled squares), and again with zero Zn (open circles).
The time required for $I_{Na}$ to reach its peak value ($t_p$) at $V$ near zero, was used as a measure of the kinetics of the turn-on of $I_{Na}$, and as seen in Table II, $t_p$ is also unaffected by internal Ca.

Potassium current parameters were also little affected by internal Ca. Table III summarizes the results on potassium current parameters of perfusing with various calcium concentrations. The maximum slope conductance, $g_K$, as defined in Methods, was not appreciably changed by calcium concentrations even as high as 20 mM. The time to one-half of the steady-state $I_K$ ($t_K$) near $V = V_{Na}$ was used as a measure of potassium channel kinetics and the table shows that $t_K$ was not influenced by internal calcium.

**Internal Zinc Depolarizes the Resting Axon**

Since ZnF$_2$ is soluble, zinc may be applied internally with the preferable fluoride anion. A 1 mM zinc concentration applied internally to an axon caused a reduction of the resting potential of 20–30 mV. In the continued presence of 1 mM zinc, a reduction of the external Na concentration from 440 to 44 mM restored 16 mV of the resting potential, (four experiments on
TABLE III
POTASSIUM CURRENT PARAMETERS, Ca++ PERFUSION

<table>
<thead>
<tr>
<th>Axon</th>
<th>Internal anion</th>
<th>[Ca]</th>
<th>Change in maximum $g_K^*$</th>
<th>Recovery of $g_K^*$</th>
<th>Change in $t_K$</th>
<th>Recovery of $t_K$</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-15</td>
<td>Is^-</td>
<td>1</td>
<td>1.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-17</td>
<td>Is^-</td>
<td>1</td>
<td>1.3</td>
<td></td>
<td>1.14</td>
<td></td>
</tr>
<tr>
<td>C-25</td>
<td>Cl^-</td>
<td>1</td>
<td>1.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-28</td>
<td>Glut^-</td>
<td>1</td>
<td>1.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-29</td>
<td>Glut^-</td>
<td>1§</td>
<td>0.9</td>
<td>1.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-45</td>
<td>Glut^-</td>
<td>5</td>
<td>0.78</td>
<td>0.96</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>C-46</td>
<td>Glut^-</td>
<td>5</td>
<td>0.83</td>
<td>0.83</td>
<td>1.04</td>
<td>1.1</td>
</tr>
<tr>
<td>C-63</td>
<td>Glut^-</td>
<td>20</td>
<td>1.0</td>
<td>0.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-65</td>
<td>Glut^-</td>
<td>20</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean±SEM

<table>
<thead>
<tr>
<th>[Ca]</th>
<th>Change in maximum $g_K^*$</th>
<th>Recovery of $g_K^*$</th>
<th>Change in $t_K$</th>
<th>Recovery of $t_K$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.12±0.07</td>
<td>1.12±0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.81±0.03</td>
<td>0.91±0.08</td>
<td>1.12±0.08</td>
<td>1.1</td>
</tr>
<tr>
<td>20</td>
<td>1.0</td>
<td>0.93±0.07</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Relative to initial, zero Ca++ value.
‡ Relative to initial, zero Ca++ value; measured at $V$ near $V_Na$.
§ Order of perfusion reversed (see text).

two axons). In the absence of Zn, a similar reduction of external sodium produced only a 3-mV average hyperpolarization. In one experiment $3 \times 10^{-7}$ M tetrodotoxin (TTX) added to the external bathing medium produced a 20-mV restoration of the resting potential of a zinc-poisoned axon. In the absence of zinc only a 4–5-mV hyperpolarization is seen upon application of TTX. These results suggest that zinc causes an increase in the ratio of resting sodium conductance to potassium conductance. There was a rather variable increase in the resting conductance of three axons where this was measured, suggesting that both potassium and sodium resting conductances increased with a larger increase in sodium conductance.

Internal Zinc Depresses Sodium Current

The effect of 1 mM Zn on the peak $I_{Na}$ was a reduction of approximately 40%, as shown in Fig. 2. There was no change in $E_{Na}$ and only a slight (about 5-mV) shift along the voltage axis of the position of the peak $I_{Na}$. Even though a series resistance compensation of 3 $\Omega$-cm² was used in this experiment, the shift seen could be due to a residual uncompensated series resistance. The shift is in the opposite direction expected if zinc were screening internal negative fixed charges. The time to reach peak $I_{Na}$ ($t_p$) is shown in Fig. 3 and it is apparent that at most membrane potentials Zn caused a slight increase in $t_p$, which could only partially be explained by a simple shift along the voltage axis similar to $I_{Na}$ above. The effects of Zn on $t_p$ at zero membrane potential
Figure 3. Time to peak $I_{Na}$ ($t_p$) versus membrane potential for a squid axon perfused without (solid circles), with (open squares), and again without (open circles) 1 mM $[Zn]_i$.

Table IV
SODIUM CURRENT PARAMETERS, Zn$^{++}$ PERFUSION

<table>
<thead>
<tr>
<th>Axon</th>
<th>$[Zn]$</th>
<th>Reduction of maximum inward $I_{Na}^*$</th>
<th>Recovery of $I_{Na}^*$</th>
<th>Change in $t_p$</th>
<th>Recovery of $t_p$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-34</td>
<td>1</td>
<td>0.67</td>
<td>0.93</td>
<td>1.25</td>
<td>1.0</td>
</tr>
<tr>
<td>C-38</td>
<td>1</td>
<td>0.55</td>
<td>0.63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-42</td>
<td>1</td>
<td>0.57</td>
<td>0.75</td>
<td>1.38</td>
<td>1.26</td>
</tr>
<tr>
<td>C-51</td>
<td>1</td>
<td>0.56</td>
<td>0.91</td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td>C-58§</td>
<td>1</td>
<td>0.68</td>
<td>0.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean±SEM</td>
<td></td>
<td>0.61±0.03</td>
<td>0.81±0.07</td>
<td>1.2±0.08</td>
<td>1.09±0.09</td>
</tr>
<tr>
<td>C-33</td>
<td>10</td>
<td>0.07</td>
<td>1.0</td>
<td>2.0</td>
<td>1.05</td>
</tr>
</tbody>
</table>

* Relative to initial, zero Zn$^{++}$ value.
† Relative to initial, zero Zn$^{++}$ value; measured at $V = 0$.  
§ Internal anion was glutamate.

and maximum peak $I_{Na}$ are tabulated in Table IV. This table also shows that 10 mM Zn reduced $I_{Na}$ by more than 90%. The time constant of sodium inactivation, $\tau_n$, was unchanged in the two experiments in which it was measured.
Internal Zinc Slows Potassium Channel Kinetics

Fig. 4 shows families of current curves for a TTX-treated axon before, during, and after perfusion with 1 mM zinc. This concentration of zinc produced a dramatic slowing of the rise of $I_K$. The time scale for the zinc traces is compressed 10 times relative to the other traces. Even at the compressed time scale, $I_K$ does not reach a steady value at the end of a 100-ms depolarization. Under these circumstances, $t_K$, as defined above cannot really be measured. However, some quantitative measure of the degree of slowing of the potassium channel kinetics is needed. We therefore measured the time required for $I_K$ to reach half of its value at the end of the longest test pulse used, and we call this quantity $t_K'$ to distinguish it from $t_K$. Clearly, $t_K'$ is an underestimate of $t_K$. Fig. 5 is a plot of $t_K$ (in the absence of zinc) and $t_K'$ as functions of $V$. Both $t_K$ and $t_K'$ decrease as $V$ is made more positive, and at any value of $V$, $t_K'$ is a factor of 4 greater than $t_K$. Changes in $t_K$ ($t_K'/t_K$) at $V$ near $V_{Na}$ (that is, near $V = 50$ mV) are summarized in Table V for a number of experiments like the one shown in Figs. 4 and 5.

In attempting to understand the mechanism of action of zinc, we tested
**TABLE V**

POTASSIUM KINETICS, Zn$^{++}$ PERFUSION

<table>
<thead>
<tr>
<th>Axon</th>
<th>[Zn]</th>
<th>Change in $I_K$</th>
<th>Recovery of $I_K$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-37</td>
<td>1</td>
<td>4.4</td>
<td>0.82</td>
</tr>
<tr>
<td>C-41</td>
<td>1</td>
<td>4.0</td>
<td>1.13</td>
</tr>
<tr>
<td>C-54</td>
<td>1</td>
<td>3.3</td>
<td>1.1</td>
</tr>
<tr>
<td>C-58</td>
<td>1</td>
<td>1.5†</td>
<td></td>
</tr>
</tbody>
</table>

Mean±SEM    3.3±0.64     1.02±0.1

* Relative to initial zero Zn$^{++}$ value; measured at $V$ near $V_{Na}$ without TTX or near $V = 45$ mV with TTX.
† Glutamate was the internal anion.

The effect of hyperpolarizing pulses applied before the test pulse on the kinetics in the presence of zinc. It was found that a large hyperpolarizing prepulse increased the rate of rise of $I_K$ significantly, as illustrated in Fig. 6, but by no means restored the rate to its value in the absence of zinc. Part a of the figure shows the effect of a prepulse in the absence of zinc. The main...
FIGURE 6. Upper trace shows potassium current in the absence of internal Zn without (solid lines) and with (broken lines) a -100-mV prepulse, shown in the bottom trace. The middle trace shows the effects of the prepulse in the presence of zinc.

The effect is to prolong the foot of the rising phase of $I_K$ as described first by Cole and Moore (1960). In the presence of zinc the effect is the opposite: $I_K$ rises more rapidly after a prepulse.

**Effects of Other Divalent Cations**

Three experiments with Co, one with Ni and one with Cd show that these cations are very similar to Zn in their effects. Table VI summarizes the effects on $I_{Na}$ and on potassium kinetics. The increase of $t_K$ is almost as striking as the increase of $t_{Na}$.

<table>
<thead>
<tr>
<th>Axon</th>
<th>Cation</th>
<th>[cation]</th>
<th>Change in maximum inward $I_{Na}$*</th>
<th>Recovery of $I_{Na}$*</th>
<th>Change in $t_{K}$†</th>
<th>Recovery of $t_{K}$†</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-35</td>
<td>Co</td>
<td>2</td>
<td>0.56</td>
<td>0.51</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.39</td>
<td></td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>C-52</td>
<td>Co§</td>
<td>1</td>
<td>0.66</td>
<td>0.76</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.53</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-33</td>
<td>Co</td>
<td>10</td>
<td>0.48</td>
<td>0.61</td>
<td>1.7</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>Ni</td>
<td>5</td>
<td>0.73</td>
<td>0.46</td>
<td>6.8</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>Cd</td>
<td>1</td>
<td>0.23</td>
<td>0.32</td>
<td>2.2</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* Relative to initial, zero concentration value.
† Relative to initial zero concentration value; measured at $V$ near $V_{Na}$.
§ TEA (15 mM) used, so no data is available on $t_{K}$ for this axon.
here as it is with Zn, but the effects on $I_{Na}$ are more variable. Recovery from exposure to these cations is not as complete as that from zinc.

**DISCUSSION**

We have shown that axoplasmic concentrations of Ca up to 10 mM have little effect on the ionic currents of voltage clamped squid axons. This is reasonably consistent with the finding of Tasaki et al. (1967) that [Ca], as large as 5 mM in KCl solutions (see Table I for a comparison of free calcium in glutamate, isethionate, and chloride solutions) cause a small reversible decrease in the height of the action potential of squid giant axons. The reduction in spike height could be a result of a slight decrease in $I_{Na}$ and a slight increase in $I_K$ (see Table III) and $I_L$. Tasaki et al. (1967) found approximately a two-fold increase in resting conductance, which may reflect an increased $I_L$.

The lack of effect of internal calcium in the present experiments shows that an increased axoplasmic calcium concentration produced by a Ca influx during an action potential cannot activate the potassium conductance of squid giant axons as has been suggested for cat motoneurons by Krnjević and Lisiewicz (1972).

The presence of low concentrations of Zn (or Ni, Cd, Co) in the perfusion fluid substantially slowed the potassium channel kinetics. In contrast to the many drugs and toxins that reduce the magnitude of the ionic currents, very few agents applied internally or externally alter the kinetics of the ionic currents. Local anesthetics (Narahashi, 1971) and decreased internal pH (Ehrenstein and Fishman, 1971) increase the time to peak $I_{Na}$ of squid axons, but to a considerably lesser degree than the three-fold slowing of $I_K$ observed here. Experiments of Dodge (1961) on voltage clamped node of Ranvier and Blaustein and Goldman (1968) on voltage clamped lobster axons showed that the presence of Ni in the external medium produced slowing of $I_K$.

Zinc (and the other divalent cations except Ca) affect the kinetics of the ionic currents and since the kinetics are related to the gating properties of the axon, the present results suggest that Zn alters the gating structure of the ionic channels, with a preference for potassium channels occurring at low Zn concentrations. We would therefore expect that zinc would alter the gating currents and indeed the experiments of Armstrong and Bezanilla (1973) showed that zinc (10 mM) blocked the sodium gating current.

There are two general mechanisms by which divalent cations may alter the ionic current properties: (a) The cation may interact electrostatically with any charge present on the membrane surface, thereby altering the potential within the membrane and thus altering the potential-dependent ionic current properties. (b) The divalent cation may react chemically with the membrane constituents (for example proteins or phospholipids) responsible for the ionic currents.
A significant contribution of the first mechanism is unlikely since we observe only very small shifts of the voltage-dependent parameters of the sodium channel. The shifts of both sodium and potassium parameters are in the opposite direction expected if the divalent cations were screening internal negative fixed charges as discussed by Chandler et al. (1965). These shifts are, however, in the direction of a small uncompensated series resistance. Also, if this were the mechanism, then Ca would be expected to be as effective as Zn. This is clearly not the case; a concentration of Ca (20 mM) 40 times the lowest effective Zn concentration (0.5 mM) had negligible effects. The second of the above possibilities is therefore the likely mechanism, with the added burden of explaining why calcium is unique among the divalent cations tested (of course, Ca is the only one whose fluoride salt is insoluble).

A possible way in which calcium may differ from these other divalent cations is the binding affinities to membrane phospholipids and proteins. Blaustein (1967) has shown that zinc binds 2.2 times more strongly to phosphatidyl serine than does calcium. Therefore if the divalent cations act by binding to phospholipids, a calcium concentration two to three times that of zinc should produce equivalent effect. We found that a calcium concentration 40 times that of zinc was insufficient to produce any effects on \( t_K \) which approached those of zinc. Papahadjopoulos (1968) found no appreciable interaction of divalent cations with phospholipids except for the acidic phospholipids phosphatidyl serine, phosphatidyl inositol, and phosphatidic acid, all of which interacted with the divalent cations, Ca, Mg, and Ba to about the same extent. On the other hand, Gurd and Wilcox (1956) have shown that calcium binds much less strongly to proteins than the other divalent cations used in this study. The comparative binding affinities to acetate, imidazole, ammonia, and sulfhydryl groups may be used as measures of binding to acidic, histidine, basic, and cysteine residues of proteins, respectively. Table VII lists the divalent cation-binding affinities of the model compounds relative to calcium which has been standardized at unity. If the action of the divalent cations is a result of their binding to membrane proteins, the data of Table VII show that a more than 40-fold greater concentration of calcium would be necessary to duplicate the effects of the other divalent cations, as we observed. Therefore we conclude that the action of

<table>
<thead>
<tr>
<th>Cation</th>
<th>Acetate</th>
<th>Imidazole</th>
<th>NH₃</th>
<th>-SH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Cd</td>
<td>6.3</td>
<td>490</td>
<td>280</td>
<td>( \sim 6.3 \times 10^7 )</td>
</tr>
<tr>
<td>Zn</td>
<td>3</td>
<td>234</td>
<td>148</td>
<td>( \sim 6.3 \times 10^6 )</td>
</tr>
<tr>
<td>Ni</td>
<td>–</td>
<td>1170</td>
<td>600</td>
<td>–</td>
</tr>
<tr>
<td>Co</td>
<td>–</td>
<td>–</td>
<td>79</td>
<td>( \sim 6.3 \times 10^4 )</td>
</tr>
</tbody>
</table>
zinc, cobalt, cadmium, and nickel may be due to their binding to membrane proteins responsible for control of ionic currents, and that the potassium gating proteins are more susceptible than sodium gates to reaction with these cations.

We thank Dr. Francisco Bezanilla and Dr. Clay Armstrong for encouragement, for many valuable discussions and for space in their laboratory at the Marine Biological Laboratory, Woods Hole, Massachusetts. We are indebted to Dr. Lorin Mullins and Dr. Donald Geduldig for many helpful suggestions during preparation of the manuscript and to Joann Chambers and Linda Purdham for invaluable secretarial assistance.

This research was supported in part by grants from the National Institutes of Health (AM01004-09 and NS-05846) and from the National Science Foundation (GB-24585).

Received for publication 28 January 1974.

REFERENCES


BEGENISCH AND LYNCH  Divalent Cations in Squid Axons


