Zn\textsuperscript{2+} blocks the voltage activated calcium current of \textit{Aplysia} neurons

D. Büsselberg\textsuperscript{1,2} M.L. Evans\textsuperscript{1}, H. Rahmann\textsuperscript{2} and D.O. Carpenter\textsuperscript{1}

\textsuperscript{1}Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY 12209-0509 (U.S.A.), School of Public Health, State University of New York at Albany, Albany, NY 12227 (U.S.A.) and \textsuperscript{2}Universität Stuttgart-Hohenheim, Institut für Zoologie, Stuttgart (F.R.G.)

(Received 4 January 1990; Revised version received 2 April 1990; Accepted 21 May 1990)

Key words: Zinc; Calcium current; Voltage clamp; Fixed charge site

We have investigated the effect of Zn\textsuperscript{2+} on voltage-activated calcium currents of \textit{Aplysia} neurons, using conventional two-electrode voltage-clamp techniques. The peak of these currents was reversibly reduced by Zn\textsuperscript{2+} (50\% reduction at 3.75 mM; total block at 20 mM), while the current-voltage relation and the activation and inactivation curves were shifted to depolarized voltages. The effects of Zn\textsuperscript{2+} were concentration-dependent. The Hill coefficient was 1.62. The high concentrations required, the shift of the current-voltage relation and the effects on activation and inactivation are best explained by a charge-screening effect combined with a specific binding site for Zn\textsuperscript{2+} near the entrance of the channel.

Zinc ions have a variety of actions on nerve cells. Yokoyama et al. [20] reported that even small amounts of this heavy metal cation are extremely toxic to neurons. On the other hand there are naturally high concentrations of zinc attached to membranes, especially in the nervous system [2], while at some sites including hippocampus and piriform cortex there are high concentrations of Zn\textsuperscript{2+} in presynaptic terminals, together with evidence that Zn\textsuperscript{2+} is released along with transmitter substances [1, 18]. Zn\textsuperscript{2+} has been shown to modulate transmitter release [15, 19] and block channels activated by N-methyl-D-aspartate [11, 16]. In addition Gilly and Armstrong [7, 8] reported that Zn\textsuperscript{2+} alters the kinetics of sodium and potassium currents.

Numerous studies have shown that divalent cations such as Co\textsuperscript{2+} and Cd\textsuperscript{2+} are potent antagonists of voltage-dependent calcium currents on a variety of nerve cells [see 9]. There has, however, been little study of the effect of Zn\textsuperscript{2+} despite the fact that, unlike several other divalents, Zn\textsuperscript{2+} has a physiologic function in the nervous system [1, 19].

Abdominal ganglia of \textit{Aplysia californica} (120–200 g) were removed and pinned to a Sylgard-coated chamber in artificial seawater (ASW). The capsule was then
opened, the connective tissue sheath removed and the neurons exposed. The cells were washed with ASW for at least 30 min before penetration. All solutions were applied via a rapid perfusion system that results in complete change of the solution surrounding the cell under investigation within 1 s [17].

Neurons were impaled with two independent microelectrodes (4–8 MΩ). In order to record voltage-activated calcium currents the ASW was replaced with a solution of the following composition (in mM): MgCl₂ 45, KCl 10, CaCl₂ 20, tetraethylammonium bromide 200 [21], 4-aminopyridine 1 [10], Tris-(hydroxymethyl)amino-methane-hydrochloride (Tris) 234, and Tris base 59 mM. Final pH was adjusted to 7.6.

The voltage electrode was filled with 3 M potassium acetate and the current electrode was filled with 4 M cesium chloride (Aldrich, 99.9995% purity) to block the outward potassium currents. Zinc (II) chloride (Aldrich, 99.999% purity) was dissolved directly in the seawater replacement solution just before each experiment to avoid precipitation of zinc salts. No precipitation was observed with Zn²⁺ concentrations of 40 mM or less.

Neurons were routinely clamped at −40 mV, which is close to the resting membrane potential. The standard voltage step was to +20 mV for 70 ms. All currents were recorded on tape via a digitizing unit and analyzed using a P/3 protocol [5] for leakage subtraction. This procedure avoids errors due to activation of chloride currents by the hyperpolarization [6].

![Figure 1](image-url)

Fig. 1. A: the effect of different concentrations of Zn²⁺ on calcium current amplitude. The records are superimposed for calcium currents produced by 70 ms command steps to +20 mV from a holding potential of −40 mV. The control record was taken immediately before change of the solution, and the other records after 5- to 10-min application of the different concentrations. Note that the calcium current peaked much later during application of Zn²⁺. B: dose–response relationship for the inhibitory effect of Zn²⁺. Each point is the mean (±S.E.M.) of several determinations of the reduction by Zn²⁺ of peak calcium current evoked by a step from −40 to +20 mV. The number of determinations for each point: 0.25 mM, 11; 0.5 mM, 12; 1 mM, 9; 2.5 mM, 9; 5 mM, 10; 10 mM, 8; and 20 mM, 6. The continuous line is the computer-generated fit to the function \( I = \frac{1}{1 + (K/A)^n} \) where \( I \) is the % inhibition at a given dose \( A \), \( K \) is the apparent dissociation constant, and \( n \) is the Hill coefficient. \( n = 1.62; K = 3.75 \text{ mM} \).
Voltage jumps were not applied at intervals more frequent than 20 s, and under these circumstances the induced currents were of constant amplitude. The peak amplitude of the voltage-dependent calcium current was reduced in a dose-dependent fashion when the preparation was perfused with ASW to which zinc chloride (0.25–20 mM) was added. Fig. 1A shows currents recorded in different concentrations of Zn\(^{2+}\). Note that the time to peak current became greater with increasing Zn\(^{2+}\) concentrations. Without Zn\(^{2+}\) the current peaked at 3–5 ms, but in 20 mM Zn\(^{2+}\) the peak occurred after more than 20 ms. Fig. 1B shows the dose–response relation obtained from 65 applications of Zn\(^{2+}\) to more than 30 different neurons. The threshold concentration for effects of Zn\(^{2+}\) was about 0.25 mM, while 5 mM gave about 58% reduction of peak current. Near complete (>80%) inhibition was observed at Zn\(^{2+}\) concentrations greater than 10 mM. The calculated Hill coefficient was 1.62. These studies were done on a variety of identified (RB, R2, R15, L2-6) and unidentified neurons of the abdominal ganglion, and no differences were found in either the characteristics of the voltage-dependent calcium current or the effects of Zn\(^{2+}\) among these cells.

Fig. 2 shows the time course of Zn\(^{2+}\) blockade and reversal upon perfusion of control ASW. The calcium current reduction was already apparent with the first voltage jump applied 20 s after initiation of the perfusion of Zn\(^{2+}\). It increased to reach a steady-state within 5–10 min, and recovered to within 10% of control in all but 2 of 65 experiments with an approximately similar time course. The time required to reach steady state and recovery (especially recovery) was greater with higher concentrations of Zn\(^{2+}\). With 20–40 mM Zn\(^{2+}\) complete recovery sometimes required 30–45 min.

The effects of Zn\(^{2+}\) on calcium currents might be direct channel blockade or rather

![Figure 2](image)

**Fig. 2.** Time course of reduction of the peak calcium current during application of 5 mM Zn\(^{2+}\). The current is clearly reduced even at the first time point in presence of Zn\(^{2+}\). Within several minutes a steady state is reached. Upon wash the current came back to control.
a consequence of an alteration of the kinetics of the response. Fig. 3A shows the effects of three concentrations of Zn$^{2+}$ (1, 2.5 and 5 mM for 10 min) on the current-voltage relation. In the control the largest current was elicited by a jump to +20 mV. With jumps to less positive potentials the current amplitude was smaller, reflecting the voltage-dependent activation of the calcium current, while with jumps to more positive values the current was reduced as a result of approaching the equilibrium potential for calcium. In the presence of Zn$^{2+}$ the voltage at which the maximal current was generated shifted in a depolarizing direction in a dose-dependent fashion. In 5 mM Zn$^{2+}$ the peak current was elicited at a potential about 15 mV more depolarized than control.

Fig. 3B illustrates the voltage dependence of activation and inactivation in a con-
trol ASW containing half the normal Ca$^{2+}$ concentration and in the presence of 5 mM Zn$^{2+}$ and half normal Ca$^{2+}$ ASW. The lower Ca$^{2+}$ concentration was used in this experiment to maximize the effect of Zn$^{2+}$ on the kinetics of the response. Both activation and inactivation curves were shifted in depolarizing directions in the presence of Zn$^{2+}$.

The concentration of Zn$^{2+}$ required for effect, the rapid onset and washout, and the alteration of the current-voltage relation and of activation and inactivation strongly suggest a binding site external to the calcium channel. There are two possible mechanisms by which this might occur: a general binding of cations to negative charges of the surface (charge screening) or a specific binding to (a) site(s) external to the channel. General charge screening should be not specific for a single channel type, while a more specific site external to a channel might be more selective. The high concentration of Zn$^{2+}$ used in this study, which probably does not occur physiologically, makes a general charge screening effect most likely. The increase of time to the peak of the current, the shift of the current-voltage relation and of activation and inactivation to depolarized voltages would all be expected as a result of charge screening. This has been demonstrated to occur when Ca$^{2+}$ concentration is increased [4, 12, 13]. However, when Ca$^{2+}$ concentration is increased the current flowing through Ca$^{2+}$ channels is increased, in addition to the changes induced in the kinetics of the response. With Zn$^{2+}$ the kinetics change, but the current decreases. It is, however, difficult to explain the degree of channel blockade solely on the basis of charge screening, which suggests the possibility of an additional binding site at the entrance of the channel which when occupied blocks calcium entry.

Zn$^{2+}$ has been previously shown to alter movement of ions through other channels. Gilly and Armstrong [7, 8] showed that Zn$^{2+}$ changed the opening and closing kinetics of sodium and potassium channels, probably by binding to a specific site external to these channels. In their experiments [7] Zn$^{2+}$ slowed the rate of opening of the voltage-dependent sodium channel in squid axon without changing the closing properties. They also showed [8] that Zn$^{2+}$ and Hg$^{2+}$ slowed the opening of the potassium channel and accelerated its closing at higher doses. The authors concluded that Zn$^{2+}$ binds to specific negatively charged sites associated with the opening and closing apparatus. The distribution of these binding sites is different at different channels, and this may explain why not all channels are affected in the same way. A somewhat similar alteration of kinetics by Zn$^{2+}$ was described by Mayer and Sugiyama [14] at the ‘A’-channel, which conducts the transients potassium current in sensory rat neurons.

We cannot exclude the possibility that Zn$^{2+}$ has a direct channel-blocking action, competing with Ca$^{2+}$ for a binding site within the channel. However, any actions of Zn$^{2+}$ within the channel cannot be very strong. Otherwise we would need much lower concentrations of Zn$^{2+}$ to block the channel, as previously shown to be the case of Pb$^{2+}$ blockade of calcium currents in this preparation [3]. The changes in the current-voltage relation and in the kinetics of activation and inactivation indicate effects at fixed charge sites, while the blockage of Ca$^{2+}$ currents by Zn$^{2+}$ can be satisfactorily explained without invoking an action within the calcium channel.
This research was supported by NIH Grant ES05203 to D.O.C.