DIVALENT CATION MODULATION OF A-TYPE POTASSIUM CHANNELS IN ACUTELY DISSOCIATED CENTRAL NEURONS FROM WIDE-TYPE AND MUTANT DROSOPHILA

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Drosophila mutants provide an ideal model to study channel-type specificity of ion channel regulation in situ. In this study, the effects of divalent cations on voltage-gated K\(^+\) currents were investigated in acutely dissociated central neurons of Drosophila third instar larvae.

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using the whole-cell patch-clamp recording. Our data showed that micromolar Cd$^{2+}$ enhanced the peak inactivating current ($I_A$) without affecting the delayed component ($I_K$). The same results were obtained in Ca$^{2+}$-free external solution, and from $slo^1$ mutation, which eliminates transient Ca$^{2+}$-activated K$^+$ current. Micromolar Cd$^{2+}$ and Zn$^{2+}$, and millimolar Ca$^{2+}$ and Mg$^{2+}$ all shifted the steady-state inactivation curve of $I_A$ without affecting the voltage-dependence of $I_A$ activation, whereas millimolar Cd$^{2+}$ markedly affected both the activation and steady-state inactivation curves for $I_A$. Divalent cations affected $I_A$ with different potency; the sequence was: Zn$^{2+} >$ Cd$^{2+} >$ Ca$^{2+} >$ Mg$^{2+}$. The modulation of $I_A$ by Cd$^{2+}$ was partially inhibited in ShM, a null Shaker (one of $I_A$-encoding genes) mutation. Taken together, the channel-type specificity, the asymmetric effects on $I_A$ activation and inactivation kinetics, and the diverse potency of divalent cations all strongly support the idea that physiological divalent cations modulate A-type K$^+$ channels through specific binding to extracellular sites of the channels.

**Keywords:** $I_A$; A-type K$^+$ channels; Cd$^{2+}$; Zn$^{2+}$; Ca$^{2+}$; and Mg$^{2+}$ ions; $slo^1$, Sh$^M$ mutations; larval neurons; whole-cell patch-clamp

**INTRODUCTION**

Divalent metal ions have long been studied for their effects on a variety of ion channels because of their physiological importance. For example, divalent cations are known to affect the gating properties of K$^+$ channels. In particular, divalent cations are important modulators of the transient K$^+$ in ventricular myocytes (Agus et al., 1991; Stengl et al., 1998), müller cells (Bringmann et al., 1999), neurons (Mayer & Sugiyama, 1988; Harrison et al., 1993; Huang et al., 1993; Talukder & Harrison, 1995; Easaw et al., 1999), as well as in other tissues (Davidson & Kehl, 1995). Delayed rectifier K$^+$ channels and ATP-dependent K$^+$ channels are also modulated by divalent cations (Spires & Begenisich, 1992; Kwok & Kass, 1993). Plausible mechanisms for such modulation depend on specific binding sites on channel proteins, or alternatively, on the screening of bulk negative charge on the membrane surface. Early studies showed that divalent cations change the voltage dependence of activation and inactivation of Na$^+$ and Ca$^{2+}$ channels, presumably by altering the membrane surface charge (Frankenhaeuser & Hodgkin, 1957; Hille et al., 1975; Hahin & Campbell, 1983; Kass & Krafte, 1987). However, this hypothesis was contradicted by the findings that divalent cations directly modulate some channel proteins, especially transient K$^+$ channels (Agus et al., 1991; Huang et al., 1993; Talukder & Harrison, 1995;
Stengl et al., 1998), which supports the existence of a discrete binding site theory on channel protein. Further support to binding site theory comes from site-directed mutation studies of \( \gamma \)-aminobutyric acid A receptors (Wang et al., 1995; Wooltorton et al., 1997), ATP-gated channels (Nakazawa & Ohno, 1997), and nicotinic acetylcholine receptors (Eddins et al., 2002).

Most studies addressing specific modulation of ion channels use in vitro neuronal cultures or expression systems such as cell lines or *Xenopus* oocytes, in which the properties of channel proteins may change due to the different cellular and/or membranous environment of culture system (Oliver et al., 2004). *Drosophila* mutants provide an ideal model to study channel-type specificity of ion channel regulation in situ. In *Drosophila*, at least four outward components underlie the whole-cell K\(^+\) currents (Broadie & Bate, 1993): the fast voltage-gated (\( I_A \)) and calcium-dependent (\( I_{CF} \)) currents; the delayed voltage-gated (\( I_K \)) and the calcium-dependent (\( I_{CS} \)) currents. A further study suggests that \( I_K \) also consists of two components: \( I_{KS} \) and \( I_{KF} \) (Singh & Singh, 1999). At least four genes are known to encode voltage-gated K\(^+\) channels in *Drosophila*. They are *Shal* and *Shaker* (\( Sh \)), encoding \( I_A \), and *Shab* and *Shaw*, encoding \( I_K \). In addition, the *slowpoke* (\( slo \)) was identified to encode fast \( I_{CF} \) (Atkinson et al., 1991).

Recently, we adopted an acute dissociation approach that makes the small CNS neurons in *Drosophila* more easily accessible for electrophysiological assays (Wu et al., 2001). Compared with the well-used culture cells (Wu et al., 1983), this approach allows us to study ion channel regulation in a systematical way, i.e., studying channel regulation with numerous *Drosophila* mutants under physiological conditions. In this report, we examined the effects of divalent cations on \( I_A \) in *Drosophila* central neurons in the presence of divalent cations (2.5 mM Ca\(^{2+}\) and 5 mM Mg\(^{2+}\)), which minimize surface charge effects. Our results suggest that divalent cations modulate the gating of *Drosophila* K\(^{+}\) channels in a channel type-dependent way by binding to specific site(s) on the channels.

**MATERIALS AND METHODS**

**Drosophila Stocks**

Stocks of *Drosophila melanogaster* were maintained on cornmeal-yeast dextrose media at 21–23°C and ~60% humidity with 12 hr dark/light cycles. The Canton S and Oregon R strains were used as wild types (WT). The mutant *slo* \(^ I \) induced by ethyl methanesulfonate becomes paralyzed when exposed to 38°C (Wu et al., 1978). The mutant *Sh* \(^ M \),
a null mutant, was isolated in an X-ray-irradiation screen and is associated with an insertion containing a 2.2 kb DNA and eliminates all Sh gene products (Kamb et al., 1987). ShM exhibits vigorous leg-shaking under ether anesthesia (Kaplan & Trout, 1969). All the strains were obtained from the Department of Biological Sciences, University of Iowa, IA.

Preparation-Containing Solutions

The Ca$^{2+}$- and Mg$^{2+}$-free dissection solution contained (mg/100 ml): 800 NaCl, 20 KCl, 5 NaH$_2$PO$_4$, 100 NaHCO$_3$, 100 glucose. The pH was adjusted to 7.25 with 1 mol/L HCl. The composition of the patch pipette solution was (mM): 130 KCl, 4 MgCl$_2$, 10 EGTA, 10 HEPES, 1 CaCl$_2$ and 2 ATP. Recording solution contained (mM): 130 NaCl, 5 KCl, 2.5 CaCl$_2$, 5 MgCl$_2$, 10 HEPES, 10 glucose. In 10 mM and 20 mM Ca$^{2+}$ or 20 mM Mg$^{2+}$ testing solutions, 15 and 30 mM NaCl were omitted to keep the optimal osmolarity, respectively. In 0 mM Ca$^{2+}$, Mg$^{2+}$ solution, osmolarity was adjusted with sucrose. Other concentrations of divalent cation solutions were obtained with a direct addition or deletion of the related cations. pH of all the recording solutions was adjusted to 7.25 with Tris. All chemicals were from Sigma.

Acute Dissociation of Drosophila CNS Neurons

Cell preparation was achieved by a mechanical dissociation method (Wu et al., 2001). Several third instar larvae were collected on a slide. After rinsing three times with distilled water, the brains were dissected with a pair of sharp needles in the Ca$^{2+}$- and Mg$^{2+}$-free dissection solution. The tissue were then rinsed by the recording solution twice and transferred into a culture dish (NUNC™ Brand Products, Denmark) containing 3 ml recording solution. A U-shape grid made of metal filament and nylon fibers was used to mount the Drosophila brains onto the bottom of the culture dish. A vibrator with a dissecting pipette was fixed on a manipulator and the dish with mounted tissues was laid on the stage of the dissecting microscope (XTL-II, Tech Instrument Co. Ltd, Beijing, China). The dissecting pipette (about 6 μm in diameter) was lightly placed on the surface of the Drosophila brains with a manipulator. A continuous (5 Hz) electric square pulse was applied to the vibrator using a pulse generator (EE 1641B, Nanjing, China), producing a series of fast horizontal movement of steps of about 100 μm of the dissecting glass pipette. The
acutely isolated neurons attached to the bottom of the culture dish within ten min. All drugs were applied via the “Y-tube” method throughout the experiment. The tip of the drug delivery tube was about 50–100 μm away from the patched neurons, thus allowing a complete exchange of external solution surrounding a neuron within 20 ms (Wu et al., 2001).

Data Acquisition and Analysis

Whole-cell recordings were carried out under voltage-clamp conditions with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA) at room temperature (22–25°C). The small size of Drosophila CNS neurons enabled us to attain gigaohm seal (>1GΩ) with or without a gentle suction. Patch pipettes were pulled from glass capillaries (Narishige, Tokyo, Japan) on a two-stage puller (PP-830, Narishige, Tokyo, Japan). The resistance of the recording electrode filled with pipette solution was 7–9 MΩ. Data collection and analysis were performed with PCLAMP software (version 8.0.1, Axon Instruments, Foster City, CA). The currents were sampled at 5 kHz and filtered at 2 kHz (4-pole Bessel filter). Unless otherwise noted, the membrane potential was held at −70 mV in the voltage-clamp studies. The steady-state inactivation curves were fitted by a Boltzmann equation:

\[
I = \frac{I_{\text{Max}}}{1 + e^{\left(V_{\text{mid}} - V\right)/k}} + C
\]

\(I_{\text{Max}}\) is the maximal current, \(V_{\text{mid}}\) is the voltage at which the current is half-inactivated, and \(k\) is slope factor. The inactivation of \(I_A\) was fitted by two-exponential function, \(I = I_s + A_1 \exp(-t\tau_1) + A_2 \exp(-t\tau_2)\), in which, \(\tau_1\) and \(\tau_2\) are the fast and slow decay time constants for the two inactivating components \(A_1\) and \(A_2\), respectively. Data are expressed as means ± standard error (S.E.M). Significant testing was performed using \(t\)-tests or one-way ANOVA.

Separation of \(I_A\) and \(I_K\) Components

Under our experimental conditions, no Na\(^+\) currents were observed in standard external solution and with Cs\(^+\) in the pipette solution (Xu et al., 2002). To exclude the effects of divalent cations on Ca\(^{2+}\) currents and Ca\(^{2+}\)-activated K\(^+\) current, we only selected the neurons exhibiting more than 90% of peak current after applying Ca\(^{2+}\)-free external solution for analysis. Ca\(^{2+}\)-free solution was applied within 1.5 min after establishing the whole-cell configuration, and the less than 10% decrease of peak
current could be considered as the current run-down due to the small size of the patched neurons. Therefore, the outward whole-cell currents analyzed here were most likely uncontaminated voltage-gated K⁺ currents: IA and IK.

For isolating the early IA from the delayed IK component, we used a prepulse paradigm (Wu et al., 2001) (Fig. 1Aa and 1Ab). At a holding potential (V_H) of −70 mV, IA was inactivated by a conditioning prepulse of −40 mV, whereas IK was relatively unaffected. Subtraction of IK

Figure 1. Selective modulation of A-type K⁺ current by micromolar Cd²⁺ in Drosophila CNS neurons. A, Two components of the voltage-gated K⁺ currents, IA and IK were separated with a 200 ms prepulse of −40 mV, which only elicited the sustained component IK (b). By subtracting IK from peak currents (a), IA was isolated (a–b). The voltage protocol used for evoking current was shown in the inset. After hyperpolarizing to −90 mV for 200 ms, outward currents were elicited by 20 mV depolarizing steps for 500 ms, from −90 to 70 mV. Holding potential was −70 mV. B, Examples of IA recorded under control condition (Cont), and after (bottom) exposure to Cd²⁺ (200 μM). C, I-V curves of IA under control condition and in the presence of different concentration of Cd²⁺. Micromolar Cd²⁺ increased IA reversibly in a concentration-dependent manner. D, I-V curves of IK under control condition and in the presence of different concentration of Cd²⁺. IK was measured at the end of the 500 ms test pulse.
(Fig. 1A, b) from the currents evoked from a prepulse of −90 mV (Fig. 1A, a) resulted in $I_A$ (Fig. 1B, a–b).

**RESULTS**

Consistent with previous reports (Wu et al., 1983; Wu et al., 2001), acutely dissociated neurons from either wild-type (WT) or mutants ($Sh^M$ and $slo^I$) of *Drosophila* brains could be classified into three types according to their morphology and size: large (>8 μm) round neuroblast-like type I neurons; small (2–5 μm) type II neurons and intermediate (5–8 μm) type III neurons. Type II and type III neurons were the majority of *Drosophila* neurons found in culture and were selected for electrophysiological recording in the present study.

**Micromolar Extracellular Cd$^{2+}$ Selectively Modulates Wild-Type $I_A$ Depending on Concentration and Prepulse Potentials**

To minimize the surface charge effects, 2.5 mM Ca$^{2+}$ and 5 mM Mg$^{2+}$ were present in the recording medium. As shown in Fig. 1B, Cd$^{2+}$

<table>
<thead>
<tr>
<th>mM</th>
<th>Tp (ms)</th>
<th>$\tau_1$ (ms)</th>
<th>$V_{mid}$ (mV)</th>
<th>$I_A$ (%)</th>
<th>$I_K$ (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd$^{2+}$ 0.05</td>
<td>0.0 (0.0)</td>
<td>0.1 (0.0)</td>
<td>2.1 (0.5)</td>
<td>102.0 (2.1)</td>
<td>97.3 (1.7)</td>
<td>13</td>
</tr>
<tr>
<td>0.1</td>
<td>0.1 (0.0)</td>
<td>0.3 (0.0)</td>
<td>5.7 (0.8)*</td>
<td>110.7 (2.2)</td>
<td>108.0 (2.1)</td>
<td>13</td>
</tr>
<tr>
<td>0.2</td>
<td>0.2 (0.0)</td>
<td>0.8 (0.1)</td>
<td>10.9 (1.0)*</td>
<td>124.3 (2.4)*</td>
<td>109.7 (1.9)</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>1.9 (0.0)*</td>
<td>6.4 (0.6)**</td>
<td>42.9 (2.6)**</td>
<td>107.9 (4.0)</td>
<td>111.1 (1.8)</td>
<td>7</td>
</tr>
<tr>
<td>Zn$^{2+}$ 0.05</td>
<td>0.0 (0.0)</td>
<td>0.1 (0.0)</td>
<td>2.1 (0.4)</td>
<td>106.6 (2.3)</td>
<td>105.9 (1.3)</td>
<td>7</td>
</tr>
<tr>
<td>0.1</td>
<td>0.1 (0.0)</td>
<td>0.2 (0.0)</td>
<td>7.8 (0.7)*</td>
<td>118.1 (3.1)*</td>
<td>111.9 (1.8)</td>
<td>7</td>
</tr>
<tr>
<td>0.2</td>
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<td>0.9 (0.1)</td>
<td>13.0 (1.0)**</td>
<td>135.5 (3.2)**</td>
<td>119.7 (2.6)*</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>2.4 (0.0)*</td>
<td>7.8 (0.7)**</td>
<td>52.1 (3.2)**</td>
<td>98.3 (3.9)</td>
<td>141.0 (2.8)**</td>
<td>6</td>
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<tr>
<td>Ca$^{2+}$ 5</td>
<td>0.0 (0.0)</td>
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<td>2.7 (0.6)</td>
<td>109.7 (2.2)</td>
<td>102.7 (1.5)</td>
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<td>10</td>
<td>0.1 (0.0)</td>
<td>0.4 (0.0)</td>
<td>7.1 (0.8)*</td>
<td>124.1 (3.1)*</td>
<td>108.4 (2.1)</td>
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</tr>
<tr>
<td>20</td>
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<td>0.9 (0.1)</td>
<td>12.5 (1.5)*</td>
<td>137.2 (3.7)*</td>
<td>110.1 (2.1)</td>
<td>6</td>
</tr>
<tr>
<td>Mg$^{2+}$ 5</td>
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<td>−0.0 (0.0)</td>
<td>−0.0 (0.3)</td>
<td>103.2 (1.8)</td>
<td>98.9 (1.4)</td>
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<tr>
<td>10</td>
<td>0.1 (0.0)</td>
<td>0.3 (0.0)</td>
<td>2.9 (0.3)</td>
<td>111.6 (2.1)</td>
<td>103.3 (1.2)</td>
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<td>20</td>
<td>0.24 (0.0)</td>
<td>0.7 (0.1)</td>
<td>7.9 (0.8)*</td>
<td>120.5 (2.4)*</td>
<td>107.3 (1.8)</td>
<td>5</td>
</tr>
</tbody>
</table>

Values are given as means ± S.E.M calculated at 30 mV except for $V_{mid}$ indicates values of control condition subtracted from those of different divalent cations. $V_{mid}$, right shift of $V_{mid}$ of steady-state inactivation curves. $^*p < 0.05$, $^{**}p < 0.01$. % shows normalized $I_A$ amplitudes of different divalent cations to those of control. Numbers of patched neurons (n) are showed in the right column.
(200 μM) enhanced the amplitude of IA in WT neurons. Within micromolar range, the effect of Cd²⁺ on IA was concentration-dependent (Fig. 1C). At 30 mV, IA was increased to 124.3% by 200 μM Cd²⁺ (p < 0.05; Table I). The enhancement was reversible upon washout of Cd²⁺. IA activated and inactivated rapidly, whereas IK did not inactivate even after a prolonged depolarization as long as 500 ms (Wu et al., 2001). Unlike the effect on IA, Cd²⁺ had little effect on IK measured at the end of the 500 ms test pulse (Fig. 1D, Table I).

To understand the mechanisms of the selective effect of Cd²⁺ on IA, we performed experiment to study the voltage-dependence of IA activation. Cd²⁺ (200 μM) had little effect on the normalized activation curves, indicating that it did not affect the voltage-dependence of IA activation (Fig. 2A). Other kinetic properties of IA were also examined. Both the time-dependent activation (Tp, time to peak) and inactivation (fast-inactivation time constant, τ) of IA were voltage-dependent. At potentials between 10 mV and 70 mV in control solution, the Tp and τ were relatively constant, varying between 2.8 ms and 2.1 ms, and 9.5 ms and 7.9 ms, respectively. At hyperpolarized potentials (more negative than −10 mV), both Tp and τ increased rapidly, reaching 3.4 ± 0.4 ms and 13.6 ± 3.4 ms at −10 mV. Cd²⁺ (200 μM) did not significantly alter the voltage-dependence and the values of Tp and τ of IA (Fig. 2B, Fig. 2C and Table I).

A conditioning prepulse potential of −40 mV inactivated IA without affecting IK. However, in the presence of 200 μM Cd²⁺, transient IA was observed even with a prepulse of −40 mV (Fig. 3A). When IA was obtained by subtraction of IK from peak current on the same trace, IA was increased to 429.6% at 30 mV (p < 0.005; Fig. 3B), which was much more pronounced than that observed at the prepulse of −90 mV (cf. Fig. 1B). Since the effects of Cd²⁺ appeared to depend on prepulse voltage, the steady-state inactivation of IA was examined under control condition and after exposure to 200 μM Cd²⁺ (Fig. 3C). When the normalized peak amplitudes (I/I_{max}) were plotted against the prepulse potentials, the external Cd²⁺ shifted the steady-state inactivation curves positively.

Figure 2. Effects of Cd²⁺ on IA kinetics. A, Normalized I-V curves of IA at prepulse of −90 mV under control condition and in the presence of Cd²⁺. B, Voltage dependence of time to peak (Tp) under control condition and in the presence of 200 μM Cd²⁺. C, Voltage dependence of fast inactivation time constant (τ) under control condition and in the presence of 200 μM Cd²⁺. The inactivation time course of IA was fitted by a double exponential function (see Materials and Methods).
Figure 3. Extracellular Cd\(^{2+}\) (200 μM) increased \(I_A\) by moving steady-state inactivation curves of \(I_A\) positively. A and B, Micromolar Cd\(^{2+}\) increased \(I_A\) in a concentration-dependent manner when the prepulse was set at \(-40\) mV for 200 ms. A, Current examples were exhibited before (top) and after applying 200 μM Cd\(^{2+}\) (bottom), and B showed the \(I-V\) curves. C and D, Modulation of steady-state inactivation of \(I_A\) by Cd\(^{2+}\). C, Current traces were in response to a test potential to 40 mV after prepulses to a range of membrane potentials between \(-120\) and \(-30\) mV in 10 mV increments (inset protocol) before (top), and after (bottom) exposure to 200 μM Cd\(^{2+}\). Arrows indicate the response to the prepulse to \(-80\) mV. D, Steady-state inactivation curves of \(I_A\). Noting that Cd\(^{2+}\) shifted the steady-state inactivation curve to depolarized direction in a concentration-dependent manner.
in a concentration-dependent manner (Fig. 3D and Table I). However, the slopes of the inactivation curves remained unchanged. These results suggest that Cd\(^{2+}\) selectively enhanced \(I_A\) by shifting the steady-state inactivation curve of \(I_A\) positively.

To ascertain that Cd\(^{2+}\) produces its effect extracellularly, we introduced Cd\(^{2+}\) into the pipette solution. When Cd\(^{2+}\) (200 \(\mu\)M) was added to the intracellular solution, there was no significant change of \(I_A\) amplitude, activation and steady-state inactivation parameters (Fig. 3E and Fig. 3F). In addition, intracellular Cd\(^{2+}\) did not affect the modulation of \(I_A\) by extracellular Cd\(^{2+}\). In the presence of both intracellular and extracellular Cd\(^{2+}\), \(I_A\) was increased to 129.1\% and the steady-state inactivation curve was moved positively by 12.1 mV (from \(-79.5 \pm 3.1\) mV to \(-67.4 \pm 2.9\) mV) (Fig. 3E and Fig. 3F), compatible to those obtained with extracellular Cd\(^{2+}\) alone (124.3\% and 10.9 mV, Fig 1B and Fig. 3D). Similar results were obtained with Ca\(^{2+}\)-free external solution blocking Ca\(^{2+}\) current and Ca\(^{2+}\)-activated K\(^{+}\) current in WT neurons, which showed 123.6\% increase and 10.0 mV shift (from \(-81.6 \pm 2.5\) mV to \(-71.6 \pm 2.4\) mV) (Fig. 4).

### Effects of a slowpoke Mutation

The fast-inactivating current consists of voltage-activated \(I_A\) and Ca\(^{2+}\)-activated \(I_{CF}\). The latter is encoded by the slowpoke (slo) gene, whose slo\(^{I}\) mutation specifically eliminates \(I_{CF}\) in muscles and neurons (Elkins et al., 1986; Singh & Wu, 1989; Broadie & Bate, 1993). Our data from the third instar larval neurons showed that mutation slo\(^{I}\) eliminated \(I_{CF}\) (unpublished data) without altering \(I_A\) and \(I_K\) (Fig. 5A). Thus, slo\(^{I}\) was employed to further examine the modulation of \(I_A\) by Cd\(^{2+}\). As in WT neurons, 200 \(\mu\)M Cd\(^{2+}\) enhanced \(I_A\) in slo\(^{I}\) neurons. At 30 mV, the \(I_A\) was increased to 122.6\%, compatible to 124.3\% in WT neurons (Fig. 5B). Cd\(^{2+}\) produced a positive shift of the steady-state inactivation curve of slo\(^{I}\) \(I_A\) by 10.6 mV (from \(-79.3 \pm 2.9\) mV to \(-68.7 \pm 3.6\) mV, Fig. 5C), which is similar to that observed in WT neurons (10.9 mV shift, 3DIVALENT CATION MODULATION OF K\(^+\) CHANNEL 97 J Neurogenet Downloaded from informahealthcare.com by Shanghai Institutes for Biological Sciences, CAS on 06/11/12 For personal use only.

The steady-state inactivation curves were fitted by Boltzmann equation (materials and methods). E, \(I-V\) curves of K\(^{+}\) current in control condition, with intracellular (In) Cd\(^{2+}\) (\(n = 13\)), and with both intracellular and extracellular (Ex) Cd\(^{2+}\) (\(n = 7\)). F, Steady-state inactivation curves of \(I_A\) in control condition, with intracellular Cd\(^{2+}\), and with both intracellular and extracellular Cd\(^{2+}\).
Fig. 3D). These results further confirm that 200 μM Cd^{2+} selectively enhanced the amplitude of $I_A$ by shifting its steady-state inactivation curve positively.

**Effects of a *Shaker* Mutation**

*Shaker* is one of the two $I_A$-encoding genes in *Drosophila*. A null mutation at this locus, $Sh^M$, which eliminates all *Shaker* protein, was used to study the effects of Cd^{2+} on $I_A$. $Sh^M$ significantly decreased the amplitude of $I_A$ at 30 mV ($p < 0.05$) without affecting that of $I_K$ (Fig. 5A). In the presence of 200 μM Cd^{2+}, the $Sh^M$ $I_A$ increased to

![Figure 4](image-url)
Figure 5. Effects of slo¹ and ShM on Iₐ and Iₖ and effects of 200 μM Cd²⁺ on slo¹ and ShM Iₐ
A. Means of total current amplitudes of Iₐ and Iₖ at 30 mV in WT (n = 20), slo¹ (n = 17) and ShM (n = 16) neurons. Iₐ and Iₖ were recorded in Ca²⁺-free solution. B. Effects of 200 μM Cd²⁺ on Iₐ amplitude in WT, slo¹ and ShM neurons. % showed normalized Iₐ amplitudes in the presence of 200 μM Cd²⁺ to those of control. C. Effects of 200 μM Cd²⁺ on Vₘid of steady-state inactivation curves of Iₐ in WT, slo¹ and ShM neurons. Vₘid, right shift of Vₘid of steady-state inactivation curves. *p < 0.05, WT versus ShM.
115.8% at 30 mV, less than that in WT neurons \( (p < 0.05, \text{Fig. 5B}) \). The steady-state inactivation curve was moved by 6.90 mV \((-83.3 \pm 1.3 \text{ mV to } -76.4 \pm 1.7 \text{ mV})\), less than that in WT neurons \( (p < 0.05, \text{Fig. 5C}) \).

**Effects of Millimolar Cd\(^{2+}\) on Wild-Type I\(_A\) and I\(_K\)**

Previous results showed that millimolar, even micromolar Cd\(^{2+}\) shifted both activation and steady-state inactivation curves of I\(_A\) positively (Mayer & Sugiyama, 1988; Stengl et al., 1998). Thus, one possibility is that micromolar Cd\(^{2+}\) used here is not high enough to alter other kinetic properties of I\(_A\). To address this question, 2 mM Cd\(^{2+}\) was applied to study its modulatory effects. As shown in Table I, 2 mM Cd\(^{2+}\) did not significantly affect the amplitude of I\(_A\) or I\(_K\) at 30 mV. The midpoint of activation curve was significantly altered, and shifted positively by 19 mV. The steady-state inactivation curve was shifted more significantly by 42.9 \( \pm \) 2.6 mV. In addition, T\(_p\) and T\(_{1}\) of I\(_A\) were markedly increased by 2 mM Cd\(^{2+}\) and the delay was voltage dependent. For example, at 30 mV, T\(_p\) and T\(_{1}\) were 1.9 \( \pm \) 0.1 ms and 6.4 \( \pm \) 0.6 ms, respectively. However, at \(-10 \text{ mV}\) T\(_p\) and T\(_{1}\) were 7.1 \( \pm \) 0.6 ms and 23.3 \( \pm \) 6.4 ms, respectively.

**Modulation of WT I\(_A\) and I\(_K\) by other Divalent Cations**

To compare the effects of Cd\(^{2+}\) with other divalent cations, we investigated Zn\(^{2+}\), Ca\(^{2+}\) and Mg\(^{2+}\) in WT neurons. Micromolar (200 \( \mu \text{M}\)) Zn\(^{2+}\) increased I\(_A\) to 135.5% and shifted the inactivation curve by 13.0 mV, more potently than Cd\(^{2+}\) (cf. Fig. 6A with Fig. 1B and 3D; Table I). Interestingly, significant augmentation of I\(_K\) (increased to 119.7%; \( p < 0.05 \)) was also observed in the presence of 200 \( \mu \text{M}\) Zn\(^{2+}\). Similarly to millimolar Cd\(^{2+}\), 2 mM Zn\(^{2+}\) induced a shift of I\(_A\) activation and inactivation curves by 26 and 52.1 \( \pm \) 3.2 mV, respectively. At the same time, 2 mM Zn\(^{2+}\) increased I\(_K\) to 141.0 \( \pm \) 2.8% without affecting its voltage-dependent activation. Ca\(^{2+}\) and Mg\(^{2+}\) modulated I\(_A\) only in millimolar concentration range. Ca\(^{2+}\) (20 mM) produced a 12.5 mV shift of the steady-state inactivation curve and a significant augmentation of I\(_A\) (increased to 137.2%, Fig. 6B and Table I). Mg\(^{2+}\) (20 mM) showed less potency, producing a shift of 7.90 mV and an augmentation of 120.5% (Fig. 6C and Table I). Both Ca\(^{2+}\) and Mg\(^{2+}\) had little effect
Figure 6. Modulation of activation and steady-state inactivation properties of $I_A$ by other divalent cations. $I-V$ curves $I_A$ and steady-state inactivation curves of $I_A$ in control condition and in the presence of different concentration of $\text{Zn}^{2+}$ ($n = 7$, A1 and A2), $\text{Ca}^{2+}$ ($n = 6$, B1 and B2) and $\text{Mg}^{2+}$ ($n = 5$, C1 and C2).
on $I_K$ or on the time-dependent activation ($T_p$) and inactivation ($\tau_i$) of $I_A$ (Table I). At millimolar concentrations, $\text{Zn}^{2+}$ (2 mM), $\text{Cd}^{2+}$ (2 mM), $\text{Ca}^{2+}$ (20 mM) and $\text{Mg}^{2+}$ (20 mM) shifted the steady-state inactivation curves of $I_A$ by 52.1, 42.9, 12.5, 7.9 mV (Table I), respectively, leading to a sequence: $\text{Zn}^{2+} > \text{Cd}^{2+} > \text{Ca}^{2+} > \text{Mg}^{2+}$.

**DISCUSSION**

The main finding of the present study is that divalent cations selectively regulate $I_A$ of acutely dissociated neurons from larval brains of *Drosophila*. Micromolar $\text{Cd}^{2+}$ selectively enhances $I_A$, an effect that is largely attributed to the positive shift of the steady-state inactivation curve of $I_A$. In addition, analysis from two $K^+$ channel mutants, $\text{slo}^1$ and $\text{Sh}^M$ supports the notion that the effect of $\text{Cd}^{2+}$ on voltage-gated $K^+$ currents is due to changes in $I_A$.

According to the model of screening of surface negative charges by divalent cations (Frankenhaeuser & Hodgkin, 1957; Hille et al., 1975; Hahin & Campbell, 1983; Kass & Krafte, 1987), gating of all ionic currents should be affected; both activation and inactivation curves should be shifted equally in response to a metal ion; all divalent cations should have the same potency. The data presented here were recorded with millimolar $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ to reduce the external surface potentials and prevent the low concentration of $\text{Cd}^{2+}$ from producing any further surface charge effects. Most importantly, the channel-type specificity, the asymmetric effects on $I_A$ activation and inactivation curves, and the diverse potency of divalent cations strongly support the idea that modulation of $I_A$ in *Drosophila* CNS neurons is due to the specific binding of divalent cations on A-type channel protein itself rather than on phospholipids electrically close to the gating apparatus.

**Micromolar Cd$^{2+}$ Selectively Enhances $I_A$ by Modulating its Steady-State Inactivation Property**

Micromolar Cd$^{2+}$ enhanced $I_A$ of *Drosophila* larval neurons in a concentration-dependent manner. This result was consistent with previous reports from central and peripheral neurons (Mayer & Sugiyama, 1988; Talukder & Harrison, 1995), ventricular myocytes (Agus et al., 1991; Stengl et al., 1998) and glial cells (Bringmann et al., 1999).


Cd\(^{2+}\) increased \(I_A\) by shifting the steady-state inactivation curve of \(I_A\) in the positive direction. A positive shift of the inactivation curve causes an increase in the population of channels available for activation at the same potential. The effects were only evident with Cd\(^{2+}\) applied extracellularly, suggesting that Cd\(^{2+}\) acts at the extracellular face of the membrane, as subscribed in other papers about extracellular modulation of K\(^{+}\) channel by divalent cations (Talukder & Harrison, 1995; Stengl et al., 1998; Bringmann et al., 1999).

Both \(I_{CF}\) and \(I_A\) contribute to the outward inactivating currents in \textit{Drosophila} neurons (Elkins et al., 1986), and mutant \textit{slo}\(^{l}\) eliminates \(I_{CF}\) without affecting \(I_A\) (Fig. 5A). In the present experiment, no significant difference of the enhancement by Cd\(^{2+}\) was observed between WT and \textit{slo}\(^{l}\) neurons, indicating that \(I_{CF}\) is not involved. Moreover, the modulatory effect of \(I_A\) by Cd\(^{2+}\) persisted even in Ca\(^{2+}\)-free external solution (Fig. 4). \(I_A\) is known to control the frequency of repetitive firing and the delay in action potential initiation in \textit{Drosophila} neurons (Saito & Wu, 1991). Therefore, our results suggest that micromolar concentration of Cd\(^{2+}\) may reduce \textit{Drosophila} neuronal excitability through enhancing \(I_A\).

Contrary to the effects of 200 \(\mu\)M Cd\(^{2+}\), 2 mM Cd\(^{2+}\) delayed time-dependent activation and inactivation of \(I_A\) (Table I), and did not significantly enhance \(I_A\) at 30 mV. Both the activation and steady-state inactivation curves of \(I_A\) were shifted in the depolarizing direction. A positive shift of the activation curve causes a decrease in the population of \(I_A\)-channels that can be activated upon stepping to a given potential, while a positive shift of the inactivation curve causes an increase in the population of channels available for activation at this same potential. It is possible that the augmentation of \(I_A\) was counteracted due to the depression of \(I_A\), thus no significant augmentation of \(I_A\) by 2 mM Cd\(^{2+}\) was observed. The shift of the steady-state inactivation curve was larger than that of the \(I_A\) activation curve. The mechanism underlying the positive shift of the activation curve is not clear. One possibility is that millimolar Cd\(^{2+}\) may serve as a voltage-dependent blocker of the \(I_A\) channel in addition to its modulation on channel gating. If the binding site is near the channel entrance, a conformational change would occur when more divalent cations bind to the channel mouth, resulting in the channel blockage. This assumption is supported by the observation that at millimolar concentration Zn\(^{2+}\) also affected asymmetrically activation and steady-state inactivation curves of \(I_A\) (Table I).
The $Sh^M$ Mutation Partially Diminishes the Cd$^{2+}$ Effect

Both Shal and Shaker are known to encode Drosophila neuronal $I_A$. Our results showed that the amplitude and the steady-state inactivation curve of $I_A$ of Shaker mutation ($Sh^M$) were less affected by 200 $\mu$M Cd$^{2+}$ than those of WT. It is reported that $I_A$ encoded by Shal exhibits different kinetics from Shaker type of $I_A$ (Covarrubias et al., 1991). Shaker mutations do not affect the majority of neuronal currents (Solc et al., 1987; Baker & Salkoff, 1990), and Shaker is only responsible for a small population of neuronal $I_A$ (Tsunoda & Salkoff, 1995). Our data suggest that both Shaker and Shal may be sensitive to Cd$^{2+}$ modulation. Consistent with this speculation, Cd$^{2+}$ modulates the kinetics of currents conducted by Kv4.2 (Shal-related channel) and Kv1.4 (Shaker-related channel) in Xenopus oocytes (Wickenden et al., 1999).

Divalent Specificity and Functional Implications

The effective concentration ranges of different divalents reported here are somewhat similar to those described for $I_A$ in ventricular myocytes (Agus et al., 1991; Stengl et al., 1998), and glial cells (Bringmann et al., 1999). The diverse potencies of divalent cations further support that, in addition to alteration of surface charge of neuronal membrane (Frankenhaeuser & Hodgkin, 1957; Hille et al., 1975; Hahin & Campbell, 1983; Kass & Krafte, 1987), divalent cations could affect neuronal excitability by specifically modulating certain ion channels including A-type $K^+$ channels. In particular, Zn$^{2+}$ has been widely studied because of its release from central synapses (Assaf & Chung, 1984). Cd$^{2+}$ is often employed as an efficient blocker of Ca$^{2+}$ channel and Ca$^{2+}$-activated $K^+$ channel when isolating voltage-gated $K^+$ current. Fluctuations of Ca$^{2+}$ concentrations do occur in the brain (Nicholson et al., 1977). Furthermore, experiments with Ca$^{2+}$ selective microelectrodes have revealed more global changes in extracellular Ca$^{2+}$ concentration in the brain during neuronal activity (Nicholson et al., 1977).

In conclusion, we for the first time studied the effects of divalent cations on native $K^+$ currents in acutely dissociated Drosophila central neurons and our data strongly support the idea that divalent cations modulate A-type $K^+$ channels through specific binding to extracellular sites of the channels. The acutely dissociated neurons may provide a means for studying Drosophila neurophysiology in a systematical way.
REFERENCES


