

EFFECTS OF DIVALENT CATIONS ON OUTWARD POTASSIUM CURRENTS IN LEECH RETZIUS NERVE CELLS

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EFEKTI DVOVALENTNIH KATJONA NA IZLAZNE KALIJUMSKE STRUJE RETZIUSOVIH NERNVIH ČELIJA PIJAVICE

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ABSTRACT

The present study examines the effects of divalent metals, cadmium (Cd^{2+}) and manganese (Mn^{2+}), on the outward potassium currents of Retzius cells in the hirudinid leeches *Haemopsis sanguisuga* using conventional two-microelectrode voltage-clamp techniques. The outward potassium current is activated by depolarization and plays an important role in determining both the neuronal excitability and action potential duration. A strong inhibition of the fast current and a clear reduction in the late currents of the outward current with 1 mM Cd^{2+} were obtained, which indicated that both components are sensitive to this metal. Complete blockage of the fast and partial reduction of the slow outward currents was observed after adding 1 mM Mn^{2+} to the extracellular fluid. These data show that the outward K^+ current in leech Retzius nerve cells comprises at least two components: a voltage-dependent K^+ current and a Ca^{2+} -activated K^+ current. These observations also indicate that Cd^{2+} is more effective than Mn^{2+} in blocking ion flow through these channels and that suppressing Ca^{2+} -activated K^+ outward currents can prolong the action potential in nerve cells.

Key word: Retzius neuron, cadmium, manganese, outward potassium currents

SAŽETAK

Ispitivana su dejstva dvovalentnih katjona, kadmijuma (Cd^{2+}) i mangana (Mn^{2+}) na izlazne ispravljачke kalijumske struje Retziusovih nervnih ćelija pijavice, *Haemopsis sanguisuga*, tehnikom nametnutog napona sa dve mikroelektrode. Depolarizacijom izazvane izlazne kalijumske struje imaju značajnu ulogu u determinaciji nadražljivosti nervnih ćelija kao i trajanja akcionih potencijala. 1 mM Cd^{2+} je izazvao blokadu brze i redukciju kasne komponente izlazne kalijumske struje, što ukazuje da su obe komponente senzitivne na Cd^{2+} . Drugi dvovalentni katjon, 1 mM Mn^{2+} je doveo do kompletne blokade brze i delimične inhibicije spore komponente izlazne kalijumske struje. Rezultati ovog istraživanja ukazuju da su izlazne kalijumske struje u Retziusovim nervnim ćelijama pijavice sastavljene od najmanje dve komponente: naponsko-zavisne i kalcijumsko-aktivisane kalijumske struje. Rezultati studije potvrđuju da je Cd^{2+} efikasniji blokator izlaznih kalijumskih kanala od Mn^{2+} , kao i da inhibicija kalcijumsko-aktivisanih kalijumskih struja može biti odgovorna za prolongiranje trajanja akcionih potencijala.

Ključne reči: Retziusov neuron, kadmijum, mangan, izlazne kalijumske struje.

INTRODUCTION

Divalent cations (such as Cd^{2+} , Co^{2+} , Ni^{2+} , Fe^{2+} and Mn^{2+}) are known as nonspecific calcium (Ca^{2+}) channel antagonists that block Ca^{2+} channels to varying degrees. Additionally, divalent metals are among the classical tools that have been used to investigate the activation and deactivation kinetics and the permeation of the potassium (K^+) current (1). Cadmium (Cd^{2+}) is a nonessential divalent metal ion that can cause cytotoxicity in multiple organs, includ-

ing the brain. Recently, a great amount of scientific data has been used in attempts to define the mechanism of action of Cd^{2+} in brain. However, the mechanisms underlying Cd^{2+} neurotoxicity remain not entirely understood. Multiple scientific studies have shown that the toxic effects of Cd^{2+} on the nervous system are manifold. For example, Lopez et al (2) demonstrated that in cultured cortical neurons, a low concentration of Cd^{2+} (100 nM) induced apop-



tosis, whereas higher concentrations (100 mM) produced necrotic cell death. More recent studies (3) have reported that Cd^{2+} toxicity in cerebral cortical neurons is mediated by intracellular Ca^{2+} elevation, which triggers the activation of the apoptotic signalling pathway in mitochondria. Additionally, the cellular toxicity generated by Cd^{2+} is due, in part, to the generation of reactive oxygen species (ROS) that depolarize the mitochondrial membrane potential and decrease the ATP levels (4).

Some novel studies have revealed the endoplasmic reticulum to be the cellular target of Cd^{2+} toxicity. The Cd^{2+} -induced release of Ca^{2+} from endoplasmic reticulum occurs via the inositol trisphosphate (IP_3) pathway (5). In contrast, Cd^{2+} inhibition of the electron transport chain in mitochondria generates reactive oxygen species (ROS) and activates caspase-9. These pathways appear to be simultaneously activated, and their synergistic activation can promote apoptosis through the production of ROS and Ca^{2+} -mitochondria signalling (6). According to Smith et al. (7), Cd^{2+} rapidly increases IP_3 , which is known to mobilize stored Ca^{2+} . Furthermore, they showed that Cd^{2+} and other divalent metals increased IP_3 and mobilized intracellular Ca^{2+} . Yang et al (8) demonstrated that Cd^{2+} induces necrotic cell death by increasing both the intracellular Ca^{2+} concentration and the ROS level. Experimental studies have shown that Cd^{2+} -induced apoptosis is mediated by the Ca^{2+} signalling pathway and that Ca^{2+} -mediated apoptosis occurs through the mitochondria-caspase signalling pathway (3). Recent studies have reported that, similar to other toxic metals, Cd^{2+} impairs neurogenesis and physiological signal transduction (9). Additionally, evidence is growing that Cd^{2+} exposure can alter gene expression and cause an epigenetic effect. One possible mechanistic pathway for Cd^{2+} -induced toxicity is through the modification of hormone levels by affecting the hypothalamic-pituitary-gonadal axis (10, 11).

In contrast to Cd^{2+} , which is a nonessential metal, manganese (Mn^{2+}) is an essential trace element that is ubiquitous and pivotal for normal cell function and metabolism. Nevertheless, excessive accumulation of Mn^{2+} in the brain may lead to a condition known as manganism, a neurodegenerative disorder associated with dysfunctions in the basal ganglia that causes parkinsonian-like symptoms (12-14). Recent studies have reported that Mn^{2+} -induced neurotoxicity is mediated, at least in part, by the generation of ROS, depletion of antioxidant defence mechanisms and mitochondrial dysfunction (15).

The purpose of this study was to investigate the components of the somatic outward current and determine the relationship between the Ca^{2+} and voltage-dependent K^+ currents on the basis of ion-substitution experiments. According to the activation and deactivation kinetics, the Ca^{2+} -activated K^+ current could be separated into two distinct components: a fast transient and a slow current. The leech served as an appropriate model for studying ion channels because of the extreme structural simplicity of its nervous system.

MATERIALS AND METHODS

Experimental animals

The experiments were performed utilizing *Retzius* nerve cells from the first ten abdominal ganglia of the leech, *Haemopsis sanguisuga* (commonly known as the horse-leech). The dissection method was similar to that described previously (17, 18). The 21 segmental ganglia contain ~400 neurons arranged in six packets. Retzius neurons are the largest cells (~60 μm diameter of the soma) located on the ventral side of the ganglia. They were identified by their large cell bodies, position and firing properties (17, 19). Isolated ganglia of the Retzius neurons have resting potentials that range from -40 to -60 mV and amplitude action potentials that range from 30 to 50 mV, and they fire spontaneously at a slow rate (0.2-3 APs/sec).

Electrophysiological technique

All electrophysiological recordings were made at room temperature (20-24°C) under two-electrode voltage-clamp conditions. The electrical arrangement for voltage clamping utilized was based on the theoretical principles developed for different cellular membranes (20). Retzius neurons were impaled with two electrolyte-filled microelectrodes to record membrane potential and to perform the current injection. For the intracellular recordings, we used glass micropipettes pulled from borosilicate glass (1.5 mm outside diameter, 0.6 mm inside diameter, Clark Electromedical Instruments, Edenbridge, UK) filled with 3 M KCl (resistance ~20 M Ω). Electrodes were connected to a voltage-clamp amplifier (Bioelectric Instrument model DS2C). The bath was grounded *via* an agar bridge. Command pulses were derived from a Tektronix 161 pulse generator. Voltage and current recordings were displaced on a Tektronix 564 oscilloscope. Output signals were digitized by an A/D converter (Axon Instruments, Jakarta, Indonesia) and were saved before being analysed on a computer running in-house acquisition software.

Briefly, the difference between the membrane potential and the command pulse was amplified, and the resulting voltage was used to drive the current across the membrane via the microelectrode. The membrane potential was measured between the second electrode and the ground. In the voltage-clamp recording mode, depolarization from a holding potential of -70 mV elicited outward currents that increased to steady-state values.

Solutions

During the experiment, isolated ganglia were superfused, initially with normal leech saline of the following composition (in mM): 115 NaCl, 4 KCl, 2 CaCl_2 , 1.2 Na_2HPO_4 , 0.3 NaH_2PO_4 (pH 7.2). The sodium-free Ringer contained 115 mM TRIS-Cl (Tris Ringer) instead of NaCl and phosphate buffer. CdCl_2 (1 mM) and MnCl_2 (1 mM) were

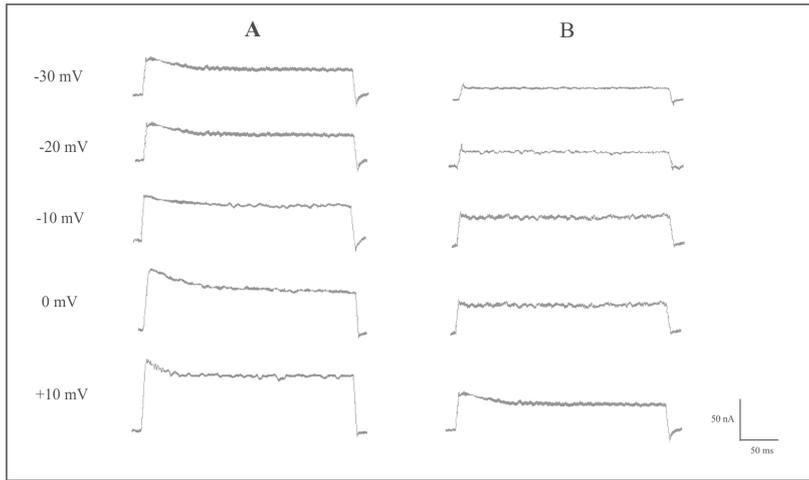


Figure 1. Representative current recordings obtained under long-lasting stimulation (300 ms) in Tris Ringer (A) and at 10 min after adding 1 mM CdCl₂ to the Tris Ringer (B) during the displacement of the holding potential from -70 mV to the potential given at each trace.

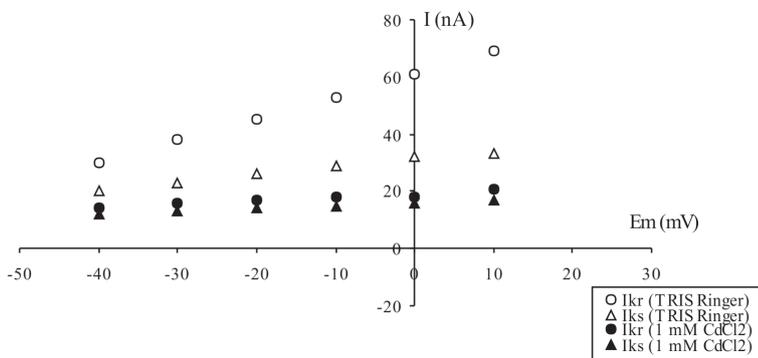


Figure 2. The current-voltage relationship at the peak of the outward K⁺ current in the absence (open symbols) and presence (solid symbols) of 1 mM CdCl₂. Ikr- rapid outward K⁺ current; Iks-slow outward K⁺ current.

used as blockers of outward potassium channels and added to the normal or Tris Ringer solution. CdCl₂ and MnCl₂ were purchased from Sigma-Aldrich (St. Louis, MO, USA). The bath volume was 2 ml, and the solution changes were completed within 30 sec.

Statistical analysis

Statistical analysis was performed using Student's t-test for paired correlated samples. Currents (in nA) in the presence and absence of CdCl₂ and MnCl₂ were compared for each Retzius nerve cell.

RESULTS

The effect of Cd²⁺ on the outward K⁺ current of leech *Retzius* nerve cells

In the first series of experiments, we examined the effect of the Ca²⁺ channel blocker, Cd²⁺ (1 mM), on the membrane K⁺ current in Retzius neurons. The outward K⁺ currents, which contribute to the resting membrane potential and repolarization of the action potential, were studied in voltage-clamped leech Retzius neurons. The K⁺ current was activated by depolarization with the sodium-free Ringer (TRIS Ringer) with long-lasting stimulation (300 ms) by

an activated outward current. From -70 mV, depolarizing steps to a potential of approximately -40 mV elicited a fast outward current. This transient part of the total outward current had rapid activation kinetics and inactivated within 50 ms. Higher depolarization produced a larger current and a slow outward current that showed little inactivation. The measurements of the outward K⁺ currents (in nA) in the control condition (TRIS Ringer) were compared with those in the Cd²⁺ condition for each Retzius cell.

The voltage-clamp experiments demonstrated that Cd²⁺ (1 mM) reduced both types of outward currents, with different efficacies. The fast outward current, activated by small depolarizations (i.e., at -35 mV), was more sensitive to Cd²⁺ than was the slow part of the outward current activated by larger depolarizations. Typical current-voltage records were obtained upon replacing sodium with Tris (A) and at 10 min after adding 1 mM Cd²⁺ (B) to Tris Ringer after displacing the membrane potential from a resting level of -40 mV, as shown in Fig. 1. A strong inhibition of the fast current and an evident reduction in the late currents of the outward current with 1 mM Cd²⁺ were observed, which indicated that both components are sensitive to this metal.

To investigate the relationship between the change in the outward potassium current with the change in voltage (I-V), the membrane potential was increased from a holding potential of -70 mV to five potentials, ranging from -40 to +10 mV in 10 mV increments (Fig. 2). In the presence



Figure 3. Patterns of voltage clamp current recordings in a leech Retzius nerve cell in Tris Ringer (A) and at 10 min after adding 1 mM MnCl_2 (B). Potentials were increased from the holding potential (-70 mV) to the potential given on each trace.

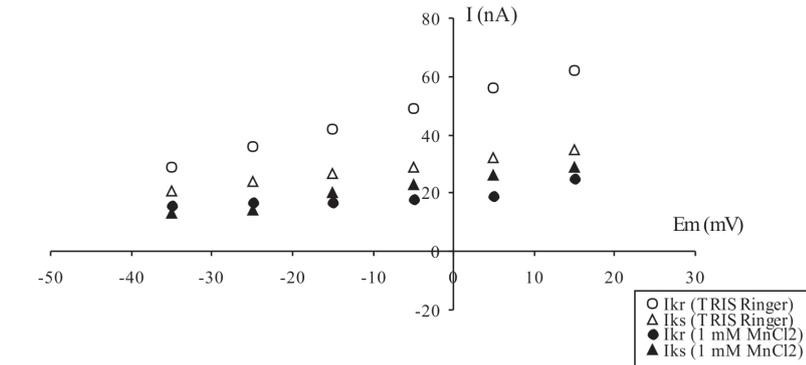


Figure 4. The current-voltage relationship at the peak of the outward K^+ current in the absence (open symbols) and presence (solid symbols) of 1 mM MnCl_2 . Ikr- rapid outward K^+ current; Iks-slow outward K^+ current.

of 1 mM Cd^{2+} , at the test potential of +10 mV, the fast and slow parts of the K^+ outward current were reduced from 69 to 21 nA (69.57%) and from 33 to 17 nA (48.49%), respectively.

The effect of Mn^{2+} on the outward K^+ current of leech Retzius nerve cells

To determine the total outward current in terms of their voltage and calcium-dependent components, experiments using another Ca^{2+} channel blocker, Mn^{2+} , were performed.

In contrast to the previous experiments with Cd^{2+} , Mn^{2+} was effective only in blocking the fast but not the late outward current. Voltage-clamp experiments were performed on freshly dissociated Retzius neurons in the TRIS Ringer (A), and at 10 min after adding Mn^{2+} (1 mM) to the Tris Ringer solution (B). Typical membrane current pattern curves that were produced by increasing the depolarization from a steady holding level of -70 mV are shown in Fig 3. Clearly, both components were affected, and the fast transient part was completely reduced.

Figure 4 shows the corresponding current-voltage (I-V) relationship that was obtained with 1 mM Mn^{2+} . In the presence of Mn^{2+} , at the test potential of +15 mV, the fast and slow parts of the K^+ outward current were reduced from 62 to 25 nA (59.68%) and from 35 to 29 nA (17.15%), respectively. The data obtained with Mn^{2+} support the view that both the fast and partially slowed outward current are Ca^{2+} activated.

DISCUSSION

The results reported in this paper show that the outward K^+ current in leech Retzius nerve cells is composed of two distinct components: a voltage-dependent current and a Ca^{2+} -activated K^+ current. The Ca^{2+} -activated K^+ current plays a key role in regulating neuronal excitability. In this study, the addition of inorganic Ca^{2+} channel blockers (Cd^{2+} or Mn^{2+}) was used to demonstrate the dependence of Ca^{2+} on outward K^+ currents.

Recent, much of our knowledge of membrane transporters has been acquired by studying the interaction of blocking agents and ionic channels. The voltage dependent conductance of electrically excitable membranes has been extensively studied by several different approaches. The classic study by Hodgkin and Huxley (16) led to a conclusion on the existence of two independent membrane conductance: early Na^+ and late K^+ conductance. However, the conductances of other tissues appeared to be more numerous than those found in the squid giant axon. In spite of the difficulties in separation, several outward currents have been identified, such as the slow and fast K^+ currents and Ca^{2+} -activated K^+ currents.

Leech ganglion cells have been recognized as a promising model system for electrophysiological studies because the size and accessibility of these neurons allow them to be readily implanted with several microelectrodes. In leech Retzius nerve cells, Ca^{2+} activated K^+ currents are large outward K^+ currents (17, 18). Electro-



physiological studies have shown that membrane transport proteins are susceptible to divalent cations (such as Cd^{2+} , Co^{2+} , Ni^{2+} , Fe^{2+} and Mn^{2+}). To date, most research has focused on the toxic effects of Cd^{2+} on the brain, with only a few experimental studies on the effects of Mn^{2+} on outward K^+ currents. Although the molecular mechanisms of action of Cd^{2+} on ion transporters are not fully understood, several hypotheses have been proposed. One possibility is that Cd^{2+} may mimic other metals (and elements) at the site of the membrane transporters or channels in the nerve cell membrane (21). Several lines of evidence suggest that Cd^{2+} is transported into neurons and other cells via transporters for naturally occurring cations, such as Ca^{2+} (“ionic mimicry”) (21, 22). Zalups and Ahmad (22) proposed the mechanism of Cd^{2+} transport into a cell. This uptake has been recently proposed to occur through a mechanism of ionic mimicry, whereby Cd^{2+} mimics the divalent cation species of one or more of these nutritive metals at the binding site of one or more carrier proteins and/or channels that transport these metals. One possible pathway for Cd^{2+} entry into a cell is via Ca^{2+} channels in the plasma membrane (including both voltage-gated and receptor-dependent Ca^{2+} channels), thus leading to the accumulation of Cd^{2+} in the cell and, finally, the induction of caspase-12-mediated apoptosis (5).

In recent years, new electrophysiological studies have indicated that Cd^{2+} may interact with ion channels and transporters. Considering nerve cell function, Cd^{2+} can attack ion channels either directly or indirectly by disrupting the physiological signal cascades (5, 23, 24). Cd^{2+} is a well-known specific blocker of Ca^{2+} channels and inhibits Ca^{2+} cellular uptake. Electrophysiological investigations in various experimental models have shown that Cd^{2+} is commonly used to block voltage-dependent Ca^{2+} currents (25) and Ca^{2+} -activated K^+ currents (26-29). In rat pyramidal neurons (26), 200 μM cadmium was found to markedly reduce the early and late components of Ca^{2+} -activated K^+ current ($I_{\text{K}(\text{Ca})}$). Cd^{2+} is classically considered a Ca^{2+} channel blocker, but previous studies have demonstrated that Cd^{2+} (200 μM) does not affect the depolarization-activated outward current (27). However, Sah et al. (30) demonstrated that Cd^{2+} at concentrations of 0.1-0.5 mM had no effect on the K^+ current of guinea pig neurons, whereas at higher concentrations (2 mM), it induced a reduction of the outward current. In the present study, we found that Cd^{2+} significantly affected the fast and late membrane currents. In contrast, Mn^{2+} blocked the fast transient outward current in leech Retzius nerve cells. The results obtained with Mn^{2+} support the theory that the fast and partially slow outward currents are Ca^{2+} activated. Our voltage-clamp data support the early proposal of Beleslin (17) and Stewart (18) on the existence of Ca^{2+} -activated K^+ channels in leech neurons. Interestingly, similar results were found by Mitra and Morad (28), who reported that nifedipine and cadmium reduced the Ca^{2+} -activated K^+ current.

Jow and Numann (31) show that 5 mM Mn^{2+} reduced large inward rectifier current [$I_{\text{K}(\text{IR})}$] by 33% in human capillary endothelial cells, and similar results in rat pyramidal neurons were obtained by Castelli et al (32) using the patch-clamp technique. Castelli et al. reported that Mn^{2+} , in addition to performing a blocking action on high-voltage-activated Ca^{2+} channels, modified the Ca^{2+} current activation and deactivation kinetics.

Taken together, we can conclude that the total outward K^+ current is composed of two distinct components in leech Retzius nerve cells, i.e., voltage dependent and Ca^{2+} -activated K^+ currents, and that the Ca^{2+} activated K^+ current is a large outward K^+ current.

Conflict of Interest

All of the authors declare no conflict of interest.

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