

Divalent cations as modulators of neuronal excitability: Emphasis on copper and zinc

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ABSTRACT

Based on indirect evidence, a role for synaptically released copper and zinc as modulators of neuronal activity has been proposed. To test this proposal directly, we studied the effect of copper, zinc, and other divalent cations on voltage-dependent currents in dissociated toad olfactory neurons and on their firing rate induced by small depolarizing currents. Divalent cations in the nanomolar range sped up the activation kinetics and increased the amplitude of the inward sodium current. In the micromolar range, they caused a dose dependent inhibition of the inward Na^+ and Ca^{2+} currents (I_{Na} and I_{Ca}) and reduced the amplitude of the Ca^{2+} -dependent K^+ outward current ($I_{\text{Ca-K}}$). On the other hand, the firing rate of olfactory neurons increased when exposed to nanomolar concentration of divalent cations and decreased when exposed to micromolar concentrations. This biphasic effect of divalent cations on neuronal excitability may be explained by the interaction of these ions with high and low affinity sites in voltage-gated channels. Our results support the idea that these ions are normal modulators of neuronal excitability.

Key terms: copper, zinc, modulators, channels, neuronal excitability

INTRODUCTION

Divalent cations affect several aspects of neuronal physiology, but the mechanism by which they affect neurons has not been elucidated for most of them (Burdette and Lippard, 2003). Copper and zinc are micronutrients that play essential roles in several cell functions in all aerobic organisms. Copper is a co-factor of enzymes that participate in the respiratory chain, therefore eukaryotic cells are not viable in its absence. Copper also is associated with enzymes like cytochrome c oxidase complex and copper and zinc are associated with enzymes like superoxide dismutase (CuZnSOD) and with metal-binding proteins like metallothioneins in the cytoplasm (Aschner, 1996).

Copper and zinc are normally present in the brain, and, based on *in vitro* electrophysiological studies, several authors have proposed that they could have an *in*

vivo modulatory role on the electrical activity of the neurons (Trombley and Shepherd, 1996; Jo et al., 2000; Horning and Trombley, 2001). There are several hints that lead us to think that copper and zinc could play this role. First, these trace metals are present in the central nervous system, but their concentrations are not homogeneous in all areas. They accumulate in some particular regions of the brain, such as the cerebral cortex, olfactory bulb, hypothalamus, and hippocampus (Donaldson et al., 1973; Kardos et al., 1989; Ono and Cherian, 1999). Second, all patients with pathologies having alterations in copper metabolism, like Menkes and Wilson's disease and idiopathic dystonia, exhibit neurological disorders (Waggoner et al. 1999; Cox, 1999; Becker et al., 2001). In addition, copper has been associated with neurodegenerative diseases such as Amyotrophic Lateral Sclerosis, Alzheimer disease and Prion disease (Waggoner et al.,

1999; Barnham et al., 2004). Although there is no conclusive evidence that zinc is involved in the etiology of neurodegenerative disorders, it has been observed that excessive extracellular zinc may contribute to neuronal cell death following ischemia and seizures (Zatta et al., 2003).

In the CNS, copper and zinc accumulate in synaptic vesicles, together with neurotransmitters, especially in glutamatergic neurons (Kardos et al., 1989; Ebadi et al., 1995; Schroder et al., 2000). There is evidence that these metal ions are co-released to the synaptic space together with the neurotransmitters during normal synaptic events. It has been suggested that their total concentration in the synaptic space may reach up to 100 and 300 μM for copper and zinc respectively (Hartter and Barnea, 1988; Kardos et al., 1989). However, their free concentration could be much lower, since they are probably bound to proteins.

It has been hypothesized that once released, copper and zinc ions diffuse in the synaptic cleft where they interact with postsynaptic neurotransmitter receptors and voltage-gated ion channels modulating their activity. Copper and zinc ions could also be re-internalized by pre- and postsynaptic neurons through membrane transporters (Kardos et al., 1989, Horning and Trombley, 2001, Colvin et al., 2003).

Most studies in the literature have focused on the effect of copper and zinc on the activity of some neurotransmitter receptors channels. Using the patch-clamp technique, Sharanova et al. (1998) studied the effect of copper on GABA(A) receptors in isolated cerebellar Purkinje cells of the rat. They showed that nanomolar Cu^{2+} induced a reversible block of GABA-mediated currents in the GABA_A receptor with an IC_{50} of 35 nM. On the other hand, Zn^{2+} was able to relieve the Cu^{2+} -induced blockade of the GABA currents. They speculate that in Wilson's patients, Cu^{2+} could trigger chronic GABA(A) receptor blockade.

More recently, Acuña-Castillo et al. (2000) demonstrated that Zn^{2+} and Cu^{2+} differentially modulate ATP-evoked

currents in the P2X₄ receptor expressed in *Xenopus* oocytes. They found that 10 μM Zn^{2+} potentiated the effect of 1 μM ATP, while 10 μM Cu^{2+} inhibited it. There is one study in which the effects of zinc and copper were tested on neuronal voltage-gated ion channels: Horning and Trombley (2001) compared the effects of micromolar Cu^{2+} and Zn^{2+} upon voltage-gated conductances in rat olfactory bulb neurons in primary culture. They found that zinc (100 μM) or copper (30 μM) inhibited TTX-sensitive sodium and delayed rectifier-type potassium currents but did not prevent the firing of evoked action potentials nor dramatically alter their kinetics.

Since the free concentration of these ions in the postsynaptic space is probably very low, in the order of nanomolar, we thought that it was important to investigate the effect of these transition metal ions on voltage-gated ion channels in a wide concentration range from nanomolar to micromolar. We included nickel as another divalent metal cation that has different chemical properties than copper and zinc and that therefore could help us to define the molecular processes involved.

To study the mechanism of action of divalent ions in neuronal excitability, we chose toad olfactory neurons whose voltage dependent conductances are very well characterized (Delgado and Labarca, 1993; Morales et al., 1994; Morales et al., 1997). These neurons can be depolarized by small currents that cause them to fire action potentials. They have voltage dependent currents that are inward Na^+ and Ca^{2+} currents and outward K^+ and Ca^{2+} -activated K^+ currents. To measure macroscopic currents, the cells were mechanically dissociated from the epithelia and patch-clamped using the whole-cell configuration.

MATERIALS AND METHODS

Biological material

Experiments were conducted on olfactory receptor neurons mechanically dissociated

from the toad *Caudiverbera caudiverbera* (Delgado and Labarca, 1993). Animals were anesthetized by cooling in ice after which they were sacrificed and pithed before dissecting out their olfactory epithelia. Extracted olfactory epithelia were cut into small pieces ($\sim 1 \text{ mm}^2$) and kept at 4°C for at most 48 hr in a solution containing (in mM): 120 NaCl, 1 CaCl_2 , 2 MgCl_2 , 3 KCl, 5 glucose, 10 HEPES (pH 7.5), 5 Na-pyruvate plus 0.1 I.U. ml^{-1} penicillin. Dissociation was accomplished by gently passing the pieces of epithelia through the tip of a fire-polished Pasteur pipette; the cells were then transferred to the experimental chamber, which contained Ringer solution (in mM): 115 NaCl, 1 CaCl_2 , 1.5 MgCl_2 , 2.5 KCl, 3 glucose, 10 HEPES, pH 7.6.

Macroscopic current measurements

To test the effect of divalent cations on the macroscopic currents and action potential firing rates, we used the whole cell and the cell attached mode of recording of the patch-clamp technique. The experimental chamber (300 μl) was perfused with six volumes of external solution supplemented with chloride salts of the cation at the required concentrations. The patch-clamp pipette was filled with a saline solution that mimicked the intracellular milieu containing (in mM) 125 KCl, 1 CaCl_2 , 1 MgCl_2 , 2 EGTA, 4 HEPES, pH 7.6. The resistance range of the patch pipettes used was 2 to 4 MOhm. Current records were made with an Axopatch 1-D or 200 B amplifier (Axon Instruments, Foster City, California) and pulse protocols were generated by a computer through and D/A convert (Scientific Solution, Solon, Ohio 44139) using pClamp 6.0 routines. Data were acquired at a sampling rate of 10 KHz, stored in a PC and analyzed using the Clampfit routine of pClamp6 software. Currents were evoked with a family of depolarizing pulses every 5 or 10 mV, starting from a holding potential of -80 mV. Under voltage clamp conditions, action currents associated with action potentials were triggered by depolarizing steps from -80 mV to 30 mV.

RESULTS

Differential effects of low concentrations of Cu^{2+} , Zn^{2+} and Ni^{2+} on whole cell currents in dissociated olfactory neurons

Figure 1A shows total current traces obtained from three different cells exposed to 0.1 and 1 μM of Cu^{2+} , Zn^{2+} and Ni^{2+} , respectively. The control traces for each cell show the inward and outward component of the whole cell currents. As mentioned above, the inward current has sodium and calcium components, and even though at first sight, the presence of the calcium current is not obvious, the shoulder observed in the outward currents is a reflection of the Ca^{2+} -dependent K^+ outward current present in these neurons, which is dependent on I_{Ca} . For an easier visualization of the effect of the divalent cations, the insets under the recordings in their presence show in an amplified scale the inward current overimposed on the control current obtained for a 50 mV depolarization. An acceleration in the activation and inactivation rate kinetics can be observed, as well as an increase in the amplitude of the inward current, most evident for copper and zinc. Figures 1B and C show the inward and outward current-voltage curves measured at the peak of the inward current and at the end of the voltage pulse, respectively. From the I-V curves for the inward current, we see that exposure to 0.1 μM of the divalent cation caused a shift in the voltage dependence of activation; this shift shows the same pattern for the three metal cations although the magnitude is quantitatively different, as seen in the corresponding I-V curves. Increasing these divalent cations to 1 μM has two effects: for the inward current, it decreases the amplitude plus the activation and inactivation rates (insets Fig. 1A and 1B) and it also decreases the outward current (Fig. 1C). At this concentration, Cu^{2+} blocks approximately 14% of the outward current measured at the end of the pulse, while Zn^{2+} and Ni^{2+} block around 50% of the outward current. We determined that the decrease in outward current corresponds almost exclusively to a loss of the calcium-activated component mediated by a blockade of the calcium current (see below).

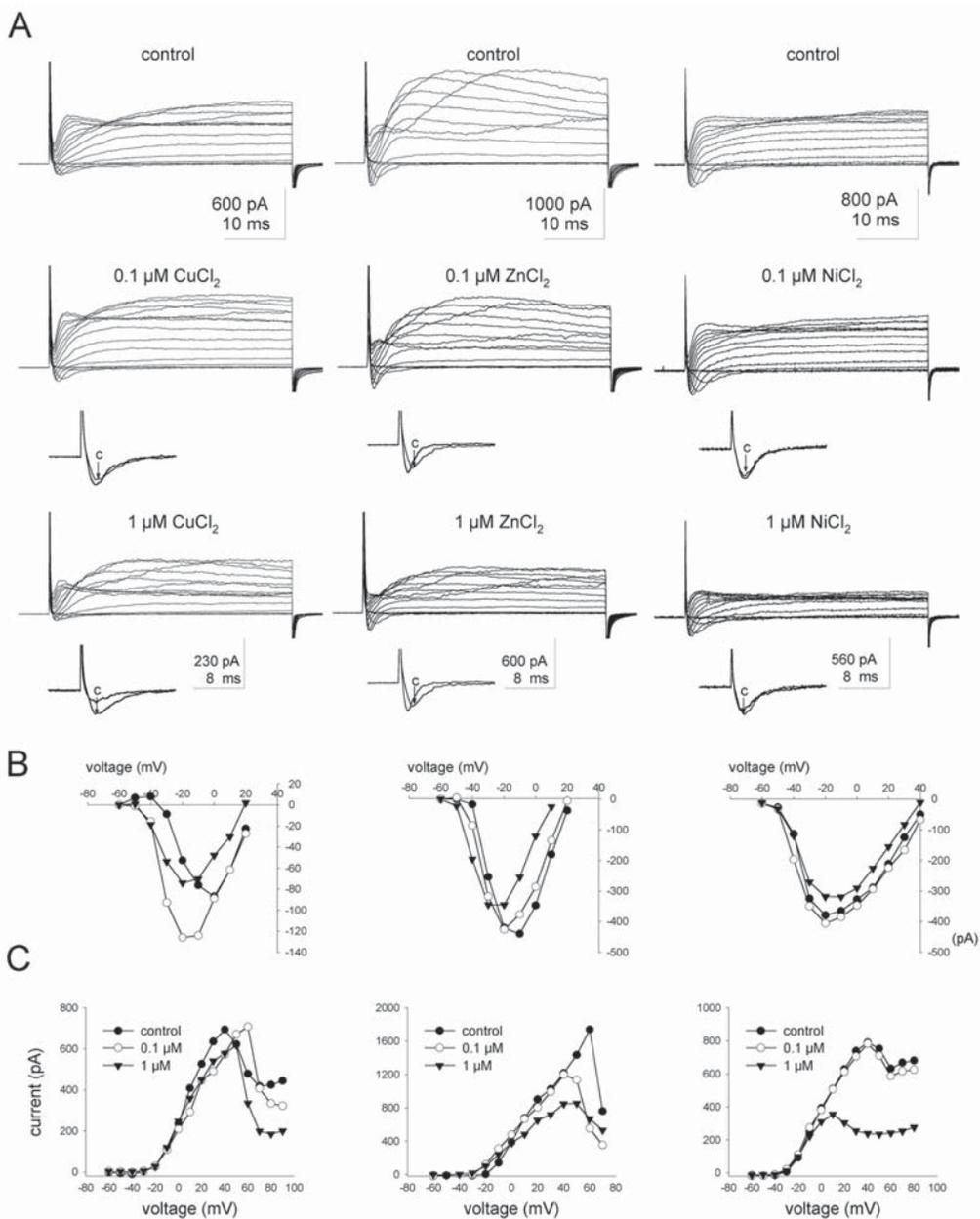


Figure 1. Differential effects of Cu^{2+} , Zn^{2+} and Ni^{2+} on whole cell currents in dissociated olfactory neurons. A: whole cell current traces from three different cells. The top traces were obtained with the cells bathed in normal extracellular saline. The middle traces were obtained after the addition of 0.1 μM Cu^{2+} , Zn^{2+} or Ni^{2+} to the external solution while the bottom traces were obtained in the presence of 1 μM of these divalent cations. The insets for the middle and bottom traces show only one of the currents recorded in the control condition (c) superimposed on the current trace obtained for the same depolarizing voltage in the presence of 0.1 or 1 μM of the divalent cation. The depolarizing voltage steps chosen for the insets are: from -80 to -30 mV. The number of cells exposed to copper, zinc and nickel were 20, 20 and 10 respectively. B: peak inward current for the traces shown in A, (●) control, (○) 0.1 μM , (▼) 1 μM . C: outward currents for the traces shown in A measured at the end of the pulse, (●) control, (○) 0.1 μM , (▼) 1 μM . The current and time scales for the total current traces is the upper one and for the insets, the lower one. Recordings are representative of 20 cells for copper, 20 cells for zinc and 10 cells for nickel.

Effects of Cu²⁺, Zn²⁺ and Ni²⁺ on the inward sodium current

Since the excitability of a neuron is to a great extent determined by the gating kinetics of sodium channels, we focused on the effect of copper, zinc and nickel on the inward sodium currents. To measure I_{Na} , we used conditions where potassium currents are greatly diminished. We replaced internal K^+ in the pipette solution with Cs^+ , a blocker of potassium channels and chose cells with low levels of calcium currents. Sodium currents in these cells presented the typical behavior: they activated at depolarizing potentials and then inactivated after a few milliseconds. Figure 2A shows the effect of low and high micromolar concentrations of divalent cations on the inward sodium currents. The peak inward current-voltage and the inactivation time constant vs. voltage plots for the current traces shown in figure 2A are shown in figures 2B and C, respectively. In the presence of 0.1 μM of the divalent cations, the current amplitude is increased and the kinetics of activation and inactivation accelerated. At higher concentrations, the current amplitude decreases, and the activation and inactivation become slower. The range of concentration where this inhibitory effect appears is not the same for all cations. At 5 μM , copper reduces the peak inward current in over 70% while nickel causes only a 40% reduction. A 20-fold higher dose of zinc (100 μM) produced only a 33% reduction of I_{Na} . The range of copper concentration that caused activation or inhibition did not vary significantly among cells but was quite variable for zinc or nickel. With 1 μM of these divalent cations, the inward current in some cells was augmented, while in others it was inhibited.

Effect of Cu²⁺, Zn²⁺ and Ni²⁺ on the inward calcium current

We replaced external Ca^{2+} by Ba^{2+} to better visualize the Ca^{2+} currents. Barium ions carry charge better through calcium channels than Ca^{2+} ions, and Ba^{2+} also blocks potassium channels (Hille, 2001).

Figure 3A shows currents recorded in response to a set of depolarizing voltage pulses in the presence of 2 mM external Ba^{2+} for two different cells. The inactivating inward sodium current and the non-inactivating inward calcium current can be recognized easily. Currents obtained for the same pulse protocol applied in the presence of micromolar Cu^{2+} or Ni^{2+} are shown in figure 3B, left or right panels, respectively. We found that the calcium current component was completely blocked by 10 μM Cu^{2+} and by 0.5 μM Ni^{2+} but, I_{Na} was only partially inhibited. These results indicate that Ni^{2+} blocks I_{Ca} more effectively than Cu^{2+} . Ten μM Cu^{2+} also decreased the amplitude of I_{Na} and slowed both activation and inactivation kinetics, as described in the previous paragraph. Nevertheless, the percent blockade of I_{Na} in the presence of barium is less than in the presence of calcium. Zinc ions have a similar effect (not shown) as copper and nickel on calcium currents measured in the presence of barium.

Effect of copper on neuronal firing rate

The subtle acceleration in kinetics and increase in amplitude of the inward sodium current triggered by low concentrations of divalent cations and the reduction in the amplitude and kinetics of I_{Na} caused by higher cation concentrations hints that the chance of firing action potentials could be higher or lower than the basal value for cells exposed to low or high micromolar concentrations of these divalent cations, respectively. To examine if this was really the case, we tested the effect of copper in the magnitude of the inward sodium current, because it was the cation that had the highest effect on the current. To be able to detect increases or decreases in firing rate, we used cells that in control solution presented low or high firing rates on which we tested the effects of copper. Because the number of cells that fire spontaneously is rather low, we gave a depolarizing pulse to neurons in the cell attached mode kept under voltage clamp conditions. We recorded the action currents that were triggered by the

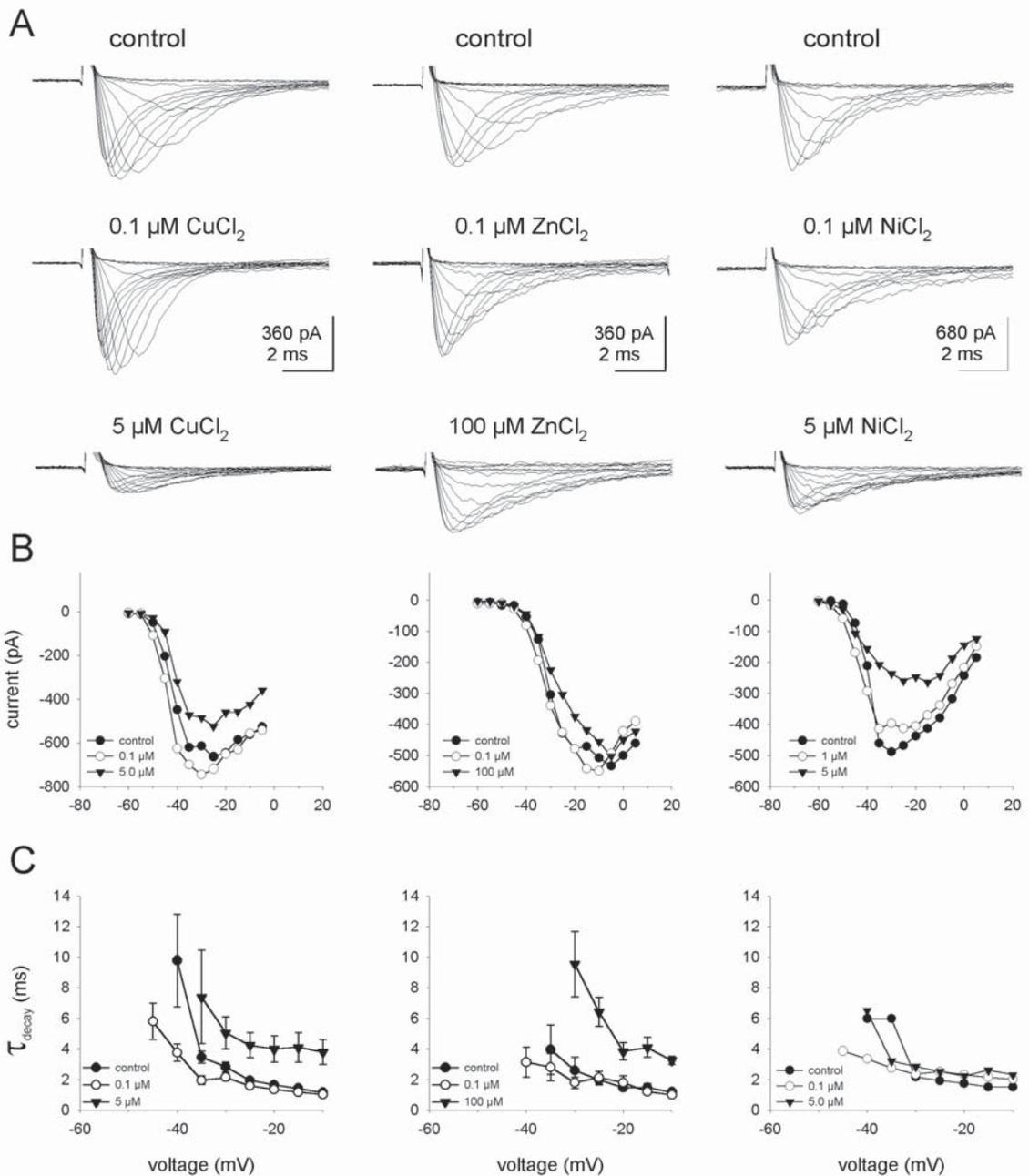


Figure 2. Effects of Cu^{2+} , Zn^{2+} and Ni^{2+} on inward sodium currents in dissociated olfactory neurons. A: top traces: Inward sodium currents from three different cells obtained when Cs^+ replaced K^+ in the pipette solution. Middle and bottom traces: Inward current traces from the same cells when the external saline was supplemented with 0.1 and 5 μM Cu^{2+} , 0.1 and 100 μM Zn^{2+} and 0.1 and 5 μM Ni^{2+} , respectively. Cells were clamped at -80 mV. B: I-V curves for peak inward current for the traces shown in A, (●) control, (○) 0.1 μM divalent cation, (▼) 5 μM copper and nickel or 100 μM zinc. C: inward current inactivation time constant (τ_{decay}) vs. voltage plots for recordings shown in A, (●) control, (○) 0.1 μM divalent cation, (▼) 5 μM copper and nickel or 100 μM zinc. These recordings are representative of 7 cells for copper, 7 cells for zinc and 4 cells for nickel.

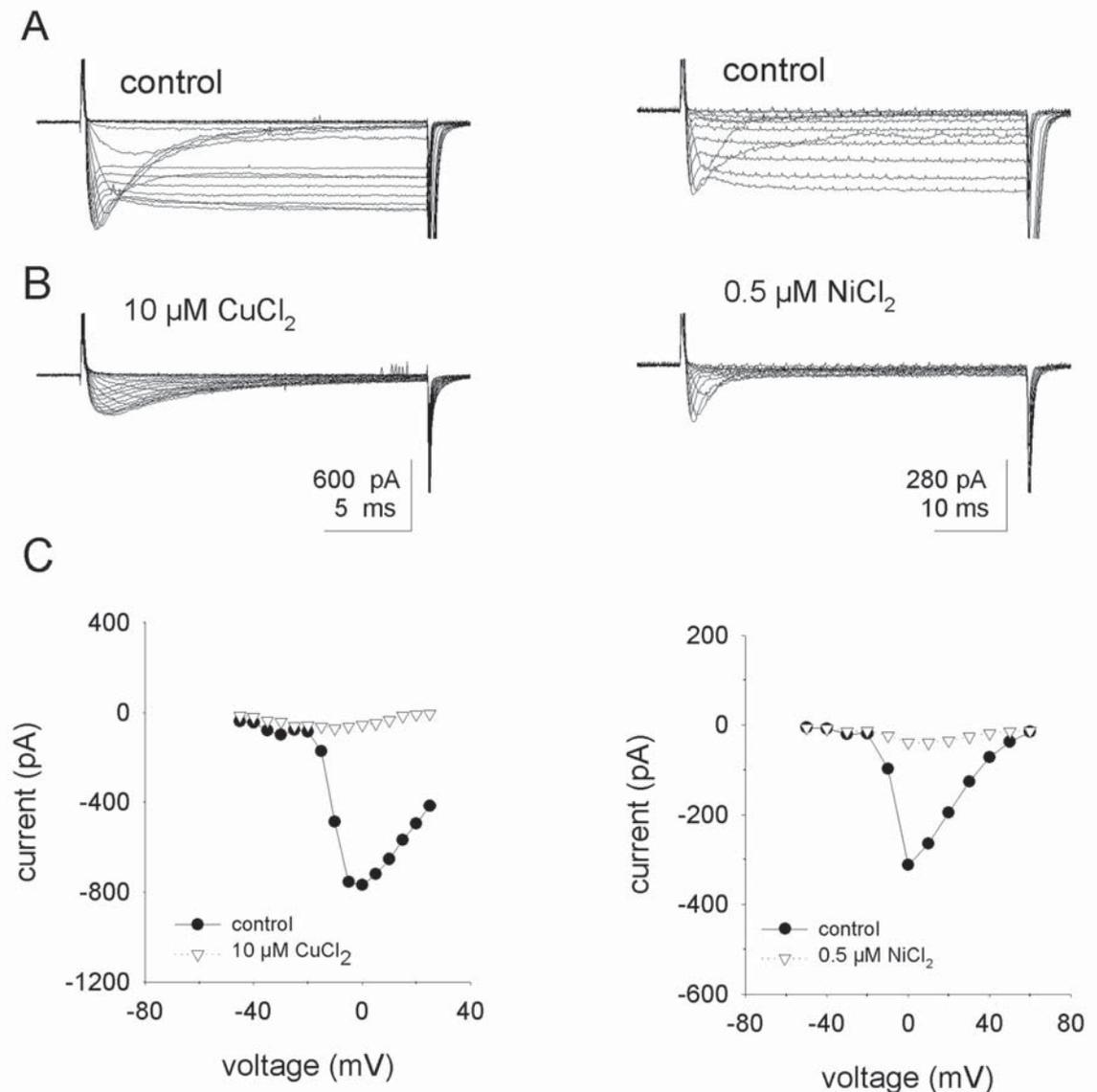


Figure 3. Comparison of the effects of Cu^{2+} and Ni^{2+} on the inward calcium current. A: representative inward current traces from two cells exposed to an extracellular saline in which Ca^{2+} was replaced by 2 mM Ba^{2+} . B: inward current traces from the same cells after $10 \mu\text{M}$ Cu^{2+} or $0.5 \mu\text{M}$ Ni^{2+} were added to the external saline. C: current-voltage curves for peak currents obtained from control and experimental ($10 \mu\text{M}$ of Cu^{2+} and $0.5 \mu\text{M}$ Ni^{2+}) current traces, respectively (●) control, (▽) $10 \mu\text{M}$ copper or $0.5 \mu\text{M}$ nickel. The recordings shown are representative of 3 cells tested under these same concentrations and of 8 tested at similar concentrations.

depolarization in normal saline or in saline supplemented with different concentrations of Cu^{2+} . In figure 4A, we see that a cell that responded to the depolarizing step (-80 mV to 30 mV) with two action potentials under control

conditions presented 14 spikes when the same pulse was repeated in the presence of 50 nM external copper. In figure 4B, we see a cell where firing was inhibited from 14 spikes to only 4 in the presence of 1 μM external copper.

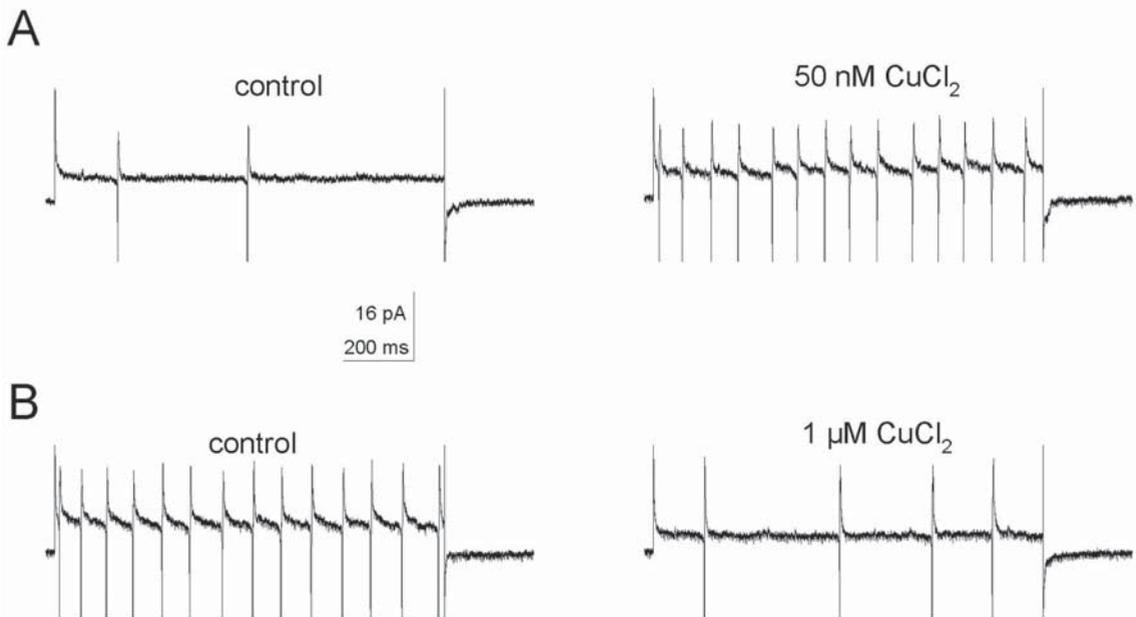


Figure 4. Effect of Cu²⁺ on neuronal firing rate. Differential effect of 0.05 μM and 1 μM Cu²⁺ concentrations on the action potential firing rate in isolated olfactory neurons. Two different cells were exposed to normal extracellular saline. Action currents were elicited by applying depolarizing voltage steps from -80 to 30 mV in the cell attached mode. The pulse was repeated in the presence of different copper concentrations. A: 0.05 μM Cu²⁺ makes the cell more excitable. B: 1 μM copper makes the cell less excitable. Recordings are representative of 8 cells tested.

DISCUSSION

The results herein presented support the idea that copper and zinc could be normal modulators of the excitable state of neuronal cells. It is quite possible that the molecular mechanisms associated to neuronal modulation by each ion would be different. Also, the physicochemical properties of these ions, as well as the concentration they reach when liberated into the synaptic clefts during synaptic activity, have to be considered. The activation and inhibition of I_{Na} at nano- and micromolar concentrations of divalent cations suggests that two sites of different affinities are involved in this effect.

Most of the previous studies regarding the role of copper and zinc on neuronal excitability have used rather high concentrations of these ions (10-100 μM) (Narahashi et al., 1994; Trombley et al., 1996; Erdelyi et al., 1998; Acuña-Castillo et al., 2000). Those values were probably

chosen after considering reports indicating that the copper and zinc concentrations reached in the synaptic space are in the order of 100 μM (Hartter and Barnea, 1988; Kardos et al., 1989). Nevertheless, it seems realistic to think that a considerable proportion of these metals are bound to proteins and that their free concentrations would be in the low micromolar or even nanomolar ranges. In order to approach a condition closer to the physiological, one should explore the effect of these ions on neuronal excitability in this lower concentration range (0.1-5 μM).

We found that, depending on their concentrations, the divalent metal ions Cu²⁺, Zn²⁺ and Ni²⁺ presented a differential effect on the kinetic and amplitude of the inward sodium current. Concentrations of 0.1 μM of these divalent cations produced a slight shift to the left for the I-V curve and an increase in the activation and inactivation rates of the inward current. On the contrary, in the

micromolar range (5-100 μM), the I-V curves were displaced to the right in the voltage axis, and they caused a concentration-dependent diminution of the inward sodium current. Even though the magnitude of the changes was slight, we found that dissociated neurons exposed to copper in the lower concentration range presented an increase in their firing rate contrasting with a diminished rate when exposed to higher concentrations of copper. We found a similar differential effect in the firing rate pattern for populations of neurons exposed to copper, zinc, nickel, or other divalent cations in isolated pieces of olfactory epithelia (unpublished results).

Our results show that copper activated and inhibited sodium currents at lower concentrations than Ni^{2+} and Zn^{2+} , indicating that both modulatory sites in sodium channels have a higher affinity for Cu^{2+} than for Ni^{2+} and Zn^{2+} . In the case of copper, we found that the SH modifying reagent dithiothreitol (DTT) reverts the inhibitory effect of copper upon neuronal firing rate (unpublished results), indicating that copper inhibition is mediated by direct or indirect oxidation of sulphhydryl groups. Also, in a previous study, we showed that micromolar Cu^{2+} induced a decrease in the open time probability in BK_{Ca} channels that could be reverted by DTT (Morera et al., 2003).

The divalent cations tested also blocked the calcium current and, therefore, indirectly decreased the calcium-activated component of the outward potassium current. Many neurons present calcium-activated currents in their somas and synaptic terminals and, given that the excitable state of a neuron depends on the balance of inward and outward currents, it seems unlikely that synaptically released copper and zinc would not affect the excitable state of the neurons around the release sites. Nevertheless, it is not easy to predict the final excitability response of a population of neurons that are exposed to copper and/or zinc. For a population of cells, the final outcome will depend on the state of electrical activity plus redox state of each neuron in the group.

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