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External Copper Inhibits the Activity of the Large-Conductance Calcium- and Voltage-sensitive Potassium Channel from Skeletal Muscle

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Abstract. We have characterized the effect of external copper on the gating properties of the large-conductance calcium- and voltage-sensitive potassium channel from skeletal muscle, incorporated into artificial bilayers. The effect of Cu^{2+} was evaluated as changes in the gating kinetic properties of the channel after the addition of this ion. We found that, from concentrations of 20 μM and up, copper induced a concentration- and time-dependent decrease in channel open probability. The inhibition of channel activity by Cu^{2+} could not be reversed by washing or by addition of the copper chelator, bathocuproinedisulfonic acid. However, channel activity was appreciably restored by the sulfhydryl reducing agent dithiothreitol. The effect of copper was specific since other transition metal divalent cations such as Ni^{2+}, Zn^{2+} or Cd^{2+} did not affect BK_{Ca} channel activity in the same concentration range. These results suggest that external Cu^{2+}-induced inhibition of channel activity was due to direct or indirect oxidation of key amino-acid sulfhydryl groups that might have a role in channel gating.

Key words: Copper — High-conductance Ca^{2+}-activated K^+ channels — Redox modulation — Sulfhydryl groups — Free radicals

Introduction

Copper is an essential trace element playing important roles in several cellular functions. It is also normally present in the central nervous system and its concentration is rather high in some regions like the cerebral cortex, hypothalamus and the olfactory bulb (Donaldson et al., 1973; Kardos et al., 1989; Ono & Cherian, 1999). In these regions, it is mainly stored in synaptic vesicles from where it is released during synaptic events generally accompanied by zinc (Donaldson et al., 1973; Hartter & Barnea, 1988; Kardos et al., 1989; Sato et al., 1994, Ono & Cherian, 1999).

There is evidence that the synaptically-released copper, which could reach extracellular concentrations up to 100 μM in the synaptic space (Kardos et al., 1989), has modulatory effects over neurons that might be physiologically relevant (Trombley & Shepherd, 1996; Horning & Trombley, 2001). Also, several findings indicate that copper may directly or indirectly participate in the pathogenesis of various human disorders like Wilson, Menkes, Parkinson, Alzheimer and prion diseases (Opazo, Ruizz-Inestroza, 2000; Rotilio et al., 2000; Strausak et al., 2001; Jobling et al., 2001; White et al., 2002).

Recent reports indicate that targets of copper action are neuronal postsynaptic neurotransmitter receptors (Narahashi et al., 1994; Trombley et al., 1996; Sharonova, Vorobjev & Hass, 1998; Erdelyi et al., 1998; Acuña-Castillo, Morales & Huidobro-Toro, 2000) and voltage-gated ion channels (Horning & Trombley, 2001; Ricardo Delgado, personal communication).

The mechanisms by which copper present in the extracellular milieu exerts its physiological or pathological effects are not known but they are being investigated in several laboratories. Assessing the effect of copper on ion channel conductance and gating kinetics at the single-channel level may give clues as to how this metal interacts with these types of membrane proteins. An interesting hypothesis is that Cu^{2+}, being a metal with oxidative properties, may alter the redox state of ion channels either by direct oxidation of radicals susceptible to oxido-reduction or indirectly as a source of reactive oxygen species. To test this assumption we investigated the effect of external Cu^{2+} on the ubiquitous large-conductance calcium- and voltage-sensitive potassium (BK_{Ca})

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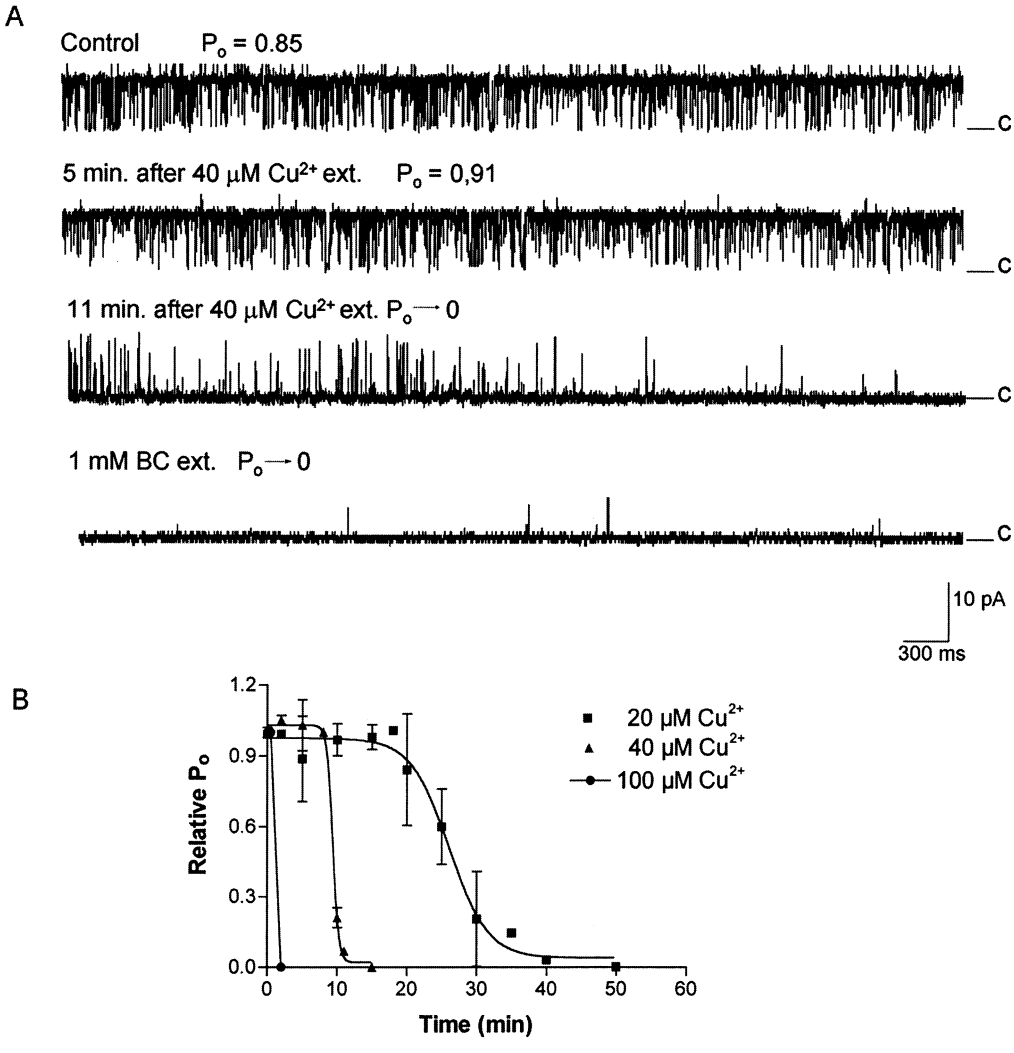


Fig. 1. Effect of external copper on BK_{Ca} channel P_o. (*A*) Single-channel current traces at 40 mV in symmetrical 100 mM KCl, 10 mM MOPS, pH 7.0 and 60 μM internal Ca^{2+}. First trace, control conditions; second trace, at 5 min after the addition of 40 μM $CuCl_2$; third trace, 11 min after copper addition; and last trace, after the addition of bathocuproinedisulfonic acid (BC). The line at the right side of the current records indicates the closed state. (*B*) Time course of the relative P_o (P_o experimental/P_o control) after the addition of 20, 40 and 100 μM Cu^{2+} to the solution. Curves in Fig. 1*B* were fitted to a polynomial using the GraphPad Prism 3.02 program and have no theoretical meaning.

channel incorporated into planar lipid bilayers under different experimental conditions. Several studies have demonstrated that these channels, present in different cells, including neurons, may be redox-modulated (Wang et al., 1997; DiChiara & Reinhart, 1997; Kourie J., 1998; Soto et al., 2002).

Our results indicate that external Cu^{2+} induces an inhibition of BK_{Ca} channel activity, which may be explained by a direct copper oxidation of external amino-acid sulfhydryl groups that affect channel gating.

Materials and Methods

Plasma Membrane Preparation

Plasma membrane vesicles from adult rat skeletal muscle were prepared using a simplified version of the method of Rosemblatt et al. (1981). Briefly, rat skeletal muscles were homogenized in a sucrose buffer supplemented with a cocktail of protease inhibitors. After three steps of centrifugation at different speeds, microsomal membrane fractions were collected and loaded on top of a 27% buffered sucrose solution that was centrifuged at 100,000 × *g*. The band that stayed in the top was collected, spun down, resuspended, divided into 10–20 μl aliquots and kept frozen at −80°C until used to incorporate BK_{Ca} channels in the bilayer experiments.

Planar Bilayers and Single-Channel Recordings

Bilayers were formed by applying a drop of a lipid mixture of phosphatidylethanolamine and phosphatydilcholine (Avanti Polar Lipids, Birmingham, AL) in decane to a 200-μm-diameter hole of a delrin cup separating two saline compartments, each containing 100 mM KCl, 10 mM MOPS-K (pH 7.0). Internal Ca^{2+} concentration was adjusted to obtain control channel open probability (P_o) values higher than 0.6.

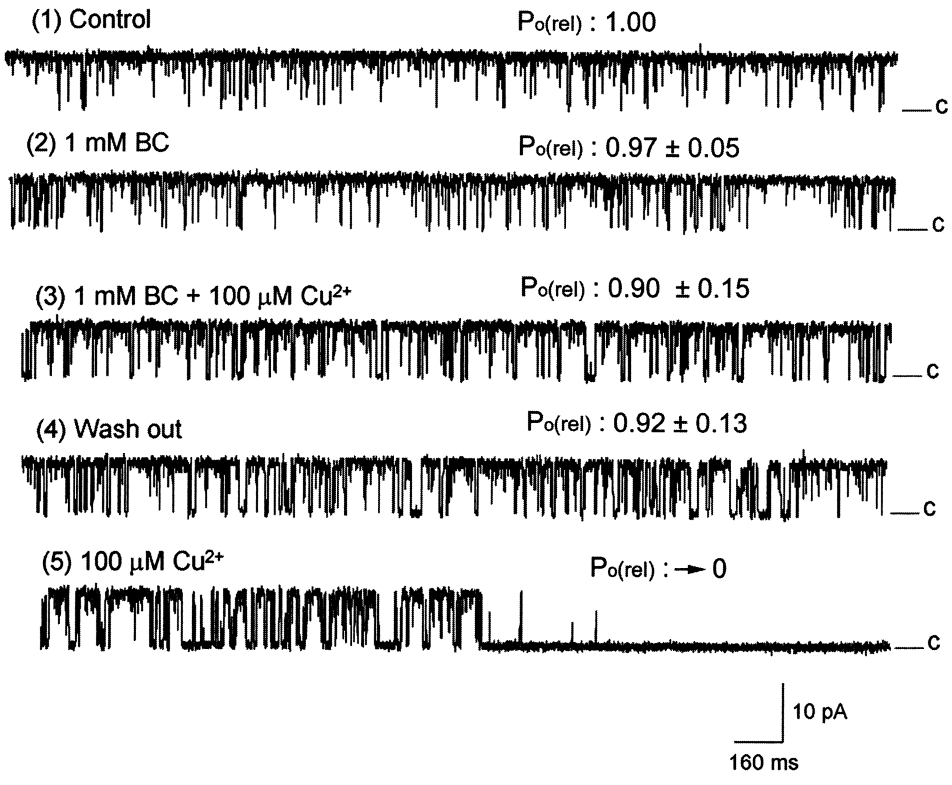


Fig. 2. Bathocuproinedisulfonic acid prevents inhibition of BK_{Ca} channel by copper. Representative three-second current traces under the following experimental conditions: (*1*) control; (*2*) 1 mM external bathocuproinedisulfonic acid (BC); (*3*) 1 mM external bathocuproinedisulfonic acid (BC) + 100 μM external Cu^{2+}; (*4*) wash; (*5*) 100 μM external Cu^{2+}. Relative P_o values determined from 3–5 minutes in each condition are shown for each trace.

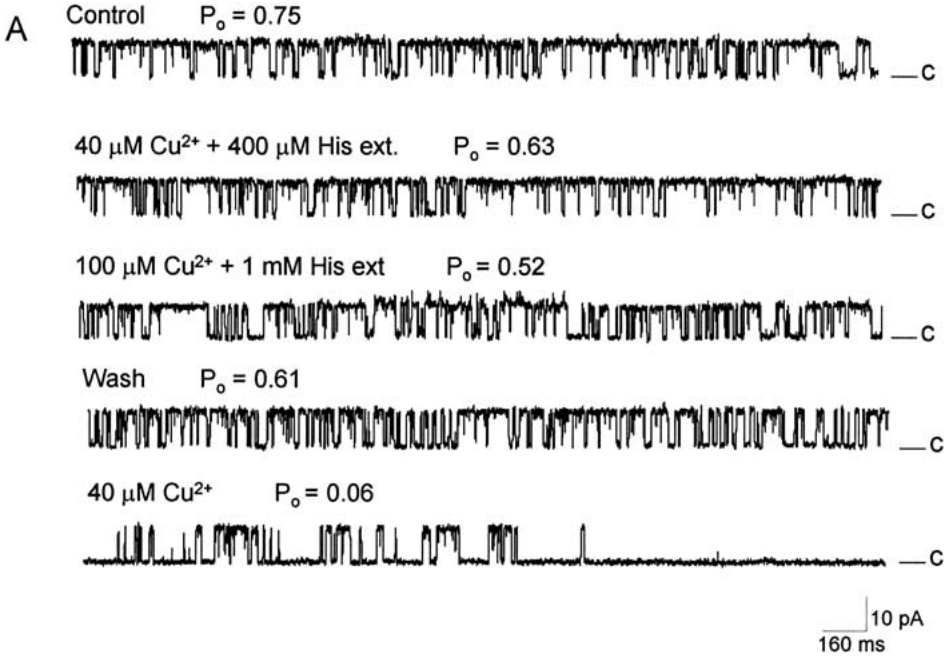
Large-conductance Ca^{2+}-activated potassium channels from rat skeletal muscle were incorporated into artificial lipid bilayers, as previously described (Vergara, Alvarez & Latorre, 1999). Channel insertion occurred spontaneously after touching the bilayer with a droplet of membrane vesicles. Appearance of single BK_{Ca} channels was detected as rapid discrete current fluctuations when a constant voltage difference was applied across the bilayer. After incorporation of a channel, control single-channel current was recorded for a few minutes; then Cu^{2+} was added to the solution bathing the channel's extracellular side. Currents were recorded with a two-electrode voltage clamp (Alvarez, Benos & Latorre, 1985). One compartment (*cis*) was connected to a voltage-pulse generator and the opposite (*trans*) to a low-noise current-to-voltage converter through Ag/AgCl electrodes. The current was amplified and stored on videotape using frequency modulation on the audio channel of a VCR (Alvarez, 1995). Current was filtered at 400 Hz and digitized at 500 μs per point. Open and closed events were identified using a discriminator located at 50% of the open channel current. P_o, mean open and mean closed times were analyzed with PClamp6 analysis software (Axon Instruments).

Results

External Copper Decreases Open Probability of BK_{Ca} Channels in a Time- and Concentration-dependent Manner

Exposure of the channel to low external micromolar copper concentrations (1–10 μM) did not appreciably affect channel open probability values (P_o = 0.94 ± 0.06; mean ± SD; n = 20) for time periods of up to 3 h. However, from 20 μM and up, copper induced a decrease in channel P_o, preceded by a lag time period that was dependent on copper concentration.

Figure 1*A* shows the effect of 40 μM copper on single-channel currents under an applied voltage of +40 mV. In this example, after a lag time of about 10 minutes, channel activity suddenly began to decrease, reaching finally a state of very low opening probability (P_o = 0.07 ± 0.06; mean ± SD; n = 6) without change in the unitary conductance. Data analysis showed that this decrease in P_o was mainly due to the appearance of a new distribution of closed times. For the control conditions that were chosen for this series of experiments, (i.e., P_o values >0.6) the mean closed time was well described by a single distribution with a mean duration of ~15 ms. After addition of copper, a second population of closed times with a longer mean duration appeared until the channel reached a point where a catastrophic event developed, leading it to a closed state, that in most cases was irreversible. The mean duration of the second distribution of closed times varied between 60 and 300 ms, depending on copper concentration and/or time elapsed since copper addition. Mean open times were described by two distributions and copper



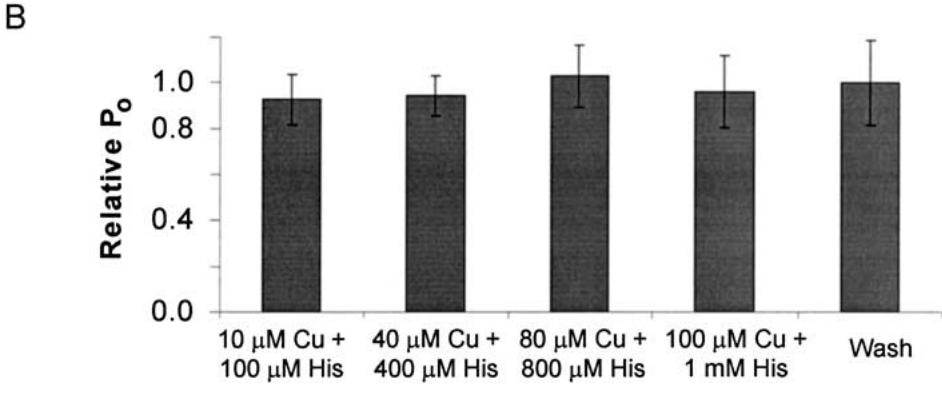


Fig. 3. Copper-histidine (Cu-His, 1:10) does not affect BK_{Ca} channel activity. (*A*) Single-channel currents without copper (control) and in the presence of 40/400 μM and 100/1000 μM Cu-His on the external side. The records were obtained in symmetrical 100 mM KCl, 10 mM MOPS, pH 7.0 and internal contaminant [Ca^{2+}]. (*B*) Effect of copper bound to histidine at four different concentrations. Values correspond to the average of three independent experiments. Error bars represent SD.

caused a decrease in the duration of the longest component.

The effect of copper was time- and concentration-dependent. Figure 1*B* shows the time course of relative P_o (P_o experimental/P_o control) change after the addition of 20, 40 and 100 μM external copper. The lag time for 20 μM copper effect is about 20 minutes, while at 100 μM, the lag time is about 1 minute. Also the time course of P_o decay is much faster at 100 μM than at 20 μM. Experimental points were fitted to a polynomial using a nonlinear fitting program.

It is highly unlikely that this effect could be attributed to a slow Cu^{2+}-induced blockade of the channel pore since we see the effect at positive potentials that would tend to drive copper ions away from it. Neither washing nor the addition of the copper chelator bathocuproinedisulfonic acid (BC), after the channel had reached this extremely low P_o, could recover channel activity. This behavior was the most common, but around 2% of the channels were insensitive to copper even up to 100 μM.

BK_{Ca}-Channel Decrease in P_o is Mediated by Free Copper Ions

Figure 2 shows that if 100 μM external copper is added in the presence of 1 mM external BC, BK_{Ca} channel activity is not affected. Trace *1* shows the channel in the initial control conditions; trace *2*, after the addition of 1 mM BC; trace *3* was obtained after adding 100 μM Cu^{2+} to the medium already containing 1 mM BC; trace *4* was recorded after returning to control

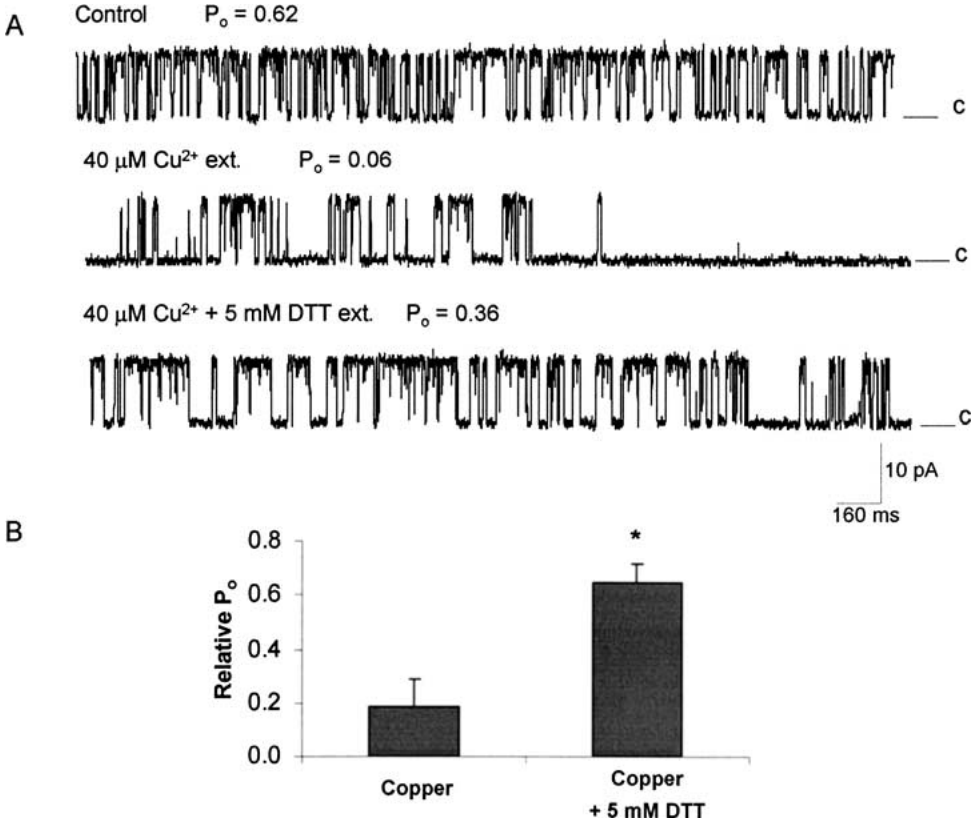


Fig. 4. External DTT reverses BK_{Ca} channel inhibition induced by copper. (*A*) Single-channel currents: control, 40 μM Cu^{2+}, and 40 μM Cu^{2+} + 5 mM DTT on the external side of the channel. The record was obtained at 40 mV in symmetrical 100 mM KCl, 10 mM MOPS, pH 7.0 and internal contaminant [Ca^{2+}]. (*B*) Summary of 6 independent experiments. Error bars represent SD. *$p < 0.005$.

conditions by washing away copper and BC and finally, trace *5* was obtained after adding again 100 μM external Cu^{2+}. The addition of Cu^{2+} after washing BC produced the inhibition of channel activity. The numbers above traces show the average relative P_o values obtained for 3–5 minutes in each condition.

Figure 3*A* shows current recordings when copper (40 and 100 μM) was added bound to histidine in a 1/10 concentration ratio. As in the previous experiment, it can be observed that BK_{Ca} channel activity is not affected. Addition of 40 μM Cu^{2+} after washing induced the typical inhibition of channel activity. Figure 3*B* summarizes the effect of copper bound with histidine at four different concentrations. Each bar represents the average of three independent experiments. These results show that as long as copper is bound, to either bathocuproinedisulfonic acid or histidine, it does not affect BK_{Ca} open probability (P_o = 0.94 ± 0.09; mean ± SD; n = 6), indicating that it is the free metal that interacts with the channel.

Does Cu^{2+} Affect the BK_{Ca} Channel as a Result of its Oxidative Properties?

Since Cu^{2+} has oxidative properties, we considered the possibility that the observed effect of this divalent

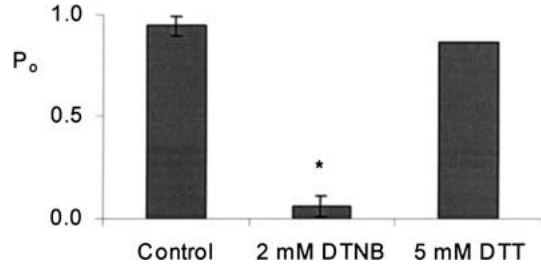


Fig. 5. DTT reversed BK_{Ca} channel inhibition induced by 2 mM DTNB. Experiments were carried out at 40 mV in symmetrical 100 mM KCl, 10 mM MOPS, pH 7.0 and internal contaminant [Ca^{2+}]. (*B*) Summary of 6 independent experiments. Error bars represent SD. *$p < 0.005$.

cation on P_o could be mediated by an oxidative reaction of some residues that could affect channel gating. To this end we tested if the decrease in P_o could be reverted by the sulfhydryl reducing agent DTT. Figure 4 shows that the addition of 5 mM DTT after the channel had entered the very low-activity mode (P_o = 0.09 ± 0.06; mean ± SD; n = 4), produced a significant reversion of P_o to control values (P_o = 0.65 ± 0.07; mean ± SD; n = 4). This result suggests that external cysteine residues could be the targets of copper ions. To demonstrate the presence

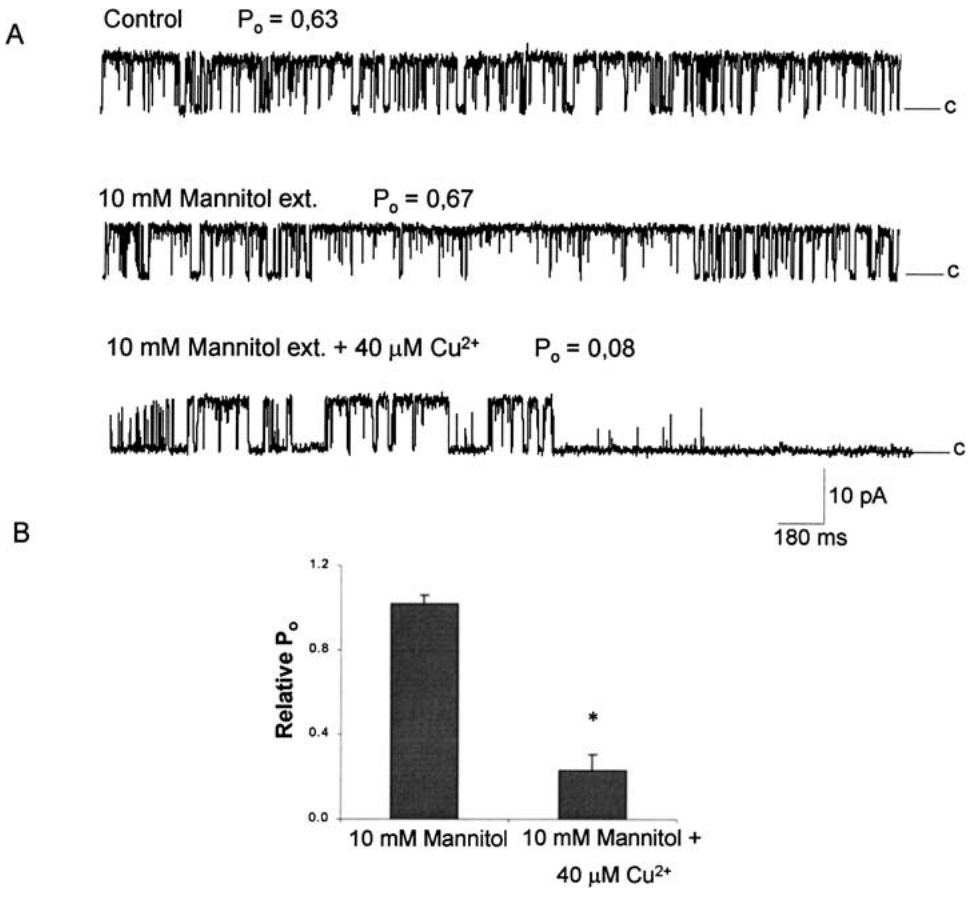


Fig. 6. Mannitol does not protect BK_{Ca} channel from copper inhibition. (*A*) Single-channel currents without copper (control), in the presence of 10 mM mannitol, and 10 mM mannitol + 40 μM Cu^{2+}. The record was obtained at 40 mV in symmetrical 100 mM KCl, 10 mM MOPS, pH 7.0 and internal contaminant [Ca^{2+}]. (*B*) Summary of 3 independent experiments. Error bars represent SD. *$p < 0.005$.

of these residues and their accessibility to oxidation we tested the effect of the SH oxidizing agent 5,5′-dithiobis(2-nitrobenzoic acid (DTNB). Figure 5 shows that 2 mM external DTNB causes a drastic reduction in P_o, similar to that produced by copper ions, from 0.95 ± 0.05 to 0.06 ± 0.05; mean ± SD; $n = 6$. The addition of DTT after washing of DTNB produced a recovery of channel activity to P_o values close to control conditions.

The Effect of Copper upon BK_{Ca} Channel Gating Apparently is not Mediated by Reactive Oxygen Species (ROS)

To assess if the effect of copper was indirectly mediated by ROS, we tested the effect of mannitol, an ROS scavenger. As shown in Fig. 6, the addition of this compound at 10 mM does not protect BK_{Ca} channel from copper inhibition, suggesting that the effect did not occur through indirect oxidations mediated by ROS present in the medium.

We also observed that copper inhibited BK_{Ca} channel activity in normoxygenated (oxygen partial pressure of 140 mm Hg) as well as in deoxygenated solutions (oxygen partial pressure of 15 to 20 mm Hg; *data not shown*). This finding also supports the idea that this inhibitory effect was not mediated by ROS.

Specificity of the Copper Effect

We compared the effect of Cu^{2+} with that of other transition metal divalent cations with similar ionic radii but different redox potentials, like Zn^{2+}, Ni^{2+}, and Cd^{2+}, to test the influence of ion charge and of their redox potentials. As shown in Fig. 7, neither Zn^{2+}, Ni^{2+} nor Cd^{2+} at 100 μM caused a significant decrease in channel P_o after 10 minutes of their addition.

Discussion

Copper is normally present in the nervous system mainly stored in synaptic vesicles and it is released during synaptic events generally accompanied by zinc (Donaldson et al., 1973; Hartter & Barnea, 1988, Kardos et al., 1989, Sato et al., 1994, Ono & Cherian, 1999). There is evidence that this synaptically released

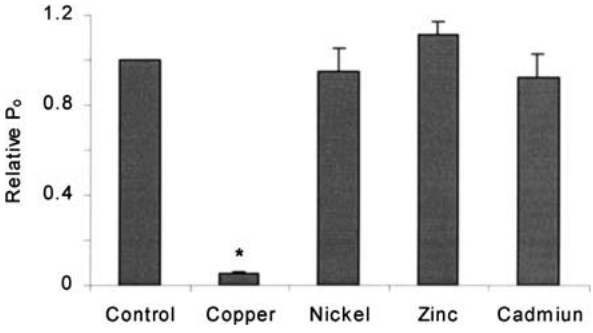


Fig. 7. Effect of Cu^{2+}, Ni^{2+}, Zn^{2+} and Cd^{2+} at 100 μM on BK_{Ca} channel activity. Each bar represents the relative P_o after 10 min of exposure to each cation added as a chloride salt. Values are means of 6–7 experiments. Error bars represent SD.

copper may modulate neuron electrophysiological activity (Trombley & Shepherd, 1996, Horning & Trombley, 2001). Recent findings indicate that postsynaptic neurotransmitter receptors and voltage-gated ion channels could be target sites of copper action. Several authors have reported about the effect of copper on either ligand- or voltage-gated macroscopic currents in isolated neurons using the patch-clamp technique (Trombley Horning & Blackemore, 1998; Sharonova et al., 1998; Erdelyi et al., 1998; Acuña-Castillo et al., 2000; Horning & Trombley, 2001). Regarding copper sensitivity of ion channel blockade, Trombley & Shepherd (1996) found that Cu^{2+} in the μM range (IC_{50} of ~20 μM) antagonized both N-methyl-D-aspartate (NMDA)- and GABA-mediated currents in rat olfactory bulb neurons. These effects were rapidly reversed by washing, suggesting a blockade mechanism. Horning and Trombley (2001) found in these neurons that 30 μM copper inhibited TTX-sensitive sodium current, delayed rectifier-type potassium current, type A potassium current and inward calcium current. The blockade of the first three currents was around 20%, while it was around 50% for the calcium current. On the other hand, Sharanova et al. (1998) reported that GABA-induced currents in dissociated Purkinje cells are blocked by copper with an IC_{50} of 35 nM. The inhibitory effect found by us in BK_{Ca} occurs in the mid-micromolar range.

Since macroscopic current measurements cannot be made directly to determine if copper is affecting single-channel conductance, opening probability, or the number of channels that contribute to the current, we decided to study the effect of copper at the single-channel level. To this aim, we tested the effect of Cu^{2+} on the BK_{Ca} channel, widely distributed in different cells and tissues including the nervous system, and whose biophysical properties are very well characterized (Oberhauser, Alvarez & Latorre, 1988; Laurido et al., 1991; Vergara et al., 1998, 1999).

We found that the exposure of BK_{Ca} channels to external Cu^{2+} caused a decrease in their P_o preceded by a lag time period. The length of the lag time and time constant of the P_o decay were highly dependent on copper concentration. (*see* Fig. 1*B*). For a concentration of 20 μM, P_o was not affected for about 20 minutes and then slowly decayed. However, at 100 μM the lag time lasted only a couple of minutes and the time course of P_o decrease was very fast. The copper effect was also specific, since other transition metal divalent cations with similar ionic radii such as Ni^{2+}, Zn^{2+} or Cd^{2+} did not decrease channel P_o. The access of copper to the groups it modifies is apparently restricted when the channel is closed. After a control recording, 100 μM Cu^{2+} was added in conditions of low opening probability during 2 to 3 min. Upon returning to control conditions, channels were no longer in the very low P_o state (*data not shown*). The fact that copper did not inhibit channel activity when it was chelated with bathocuproinedisulfonic acid or with histidine, indicates that free Cu^{2+} ions were involved in the effect. On the other hand, the decrease in activity did not seem to be produced by blockade of the channel pore by Cu^{2+}. The inhibition by copper was, however, significantly reversed by the addition of 5 mM DTT, suggesting that external cysteine residues could be involved. We found that the sulfhydryl oxidizing agent DTNB (2 mM) inhibited BK_{Ca} channel activity in a way that closely mimics the effect of copper, and its effect was also reversed by DTT, although to a higher extent. This supports the idea that extracellularly-oriented cysteines are present and susceptible to being oxidized by copper and by DTNB. The higher degree of recovery reached with DTT after DTNB exposure as compared to recovery after copper suggests that this cation affects other residues besides cysteines.

Several authors have reported that BK_{Ca} channels from different sources, studied either in situ with the patch-clamp technique or incorporated into artificial bilayers may be redox-modulated (Wang et al., 1997; DiChiara & Reinhart, 1997; Soto et al., 2002). The intracellular application of H_2O_2 or SH oxidative agents such as DTNB or thimerosal caused a strong reduction of channel activity and, similarly to what we found extracellularly, DTT partially reversed the inhibition (Soto et al., 2002). However, the application of external ROS or SH agents has shown to be less effective or without effect (Soto et al., 2002).

In the *hslo* channel, each one of the tetramer subunits has three external cysteines (C14, C141, and C277) those could be copper ions targets. Copper could generate S-S bridges by directly oxidizing SH groups if any two of the twelve external cysteines were closely located. Alternatively, it could catalyze the autooxidation of these residues. The finding that mannitol did not protect the channel from copper inhibition suggests that hydroxyl radicals (OH) are

not being produced. Moreover, the results of Soto et al. (2002), showing that external H_2O_2 up to 23 mM did not affect BK_{Ca} channel activity, support the assumption that ROS are not mediating the effect of copper.

In preliminary experiments attempting to asses the role of external cysteines on copper effect, we expressed in *X. laevis* oocytes an hslo mutant in which external cysteines were replaced by serines (C14S, C141S, C277S). In channels obtained from an oocyte membrane preparation we found that after 10 minutes of exposure to 100 μM external copper, only 40% of the channels showed a significant decrease in P_o, while for wild-type channels this occurs for 99% of them. In summary, all our results indicate that external copper affects BK_{Ca} channel activity by a redox effect.

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