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Administration of copper reduced the hyper-excitability of neurons in CA1 hippocampal slices from epileptic rats

J. LEIVA, C. INFANTE

Facultad de Medicina, Instituto de Ciencias, Biomédicas, Universidad de Chile

ABSTRACT

Copper as a trace metal is involved in several neurodegenerative illnesses, such as Menkes, Wilson's, Alzheimer's, amyotrophic lateral sclerosis (ALS), and Creutzfeldt-Jakob. Electrophysiological evidence indicates that acute perfusion of copper can inhibit long-term synaptic potentiation in hippocampal slices. The objective of this work is to determine whether Cu perfusion can perturb synaptic transmission in hippocampal slices derived from pilocarpine-treated epileptic rats.

Field potential (FP) recordings of the CA1 neurons of rats with chronic epilepsy showed voltage and response duration decrease following copper sulfate perfusion. However, voltage and response duration were higher after removing copper by washing. The discharge frequency of the CA1 neurons of hippocampal slices from non-epileptic control rats was increased after acute perfusion of 10 μM of pilocarpine. This increase was blocked by administering copper sulphate 10 μM. Krebs-Ringer solution washing re-established the discharges, with a higher frequency than that provoked by pilocarpine perfusion. We discuss the blocking effect of copper and the synaptic hyper-excitability generated by its removal.

Key words

Copper • Field potential (FP) • Hippocampus slices • Pilocarpine-induced chronic epilepsy • Rats

Introduction

Copper is an essential element in many cellular functions (Azimi and Rauk, 2011; Madsen and Gitlin, 2007) and is an integral part of several protein and enzymatic reactions. Copper binds thiolates, amines and carboxyl groups present in diverse macromolecules (Cousins, 1994; Danks, 1995; Linder and Hazegh-Azam, 1996). Copper has been linked to pathologies such as Alzheimer's, Parkinson's and amyotrophic lateral sclerosis (Bush, 2003; Sparks and Schreurs, 2003; Gaier et al., 2013; Stys et al., 2012). Recent evidence indicates that copper blocks NMDA receptors in CA1 neurons by suppressing long-term synaptic potential (Doroulee et al., 1997; Leiva et al., 2003; Leiva et al., 2009).

The aim of this study is to determine the effect of copper on synaptic excitability in CA1 neurons slices derived from pilocarpine-treated epileptic rats and non- epileptic control with acute pilocarpine and copper perfusion.

Experimental procedures

The experiments were conducted in accordance with the Guide for Care and Use of Laboratory Animals, published by the *National Institutes of Health* and the experimental protocol was approved by the Bioethical Committee of the Institute of Biomedical Sciences, Faculty of Medicine of the Universidad de Chile.

Corresponding author: Prof. Juan Leiva Rojas, Casilla 16038 Santiago 9, Chile Av. Salvador 486, Campus Oriente - Tel.: +56 2297 70546 - Email: jleiva@med.uchile.cl

Adult male Wistar rats (n=30) weighing between 200 and 250 g, were maintained at 22 ± 1°C with a light/dark cycle of 12/12 h. The animals were divided into groups of five each. All the groups received 280mg/kg of pilocarpine by intraperitoneal injection (Sigma, St. Louis, MO). Thirty minutes before administering pilocarpine, 1 mg/Kg of scopolamine (Sigma, St. Louis, MO, USA) dissolved in 0.5 ml of saline was administered to attenuate the neurovegetative effects of pilocarpine (Priel et al., 1996). The rats were monitored visually in a transparent 30x20x27 cm plexiglass cage for 10 minutes before and 60 minutes after administering pilocarpine. Twenty of the thirty animals treated with pilocarpine survived and became epileptic. They were observed for 15 to 18 days before being sacrificed and prior to sacrifice their behavior was assessed. The rats were then superficially anesthetized with ether and euthanized with a Stoelting guillotine. The brain was rapidly removed under a continuous cold Krebs-Ringer (K-R) drip and then submerged for a short period in K-R at 4º C. The hippocampus was removed and cut into 400 µM coronal slices with a McIlwain tissue chopper (Mickle Laboratory Engineering, Surrey, UK). The slices were submerged for 1 h in an oxygenated incubation chamber and then transferred to the immersion register chamber with continuous Krebs-Ringer perfusion at 31 ± 1ºC.

The K-R solution (in mM) was composed of NaCl 124.0, KCl 5.0, KH_2PO_4 1.25, MgSO4 + $7H_2O$ 2.0, $CaCl_2$ 2.0, $NaHCO_3$ 26.0 and glucose 10 at pH 7.4. The K-R solution was previously bubbled with carboxygen (95% O_2 and 5% CO_2). The immersion chamber was perfused with a continuous flow of 2 to 3 ml/min.

In vitro slice recording

Hippocampal slices were placed on a nylon net and stratum radiatum was electrically stimulated using bipolar tungsten electrodes. Extracellular recordings were made with glass micropipettes. Microelectrodes were filled with K-R solution and showed impedance of 1-2 MΩ. These microelectrodes were made for recording of extracellular spike activity and field potentials (FP) (Kirkwood and Bear, 1994). The microelectrodes were visually positioned in the CA1 with the aid of a binocular lens (Carl Zeiss). A hydraulic micromanipulator drove the micropipettes through the slice. Neural activity was amplified (Bio-Logic VF180) and displayed in an oscilloscope (Hitachi VC-6020). All recordings were tape recorded (SONY.DTC.59 E.S) for off-line analysis. A DG2 digitimer trigger generator connected to an isolated stimulator (model DS2A) generated a single stimulus of 0.05-6.0 volts and 0.030 ms in duration. Increasing voltage to twice the threshold induced unitary and or field potential (FP) response. Data were analyzed with an analogue-to-digital data convertor (Cambridge Electronic Design, Cambridge, UK). The digitized data were averaged with SIGAVG program on a Pentium microcomputer.

Statistical Analysis

Statistical changes in the voltage and duration of FP responses were determined by a one-way ANOVA and a Newman-Keuls statistical t-test with the Prisma program (www.GraftPath.com). $P< 0.05$ was considered statistically significant.

Results

The pilocarpine-treated epileptic rats showed signs of abnormal behavior the first five minutes after pilocarpine (i.p.) induction. Afterwards, these rats were followed by brief stretching and contracting movements of the fore and back limbs, followed by brief shaking of the head or body, which appeared within 3 to 20 minutes after administering pilocarpine and lasted for 5 to 15 minutes. The epileptic seizures of clonic-tonic character, sometimes associated with the aforementioned movements, lasted between 3 and 30 seconds and were accompanied by piloerection, salivating, and sustained rigidity of the tail and limbs. Similar descriptions of this model of temporary epilepsy have been provided by different authors (Priel et al., 1996; Leite et al., 1990).

Recording of the mean FP voltage in CA1 neurons of hippocampal slices derived from non-epileptic control rat (NEC)

Stimulation of the stratum radiatum at twice the threshold generated a stable FP response in pyramidal CA1 neurons. The mean voltage value was 0.63 ± 0.04, mV (n=15) and the mean duration was 6.1 ± 0.8 ms. Both values characterize the NEC responses (Figure 1.1).

Recording of the mean FP voltage in slices derived from pilocarpine- treated epileptic rats

The mean FP response in pyramidal neurons from hippocampal slices was 0.88 ± 0.12 mV (n=15), which was significantly higher than the NEC responses $F_{(3,56)}$=246, q=11.57, p<0.001 (Figure 1.2). The mean FP response displayed a complex morphology composed of four clear inflections, with delays of 3, 4, 8, and 12 ms, very different compared to the responses registered in NEC. Perfusion of 100 μM copper sulfate significantly reduced mean FP response to 0.372 ± 0.06 mV (n=15). The effect began 6-8 min after copper perfusion (Figure 1.3). This response was clearly lower than the mean FP response previously registered $F_{(3,56)}$=246, q=24.1, p<0.001 and lower than the responses registered in NEC, $F_{(3,56)}$=246, q=12.5 p<0.01. A subsequent K-R wash for 85 minutes increased the mean FP to 1.137 ± 0.081 mV (n=15) (Figure 1.4). This value was clearly higher than the FP response registered during copper perfusion $F_{(3,56)}$=246, q=36.5 p < 0.01 and the same value was significantly higher than slices registered in NEC slices $F_{(3,56)}$=246, q=31.60 p<0.001 as well as being significantly higher than the FP response registered at the beginning in pilocarpine-treated epileptic rats. $F_{(3,56)}$=246, q=12.42 p<0.001 (Figure 1.4).

The mean FP duration in CA1 hippocampal slices derived from pilocarpine-treated epileptic rats

The mean FP duration in neurons of hippocampus slices of epileptic rats was 23.4 ± 2.3 ms (n=15) (Figure 1.2), which was significantly longer than the average registered in NEC, value 6.1 ± 0.8 ms, $F_{(3,56)}$=278.3, q=29.62 p<0.001 Perfusion of 100 μM copper sulfate significantly reduced the mean FP duration to 8.28 ± 1.9 ms (n=15). This effect was observed 6-8 min after copper perfusion(Figure 1.3). Washing the slices for 85 min increased mean FP duration to 24.56 ± 3.3 ms (n=15), which was significantly longer than the mean FP duration registered in NEC slices $F_{(3,56)}$=278.3, q=31.6 and from the pilocarpine-treated epileptic slices recorded under Cu perfusion $F_{(3,56)}$= 278.3, q=27.85 p<0.01, but similar to the mean FP duration response observed in pilocarpine-treated epileptic slices $F_{(3,56)}$=278.3, q=1.9 p>0.05. (Figures 1.2 and 1.4).

Effect of copper perfusion on CA1 spike activity slices derived from non- epileptic control rat (NEC).

Stimulation of the stratum radiatum at twice the threshold generated stable unitary activity responses in pyramidal CA1 neurons, characterized by an ensemble of 3-4 spikes within 3, 5, and 8 ms after the stimulus. The spikes usually lasted 6.1 ± 0.8 ms very similar to the mean FP duration of 6.3 ± 0.4 ms observed in NEC slices (Figure 1.1 and Figure 2 B.2). The number of spikes evoked by electrical stimulation increased to 6 after acute perfusion of 10 μM of pilocarpine. Spikes latencies were 3, 5, 10, 48, 77, 93 ms, respectively (Figure 2.2). The spikes lasted longer than the mean FP response obtained from pilocarpine-treated epileptic slices 23.4 ± 2.3 ms (n=15). The spike frequency increased eight minutes after pilocarpine perfusion began. Perfusion of 10 μM copper sulfate reduce all spike activity seven minutes later (Figure 2.A.3). The effects and timing of the spike blocking was clearly similar to the FP blocking response copper-induced in pilocarpine-treated epilepsy slices (Figure 1.3). After fifty-five minutes of K-R washing, with standard electrical stimulation, twice the threshold, the number of spikes increased significantly (n=6), which was similar to the registered during pilocarpine perfusion (Figure 2.A.4). However, the number of spikes was higher than the registered from control assessment (Figures 2.B.1-4).

Discussion

The behavioral changes observed in chronically epileptic rats were similar to those described by others authors (Priel et al., 1996; Leite et al., 1990; Radley and Jacobs, 2003; Rutecki and Yang, 1998). The mean FP response of CA1 neurons of hipocampal slices derived from NEC rats was stable in electrical waveform, amplitude and latency, while the mean FP response of CA1 neurons derived from pilocarpine-treated epileptic rats was more complex, composed of several inflections, the latencies were 3, 4, 8 and 12 ms respectively (n=5). At first sight, the difference appears to be associated with plastic synaptic modification in the CA1 neurons as result of increased excitability emerging from chronic nature of the epilepsy (Mello et al., 1993; Müller-Dahlhaus et al., 2010). Increased excitability, initially induced

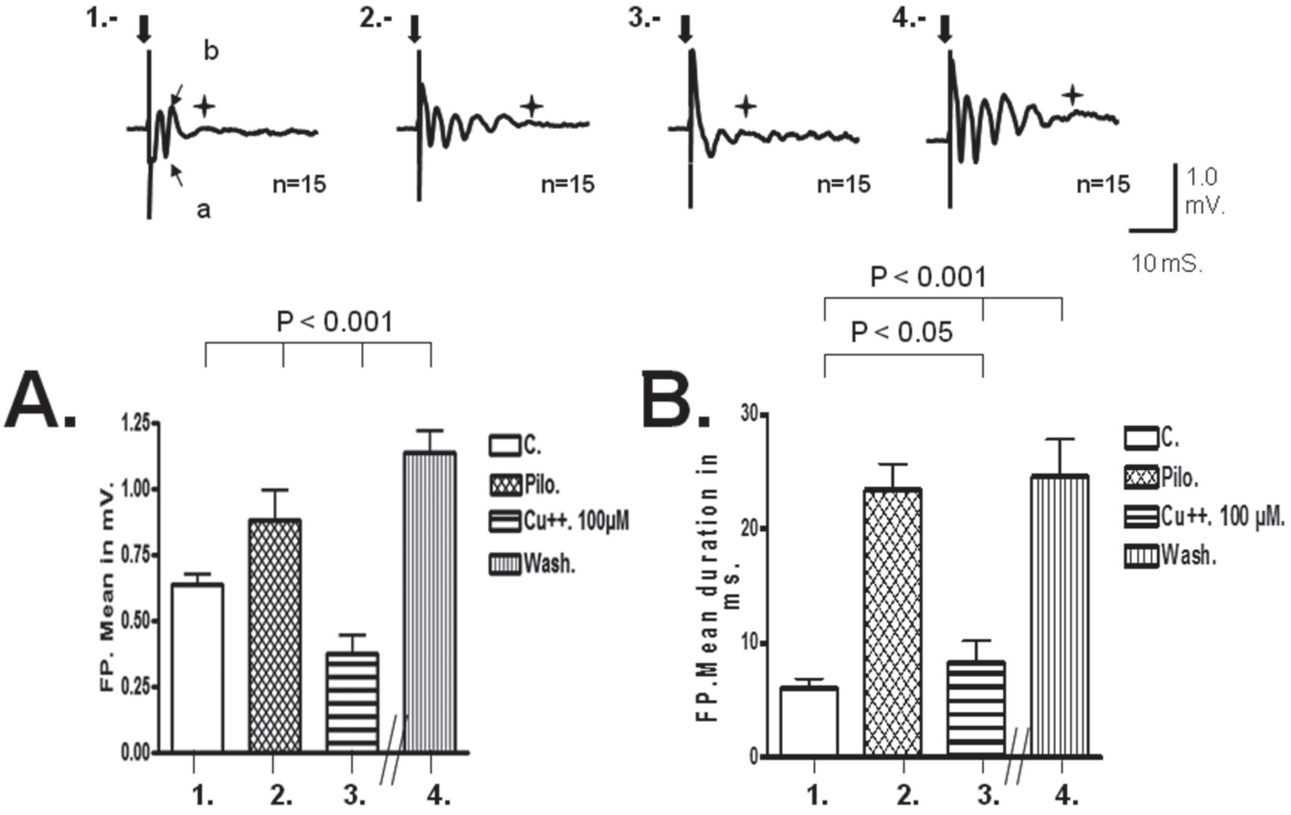

Fig. 1. - The hyper-excitable Field Potentials(FP), were blocked by copper in chronic epileptic neurons. **Top:** The recordings show the mean FP voltage responses obtained from pyramidal CA1 neurons in hippocampal slices (n=15) of control and chronic epileptic rats. The responses were obtained under four conditions: **1.** Control slices; **2.** Slices from chronically epileptic; **3.** Slices from chronically epileptic rats with 100 µM copper perfusion; **4.** Copper washed from slices of chronically epileptics. The wash recordings were made 85 min after the wash began. The recordings under conditions **2, 3** and **4** were made with the same slices. Under each condition we stimulated the stratum radiatum at twice the threshold to induce FP responses. The wide arrow indicates the duration of the stimulation. Furthermore, the wide arrow indicates the start of FP and the star marks the end of responses. Calibrations are shown. The inhibitory effect of copper can be observed. **Lower Left. A:** The graph shows the mean FP voltage (n=15) measured in the first inflection of the response (EPSP), indicated by the narrow arrows (**a, b**) in recording 1 above. There were significant differences under the four recording conditions in mean voltage responses, as indicated by a one-way ANOVA and a Newman-Keuls statistical test. **Lower Right. B:** The graph shows the mean FP duration (n=15) measured during the four situations described above. The four recording situations showed significant differences in mean FP duration, as indicated by a one-way ANOVA and a Newman-Keuls statistical test.

by pilocapine and subsequently remodeling over the next fifteen days, is evidence of persistent functional change (Priel et al., 1996; Leite et al., 1990; Rutecki and Yang, 1998; Cavalheiro et al., 1991; Park et al., 2006)**.** Acute pilocarpine administration in NEC rats significantly increased the average spike frequency, while pilocarpine- treated epileptic rats increased the amplitude of the FP response. The synaptic hyper-excitability responses could be an expression of morphological remodeling in plastic hippocampal circuitry associated to CA1. It has been hypothesized that plastic network reorganization increases excitability in the dentate gyrus and neighboring regions (Fujikawa, 1996; Isokawa et al., 1990; Tauck and Nadler, 1985; Wuarin and Dudek, 1996), which can also include cellular proliferation in the dentatus gyrus of adult rats (Mello et al., 1993; Turski et al., 1984; Cavazos et al., 2004). The hyper-excitability associated to morphological changes have also been observed in hippocampal slices employing cholinergic agonist and other epileptic agents (Radley and Jacobs, 2003; Rutecki and Yang, 1998). In line with these results, cell proliferation has been observed in the septo granular zone (SGZ) and the granular cell layer (GCL), three hours after pilocarpine-induced status epilepticus (Radley and Jacobs, 2003). In the same regard, has been described a significant increase in the sprouting of the mossy fiber in the

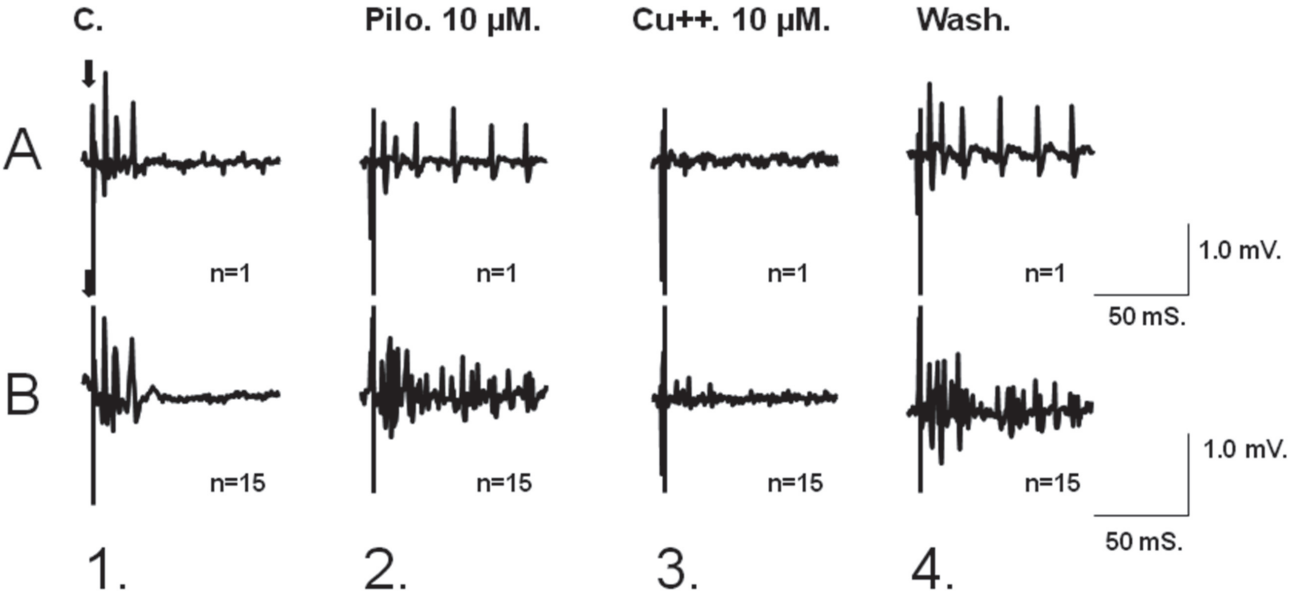

Fig. 2. - The hyper-activity in extracellular spikes were blocked by copper in acute epileptic neurons. Extracellular spike recordings obtained from pyramidal CA1 neurons in hippocampal slices (n=15) of control rats. The responses were obtained in four situations from the same slices: **1.** Control (C). **2.** Pilocarpine (10 µM) for eight to ten min. **3.** Copper sulphate (10 µM) for eight to ten min.. **4.** Wash. The recordings for the wash were made 55 minutes after the wash began. During each situation, the stratum radiatum was stimulated at twice the threshold to induce unitary responses. The wide arrow indicates the time of stimulation. Calibrations are shown on the right. The inhibitory copper effect can be observed. **Upper recording A:** Unitary recording of one sample (n=1) under all the conditions (**1**, **2**, **3**, and **4**), previously depicted from the same slice. **Lower recording B:** Average unitary recording (n=15) of the same slice under all conditions (**1**, **2**, **3** and **4**).

innermost part of the molecular layer (Cavazos et al., 2004; Károly et al., 2011). In the present work, the significant increase of frequency in the action potential after acute pilocarpine perfusion is caused by the irritation that precedes the synaptic plastic remodeling in progress, as has been documented by other authors (Rutecki and Yang, 1998; Priel et al., 1996; Cavalheiro et al., 1991). Ingesting copper sulfate or hippocampal slice perfusion clearly reduces neuronal excitability and suppresses the LTP in CA1 (Doroulee et al., 1997; Kardos et al., 1989; Leiva et al., 2000; Leiva et al., 2003; Goldschmith et al., 2005; Leiva et al., 2009). It is well known that NMDA and AMPA receptors are involved in generating LTP (Collindridge et al., 1983; Collingridge, 2003; Kandel et al., 1997; Martin et al., 2000; Revest and Longstaff, 1998). In this respect, there is evidence that copper blocks both types of receptors by interfering with NR2A sub-unit (Revest and Longstaff, 1998; Bush, 2003; Erreger et al., 2005). It was recently shown that 100 µM of copper inhibits AMPA currents and thus decreases synaptic activity, including the typical miniature synaptic currents in neurons of the rat hippocampal (Peters et al., 2011). Copper appears to interfere with the functioning of key receptors related to the excitability of synaptic neurotransmissions and plastic mechanisms. A biophysical study showed multiple effects of copper on NMDA receptor current. The copper effects was quantitatively similar in GluN1/GluN2B sub-units NMDA receptors, which were potentiated by 10 µM and inhibited by 100 µM copper (Marchetti et al., 2014). Our group has proven that the hyper-excitability FP responses generated by penicillin perfusion can be blocked in the CA1 by copper sulfate perfusion, while removing copper by K-R washing significantly increases the FP response in CA1 neurons (Leiva et al., 2013). The effects of copper remain while the copper is being perfused. However, when the copper is washed away FP hyper-excitability is restored. The effect of copper appears to be related to the synaptic micro-environment and it causes temporary modifications, probably without causing any acute anatomical synaptic damage.

The levels of epileptic hyper-excitability that we registered after washing suggest compensatory synaptic modification as a result of blocking by Cu in CA1 network. In our study, copper perfusion clearly reduced the synaptic neurotransmission. There is

evidence that copper concentration in rat brain cells decreased after the epileptic episodes PTZ-induced (Sahin et al., 2003). This suggests that epilepsy can induce chronic reductions in copper, which in turn can reinforce epilepsy. Consequently, low levels of coppers can facilitate the emergence of epilepsy. This appears to be confirmed with recent observations in children with Menkes's disease given that epilepsy is the major clinical feature of this disorder, associated to difficulties in copper metabolism (Prasad et al., 2011). In line with this, early diagnosis and regular Cu- histidine injections are currently the mainstay of treatment and have been shown to reduce the seizure susceptibility and hyper-tonicity associate with the disease (Kreuder et al., 1993; Christodoulou et al., 1998; Tumer and Moller, 2010). Recent gains in knowledge have led researchers to propose a new hypothesis, copper might affect the neuroproteostasis of CNS neurons that lead to changes in neuronal excitability. In summary, intracellular copper levels would be a delicate tuner with the proper functions of ubiquitin- proteosome system (Opaso et al., 2014). However, further experimental studies are necessary before any final conclusion can be reached.

Conclusions

Administration of copper (100 uM) blocks synaptic transmission in CA1 pyramidal neurons of hippocampal slices derived from pilocarpine-treated epileptic rats. Administration of copper (10 µM) blocks the frequency of spikes in neurons of CA1 hippocampal slices derived from non-epileptic control(NEC) rats treated with pilocarpine.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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