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Administering Copper Blocks CA1 Neuron Hyper-Excitability in Rat Hippocampal Slices*

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ABSTRACT

The aim of this study was to determine the capacity of copper to modify synaptic hyperexcitability generated by penicillin G. This epileptogenic drug was studied with CA1 neurons of the rat hippocampus. Hippocampal slices were extracted from adult male Wistar rats (n = 16). The field potentials (FP) were registered in CA1 neurons after electrical stimulation from the stratum radiatum. The mean voltage and duration of FP were measured during control, penicillin G, copper and washout stages. Copper (100 μM) significantly decreased mean FP voltage compared to the control and penicillin stages. However, during the washout stage, the mean FP voltage was significantly higher than in the penicillin stage. Regarding the FP duration, 100 μM of copper significantly decreased the mean FP during the penicillin stage. After the washing stage, the mean FP lasted significantly longer. Thus, administering copper modified CA1 synapses by blocking hippocampal neuronal excitability was generated by the epileptic agent.

Keywords: Copper; Field Potential (FP); Hippocampus; Penicillin G

1. Introduction

Copper is an integral component of several proteins and as a trace element is a necessary co-factor for diverse enzymatic reactions [1]. Copper can react with proteins by bonding to thiolate, amino and carboxylic groups present in macromolecules. Copper is associated with cytochrome oxidase, metallothionein, superoxide dismutase, dopamine β hydroxylase, tyrosinase, lysyl oxidase activities and coagulation factors V and VIII [2-4].

Recent studies have considered the role of copper in neurodegenerative diseases such as Wilson's, Menkes, Alzheimer's, Parkinson's, amyotrophic lateral sclerosis and prion disease [5-9].

According to experimental results, 100 μM of copper in the rat cortical neurons is capable of modulating neuronal activity [10,11]. We have recently shown that copper sulfate at concentration as low as 10 μM can partially reduce neuronal field potentials (FP) [12] and suppress LTP (a form of persistent synaptic voltage increase after a high rate of stimulus) observed in CA1 neurons in rat hippocampal slices [13]. In the same direction, it has been described that LTP is not induced in the CA1 neurons of rats that drank water with copper sulfate (8 to 12 mg/Kg/day) for 30 days. Under similar experimental conditions, the threshold to evoke the FP clearly increased [14,15]. On the other hand, rats with intra-peritoneal (i.p.) administration of copper sulfate (1 mg/kg/day) for 30 days did not exhibit LTP, at the CA1. However, they were successful in performing the Morris water test (16).

Studies by other authors, as well as those of our group, have strengthened the idea that copper can exert an inhibitory effect on the neuronal transmission. Taking into account the inhibitory role of copper in CA1 synaptic activity, the current study was conducted to determine the capacity of copper to modify synaptic hyperexcitability generated by penicillin G, a drug that induces epileptogenic activity [17].

2. Methods

Adult male Wistar rats (n = 16) weighing between 150 and 250 g, with a mean age of 6.7 ± 1 weeks, were maintained at 22˚C ± 1˚C with a light/dark cycle of 12/12 h. Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, published by the *National Institutes of Health*. The experimental protocol was approved by the Bioethical Committee of the Institute of Biomedical Sciences, Faculty of Medicine of the Universidad de Chile.

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The animals were deeply anesthetized with ether and decapitated with a Stoelting guillotine once tactile leg and corneal reflexes were no longer apparent. The brain was rapidly removed under a continuous cold Krebs-Ringer (K-R) drip and then submerged for a short period in K-R. The hippocampus was removed from the hemi-brain, and was cut into 400 μM coronal slices [18] with a McIlwain Tissue Chopper 5 (Mickle Laboratory Engineering, Surrey, UK). The slices were submerged in an incubation chamber for 1 h, and then transferred to the immersion register chamber with continuous K-R perfusion at 31˚C ± 1˚C.

The K-R solution was composed of (in mM) NaCl 124.0, KCl 5.0, KH_2PO_4 1.25, $MgSO_4 + 7H_2O$ 2.0, $CaCl_2$ 2.0, $NaHCO_3$ 26.0 and glucose 10 at pH 7.4, solution was previously bubbled with carbooxigen (95% O_2 and 5% CO_2). The perfusion system provided a continuous flow of 2 to 3 ml/min.

2.1. Neural Registration *in Vitro*

Hippocampal slices were placed in an immersion chamber with a nylon net on the bottom. The stratum radiatum was stimulated with bipolar tungsten electrodes through a Digitimer Trigger Generator DG2 connected to an Isolated Stimulator (model DS2A) Single pulses of 0.5 - 6.0 volt stimulus were employed at 10 s intervals. The pulses had a duration of 0.30 ms, with a frequency of 0.1 - 0.01 Hz at twice the threshold. The field excitatory postsynaptic potentials were recorded (fEPSP or FP) with glass micropipettes filled with K-R with 1 to 1.5 MΩ of impedance. The microelectrodes were visually positioned in CA1 with the assistance of a binocular magnifying glass (Carl Zeiss). A hydraulic micromanipulator allowed us to move the micropipette through the depth of the slice. Neural activity was captured with an amplifier (Bio-Logic VF180), which displayed the signal in an oscilloscope (Hitachi VC-6020). A recording was made simultaneously with a tape recorder (SONY.DTC.59 E.S) for offline analysis. Analog-to-digital data conversion was done using a Cambridge Electronic Device (CED). Digitized data were averaged using a program (SIGAVG) on a Pentium microcomputer.

2.2. Experimental Procedure

After defining the FP under control conditions, 500 - 1500 UI/ml of penicillin G was administered for 7 - 10 min. This period is termed the penicillin stage. The slide was then perfused with 100 μM of copper sulphate, for 7 - 10 min, which constitutes the copper stage. Finally, the preparation was washed out with K-R for 30 to 120 minutes, until reaching the initial control FP (n = 15). The recording period and the stimulation modality were similar to those previously described (16). We analyzed only the slices that showed stable responses over the four stages.

2.3. Statistical Analysis

The responses evoked in each stage were monitored on the oscilloscope and signals sent to an analog-to-digital data conversion device (Cambridge Electronic Design). Digitalized data were analyzed with an averaging program (SIGAVEG), and a one-way ANOVA and Newman-Keuls t tests were applied to identify significant differences in voltage and the duration of FP responses using the program Prisma (www.GraftPath.com). A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Modification of the Mean FP Voltage during the Penicillin G, Copper and Wash Periods

Electrical stimulation of the stratum radiatum at twice threshold resulted in a stable synaptic responses in pyramidal CA1 neurons. FP responses were characterized at least four positive and negative inflections as a result of synaptic activity from distinct afferent collaterals, their latencies being 4, 7, 9 and 12 ms, respectively. For comparison, the amplitude of the first inflection was considered the basal response and presented a mean amplitude of 10.87 ± 1.42 mV. Penicillin perfusion (500 IU) provoked an increase of the basal FP, which reached a mean value of 16.6 ± 1.44 mV, which was clearly higher than the FP observed during the control stage ($F(3, 56) = 932.9$, $q = 14.14$, $P < 0.001$). Eight minutes later copper sulfate perfusion at 100 μM, reduced the mean FP amplitude to 2.9 ± 0.7 mV. This value was significantly lower than the FP during the penicillin and control stage ($F(3, 56) = 932.9$, $q = 33.5$, $P < 0.001$) and ($F(3, 56) = 932.9$, $q = 19.4$, $P < 0.001$), respectively. A subsequent K-R washout for 60 - 120 minutes increased the mean FP to 32.54 ± 2.35 mV. This value was clearly higher than the FP observed during the control stage ($F(3, 56) = 932.8$, $q = 52.8$, $P < 0.001$); copper stage ($F(3, 56) = 932.9$, $q = 72.2$, $P < 0.001$), and after the penicillin stage ($F(3, 56) = 932.8$, $q = 38.7$, $P < 0.001$), see **Figure 1(A)**.

3.2. Mean FP Duration during the Penicillin G, Copper and Washout Periods

The FP duration was defined as the interval between the beginning of the stimulus and the end of the global FP response. Synaptic FP responses in pyramidal CA1 neurons presented four stable inflections with a mean duration of 22.6 ± 1.9 ms. Penicillin G perfusion with 500 IU increased the mean FP duration to 33.31 ± 3.2 ms, which

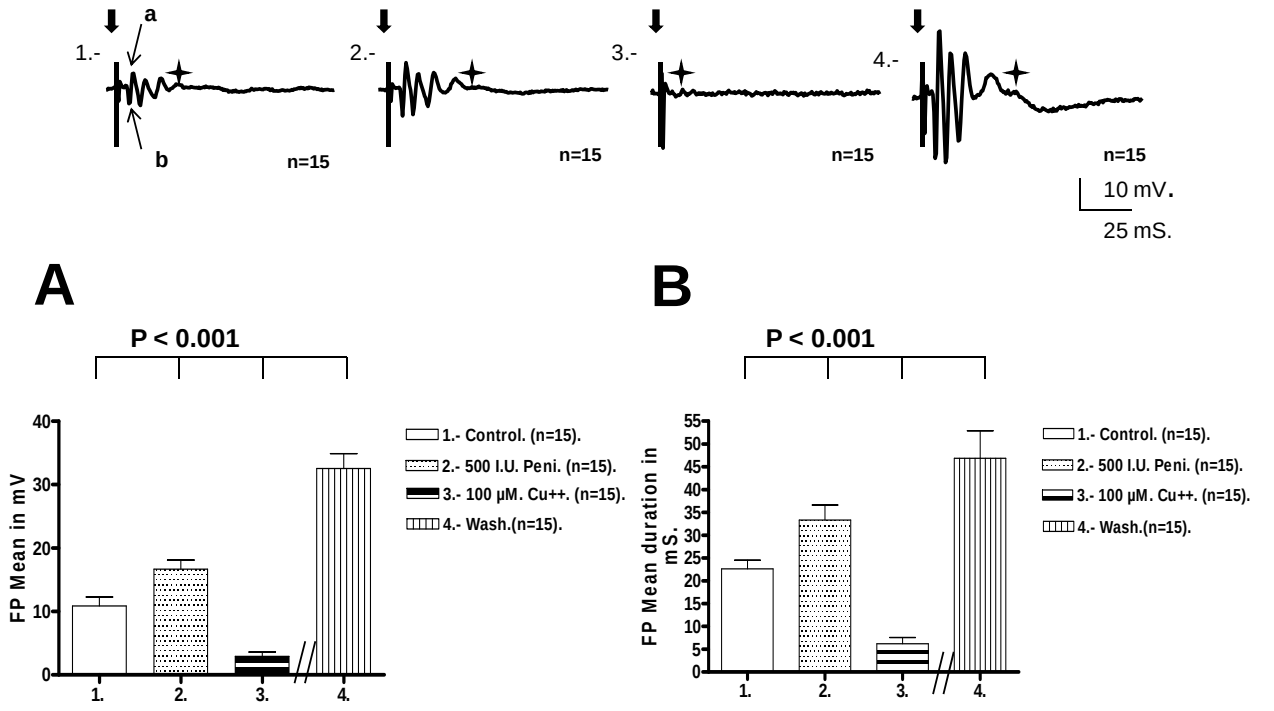

Figure 1. Top: The recordings show the mean FP voltage responses obtained from pyramidal CA1 neurons in rat hippocampal slices (n = 15) using penicillin G as an epileptic agent. The responses were obtained during four periods: 1) Control stage; 2) Penicillin G (500 UI) stage for 8 - 10 min; 3) Copper sulfate (100 µM), stage for eight to ten min; 4) Wash stage. The recordings for the wash stage were made 120 min after this period began. During each stage, we stimulated the stratum radiatum at double the threshold to induce FP responses. The broad arrow indicates the time of stimulation and the start of FP. The star marks the end of responses. Calibrations are shown. 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 among the four recording stages in mean voltage responses, as indicated by a one-way ANOVA followed by a Newman-Keuls statistical test. Lower Right. (B) The graph shows the mean duration of the FP (n = 15) measured during the four stages described above. There were significant differences in mean FP duration, as indicated by a one-way ANOVA followed by a Newman-Keuls statistical test.

was significantly higher than the FP observed during the control stage ($F(3, 56) = 339.3$, $q = 11.4$, $P < 0.001$). This increase is considered indicative of neuronal hyperexcitability because new excitatory synaptic activity can increase the FP duration. [19]. Copper sulfate at 100 µM, perfused for eight to ten min decreased the mean FP duration to 6.2 ± 1.4 ms, which was significant compared to the penicillin stage ($F(3, 56) = 339.3$, $q = 29.1$, $P < 0.001$). The mean FP duration during the copper stage was also significantly shorter than the control stage ($F(3, 56) = 339.3$, $q = 17.6$, $P < 0.001$). Recordings obtained 100 min after the beginning of the washout stage had a mean FP duration of 46.9 ± 6.0 ms, which was significantly higher than the control stage ($F(3, 56) = 339.3$, $q = 25.9$, $P < 0.001$), penicillin stage ($F(3, 56) = 339.3$, $q = 14.5$, $P < 0.001$) and copper stage ($F(3, 56) = 339.3$, $q = 43.6$, $P < 0.001$), See **Figure 1(B)**.

4. Discussion

The principal result of this work indicates that copper sulfate in physiological concentrations significantly reduces the hyperactivity of CA1 neurons, defined by changes in both the amplitude and duration of the FP. This phenomenon occurred when a hippocampal slice was perfused with penicillin G, a recognized epileptogenic substance. Before penicillin perfusion, copper reduced the hipocampal FP response, confirming previous results [12,13,15,16]. As described before, copper perfused before tetanic stimulation prevented LTP generation in CA1 neurons [13]. A similar effect has been obtained with 5-amino-phosphovalerate (a NMDA receptor antagonist) [14]. However, if 5-amino-phosphovalerate is perfused after settling down the LTP; this effect was not observed. This result suggests that the NMDA receptor antagonist did not block the biochemical machinery that supports long-term LTP consolidation. Instead, copper perfusion in different concentrations temporarily blocked FP response before and after tetanic stimulation [13,14]. 5-amino-phosphovalerate has a selective inhibitory effect on NMDA receptors, while copper exerts a non-selective effect. There is evidence that copper blocks AMPA and NMDA receptors by interfering with the NR2A subunit,

NMDA and AMPA receptor blocking by copper would prevent LTP generation [20-23]. The NMDA receptor NR1/NR2A sub-units are responsible for kinetic and pharmacological interactions with other subunits [24,25]; LTP blocking by copper probably also includes interference of AMPA receptors [21,22]. As has been described, copper inhibits AMPA currents, thus decreasing synaptic activity, including typical miniature synaptic currents [26]. It is probable that similar phenomena occurred under our experimental conditions.

In the present study, 500 I.U. of penicillin G significantly increased the voltage and duration of FP compared to the FP control. Although there is no definitive explanation for penicillin-induced hyperexcitability, it is thought that penicillin interferes with GABA-mediated inhibition [17-29]. A similar mechanism has been proposed for another epileptogenic agent, like Pentylentetrazol (PTZ) [30]. In our results penicillin-induced hyperexcitability was significantly attenuated by copper, but after the washout period the hyperexcitability response was significantly higher than those initially generated by penicillin G. A possible explanation could be in an increase of NMDA and/or AMPA receptor expression as a result of the partial blocking of the synaptic traffic through these receptors promoted by copper. Alternatively, copper could produce changes in the affinity of the AMPA receptors. [26]. The effects of copper on the FP could be related to temporal blocking of AMPA receptors; which could result in upregulation of those receptors. Accordingly, an important link has been found between AMPA and copper. In fact, it has been demonstrated that copper produces biphasic changes in neurotransmission. When copper is acutely applied to the synaptic cleft, it blocks neurotransmission, but when it is applied for 3 h to the hippocampal neurons it mainly increases the frequency and amplitude of evoked AMPAergic current, which is considered dependent on the concentration of copper [26]. Another possibility is that copper interacts with the GABA receptor, replacing zinc, which is present in high concentrations in structures of the limbic system including the hippocampus [31-34]. In this regard, it has been reported that synaptic release of zinc can interact with ion channels and receptors, including the AMPA, NMDA and GABA receptors, reducing the threshold response [31-40]. Like copper, zinc inhibits LTP, suggesting that the two trace metals share the same targets [41].

On the other hand, there is an evidence of chronic copper deregulation associated with neurodegenerative and chronic diseases [5-9], including epilepsy [42]. In our study, however, the effect of acute copper administration is in contrast with the high levels of copper present in the plasma of epileptic patients [42,43].

The result of the acute copper administration and its subsequent discontinuity can be related to the over-expression of glutamatergic receptors as a consequence of temporal blocking of the glutamatergic synapses. However, we cannot rule out that copper can also interact with other proteins related to neuronal transmission. In fact, copper can also interact with channel proteins; for instance, with K^+ and Na^+ or Ca^{++} channels. It has been demonstrated that copper reversibly inhibits BK and shaker K^+ channels in micro molar concentration [44]. As well, It has been described that administration of nano-Cu in hippocampal CA1 inhibits amplitude of Na^+ depolarizing currents [45], additionally copper has a high affinity for the Ca v 3.2 T-type calcium channels, stabilizing the closed conformation of the voltage-sensor and thereby inhibits channel opening [46]; moreover, Cu can also block Ca currents in high-voltage activated Ca channels [47]. Therefore, acute excitatory blocking can result from NMDA receptor blocking and/or blocking of depolarization currents at the hippocampus synaptic circuitry. These two general mechanisms may be involved in the up regulation of the excitatory transmission. Previous results and those that indicate a significant reduction of copper concentration in rat brain cells after administration of single and/or repeated Pentylentetrasol (PTZ) [48] suggest that chronic reductions in copper levels can facilitate the propensity to epileptic diseases; while increasing the copper concentration could attenuate this effect. If this is the case, copper could be used as an antiepileptic substance. However, further experimental studies are necessary before any final conclusions can be reached.

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