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## Effects of $\alpha$ -keto- $\delta$ -guanidinovaleric acid on inhibitory amino acid responses on mouse neurons in cell culture

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The experimentally proven convulsant  $\alpha$ -keto- $\delta$ -guanidinovaleric acid ( $\alpha$ -K- $\delta$ -GVA) was applied to mouse spinal cord neurons in primary dissociated cell culture to assess its effects on postsynaptic  $\gamma$ -aminobutyric acid (GABA)- and glycine (GLY)-responses. Intracellular microelectrode recording techniques were used.  $\alpha$ -K- $\delta$ -GVA reversibly inhibited both GABA- and GLY-responses in a concentration-dependent manner. The effect of  $\alpha$ -K- $\delta$ -GVA on GABA-responses was not antagonized by co-application of the benzodiazepine receptor antagonist CGS 9896. The results suggest that  $\alpha$ -K- $\delta$ -GVA inhibited responses to the inhibitory neurotransmitters GABA and GLY by blocking the chloride channel. This action might underlie the convulsant effect of this compound in rabbit. The possible pathophysiological importance of  $\alpha$ -K- $\delta$ -GVA in hyperargininemic patients is discussed.

### INTRODUCTION

$\alpha$ -Keto- $\delta$ -guanidinovaleric acid ( $\alpha$ -K- $\delta$ -GVA), a monosubstituted guanidino compound, has been identified in urine, serum and cerebrospinal fluid of hyperargininemic patients<sup>14,15,17</sup>. Hyperargininemia is an inborn error of metabolism characterized by a deficiency of arginase, the last enzyme of the urea cycle, which converts arginine to urea and ornithine. The first clinical and biochemical descriptions were published in 1969<sup>34</sup> and 1970<sup>35,36</sup>. Since then, 18 other cases have been reported in the literature<sup>2-4,7,10,11,19,20,24,25,28,31,32,37,38</sup>. The disease is autosomal recessive. Patients with this disorder show similar clinical symptoms: irritability, vomiting, a progressive pyramidal spasticity and epilepsy. In addition, some patients have an episodic hyperammonemia and mental retardation.

The causative factors of the neurological symptomatology are not clear. Possible factors could be the disturbance of ammonia metabolism or the accu-

mulation of arginine and its catabolites, the guanidino compounds. The presence of normal or only slightly elevated plasma ammonium levels in several patients<sup>4,19,25,31</sup> casts doubt on the former hypothesis. However, several guanidino compounds, metabolites of arginine, consistently accumulate in biological fluids of hyperargininemic patients<sup>14,15,17</sup>.  $\alpha$ -K- $\delta$ -GVA, one of these guanidino compounds, was demonstrated to be a convulsant in rabbit. After topical application of  $\alpha$ -K- $\delta$ -GVA on the sensory motor cortex, tonic-clonic seizures were evoked and epileptiform discharges were recorded on the electroencephalogram<sup>16</sup>. The experimentally observed epileptogenicity of this keto analogue of arginine might contribute to the epileptic symptomatology observed in hyperargininemic patients.

Several convulsants have been shown previously to inhibit responses to iontophoretically applied GABA on mammalian spinal cord neurons grown in cell culture<sup>13</sup>. In an attempt to determine the mechanisms through which  $\alpha$ -K- $\delta$ -GVA might produce sei-

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zures, we studied its influence on responses evoked by iontophoretically applied GABA and GLY on mouse spinal cord neurons in primary dissociated cell culture. Intracellular microelectrode recording techniques were used.

## MATERIALS AND METHODS

### *Primary dissociated cell culture*

Cultures of spinal cord neurons were prepared from dissected spinal cords and attached dorsal root ganglia from 12–14-day-old fetal mice as described previously<sup>27</sup>. The tissue was minced and then mechanically dissociated by trituration in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free balanced salt solution to a suspension of single cells and small clumps. The dissociated cells were suspended in culture medium [90% Eagle's minimal essential medium supplemented with 5.5 g of glucose and 1.5 g of  $\text{NaHCO}_3$ /5% heat-inactivated horse serum and 5% Nu-Serum II (Collaborative Research Inc.), 325 mOsm] and then plated on sterile collagen-coated 35-mm dishes. The cultures were maintained in an incubator with an atmosphere of 93% room air and 7%  $\text{CO}_2$  at 35 °C. The bicarbonate/ $\text{CO}_2$  buffer maintained pH at 7.4. 5-Fluoro-2'-deoxyuridine was added to the cultures on days 6–8 to suppress the growth of rapidly dividing non-neuronal cells. Medium was changed twice weekly. Cultures were maintained for 4–9 weeks before electrophysiological experiments.

### *Experimental procedures*

**Solutions.** All recordings were made in a Dulbecco's phosphate-buffered saline (DPBS) after removal of growth medium. The DPBS, with elevated magnesium ion concentration in order to suppress spontaneous activity, contained (in mM): NaCl 137,  $\text{Na}_2\text{HPO}_4$  8.06, KCl 2.68,  $\text{KH}_2\text{PO}_4$  1.47,  $\text{CaCl}_2$  1,  $\text{MgCl}_2$  10 and glucose 5.6 (pH 7.3–7.4). Heavy paraffin oil was applied to the surface of the bathing solution to retard evaporation.

Solutions of  $\alpha$ -K- $\delta$ -GVA were always prepared on the day of the experiment in the following manner. Dry enzymatically synthesized  $\alpha$ -K- $\delta$ -GVA was dissolved in DPBS to form 1 M stock solution. Aliquots were removed and diluted in DPBS to give concentrations between 1 nM and 100 mM.

**Experimental apparatus.** For experiments, the cul-

ture dish containing the bathing solution was placed on a stage heated by a Pellitier device with temperature regulated at 34–35 °C. The stage was mounted on a Leitz inverted microscope fitted with phase-contrast optics to facilitate micropipette placement (using Leitz micromanipulators) and to penetrate cells under direct visual control.

**Electrophysiological recordings.** Intracellular recordings were made from the somata of spinal cord neurons ( $>20 \mu\text{m}$ ) using glass micropipettes (20–25 M $\Omega$ ) filled with 3 M KCl. Use of an active bridge circuit (Dagan 8100 or W-P Instruments M707) allowed simultaneous recording of membrane potential and injection of current (for steady-state polarization or periodic stimulation) using a single micropipette. The preamplifier output was led to a 6-channel polygraph (Gould 2600S) for continuous recording.

**GABA- and GLY-responses.** GABA (0.5 M, pH 3.4) and GLY (0.5 M, pH 3.0) were applied iontophoretically using 500-ms duration rectangular current pulses at 5-s intervals. Iontophoretic pipettes were positioned to within 2  $\mu\text{m}$  of neuronal somata. The use of 3 M KCl-filled micropipettes shifted the chloride equilibrium potential from about  $-65 \text{ mV}$  to about  $-20 \text{ mV}$ . Under these conditions, an increase of chloride conductance resulted in an outward chloride current<sup>5</sup>, giving depolarizing GABA- and GLY-responses<sup>5,22</sup>. Responses of about 10–15 mV in amplitude were evoked following membrane hyperpolarization ( $-70$  to  $-90 \text{ mV}$ ) to avoid saturation at or near the reversal potential and to obtain a large transmembrane chloride ion gradient. Effects on GABA- and GLY-responses were accepted only if the responses returned to control amplitude within 5 min of removal of the  $\alpha$ -K- $\delta$ -GVA-containing micropipette.

**Drug application** For evaluation of  $\alpha$ -K- $\delta$ -GVA effects on GABA- and GLY-responses,  $\alpha$ -K- $\delta$ -GVA was applied by miniperfusion. A blunt-tipped (5–10  $\mu\text{m}$ ) micropipette filled with the test solution was positioned 15–30  $\mu\text{m}$  from the soma of the cell under study. The open end of the miniperfusion pipette was connected to a pressure regulator, set between 0.4 and 0.8 pounds per square inch (psi), by tight-fitting polyethylene tubing. Pressure pulse duration, regulated by a voltage-activated 3-way valve, was 10 s. Under these conditions, miniperfusion produced no artifacts, and application of control solution (DPBS

alone) was virtually free of effects. The concentrations reported in this study were those contained in the miniperfusion micropipettes. While it is possible that there was some reduction in the concentration of drug at the neuronal surface due to dilution in the surrounding medium, increasing durations of application or altering the position of the micropipette did not increase the effects of the drugs. Thus, while there might be some small inaccuracy in the drug concentrations, the reported concentrations are likely to be quite close to those existing at the surface of the neuron. For assessment of the possible antagonist effect of the pyrazoloquinoline CGS 9896 (a pure benzodiazepine receptor antagonist<sup>6</sup>) on  $\alpha$ -K- $\delta$ -GVA-induced changes of GABA-responses, CGS 9896 (1  $\mu$ M) was applied by diffusion from large-tipped (10–20  $\mu$ m) micropipettes before application by miniperfusion of  $\alpha$ -K- $\delta$ -GVA. As control, DPBS alone applied by diffusion was without effect in this paradigm. The miniperfusion pipettes and recording micropipettes were held by Leitz micromanipulators. To decrease leakage of drugs into the bathing medium, the tips of the miniperfusion pipettes were kept in the oil phase between drug application trials. They were lowered into the aqueous phase only when drug application was desired.

**Drugs.** CGS 9896 (2-(4-chlorophenyl)-2,5-dihydropyrazolo(4,3-C)quinoline-3(3-H)-one) was provided by Ciba-Geigy-Corp., Summit, New Jersey, U.S.A. GABA and GLY were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A.

$\alpha$ -K- $\delta$ -GVA was synthesized enzymatically by a modification of the method of Meister<sup>18</sup>. Arginine (1000 mg), free-base, and 355 mg boric acid were dissolved in 50 ml water. L-amino acid oxidase (17.5 U) (Sigma A5147; 0.35 U/mg, solid one unit will oxidatively deaminate 1.0  $\mu$ mol of L-phenyl-alanine/min at pH 6.5 at 37 °C) were added to this solution. The H<sub>2</sub>O<sub>2</sub> formed was decomposed by adding 10<sup>6</sup>,000 units catalase (Aldrich; 120,000 units/ml solution). The pH of the solution, originally at pH = 9.2, was brought to a pH of 7.6 with approximately 6 ml 1 N HCl. The vessel containing this solution was placed in a waterbath at 37 °C. An O<sub>2</sub> stream was continuously bubbled through the solution. The enzymatic oxidative deamination occurred over a 24-h period. Every 6 h, 17.5 U of L-amino acid oxidase and 108,000

U of catalase, were added to the solution and the pH was also controlled. The solution was deproteinized by dialysis against water. Boric acid was eliminated by passing the deproteinized solution over a cation exchange column (H<sup>+</sup> form), and the resin was washed with water.  $\alpha$ -K- $\delta$ -GVA was eluted from the column by passing 0.5 N NH<sub>4</sub>OH solution through the column. The eluate was concentrated by evaporation with a rotavapor under vacuum to about 15 ml. Out of this solution a first crystallization was performed. A white powder was obtained. A second crystallization from hot water gave well-formed crystals. The purity of the synthesized compound was controlled by liquid chromatography and nuclear magnetic resonance.

**Algebraic and statistical methods.** At all applied concentrations, mean values and standard deviations were calculated for the effects on GABA- and GLY-responses. The effects were expressed as percentage decrease of GABA- or GLY-responses. The statistical significance of differences between control and drug groups was calculated using the two-tailed Student's *t*-test; *P* < 0.05 was considered statistically significant.

## RESULTS

Miniperfusion of  $\alpha$ -K- $\delta$ -GVA did not alter resting membrane potential or conductance. Miniperfusion of DPBS alone (*n* = 10) gave non-significant enhancement (0.8  $\pm$  1.9%) of GABA- and GLY-re-

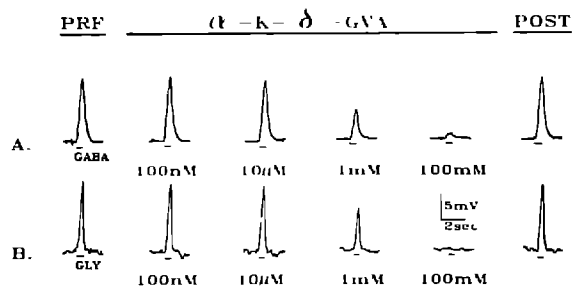


Fig 1 Reversible, concentration-dependent effects of  $\alpha$ -K- $\delta$ -GVA on GABA-responses (A) and on GLY-responses (B) on spinal cord neurons. Row A and B show responses obtained on one cell. PRE shows stable GABA- and GLY-responses before drug application. The 4 middle responses show the effect of the miniperfused drug. GABA- and GLY-responses returned to control values (POST) within 2 min following removal of the  $\alpha$ -K- $\delta$ -GVA containing micropipette. Iontophoretic application of GABA or GLY is indicated with a dash.

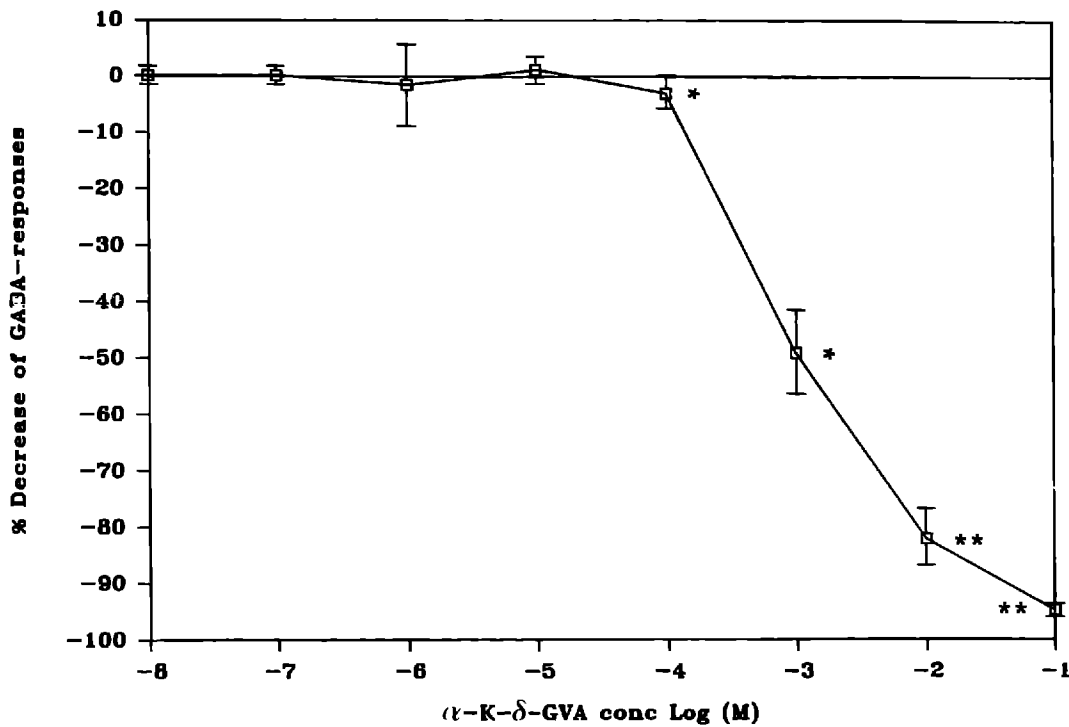


Fig. 2. Concentration-dependent decrease of GABA-responses in spinal cord neurons by  $\alpha$ -K- $\delta$ -GVA. Effects are expressed in percentage decrease of the original GABA-response. Data shown are means and standard deviations. Three to six cells have been studied for each concentration tested. \* $P < 0.01$ , \*\* $P < 0.001$  from control GABA-response.  $\alpha$ -K- $\delta$ -GVA concentrations on the abscissa are logarithm molar.

sponses. Reversible effects of  $\alpha$ -K- $\delta$ -GVA on GABA- and GLY-responses are shown in Fig. 1.  $\alpha$ -K- $\delta$ -GVA reversibly decreased GABA-responses (Fig. 1A) as well as GLY-responses (Fig. 1B). The effects of  $\alpha$ -K- $\delta$ -GVA on GABA-responses (Fig. 1A) and GLY-responses (Fig. 1B) were concentration-dependent. The concentration dependency of the effect of  $\alpha$ -K- $\delta$ -GVA on GABA-responses is further illustrated in Fig. 2. A significant  $3.6 \pm 2.9\%$  reduction ( $P < 0.01$ ) was obtained at  $100 \mu\text{M}$  and a  $94.4 \pm 1.4\%$  decrease was obtained at  $100 \text{ mM}$  ( $P < 0.0001$ ). Non-significant effects were observed at concentrations ranging from  $10 \text{ nM}$  to  $10 \mu\text{M}$ . The concentration-dependency of the effect of  $\alpha$ -K- $\delta$ -GVA on GLY-responses is shown in Fig. 3. At  $100 \mu\text{M}$ , a non-significant  $2.5 \pm 5.5\%$  decrease of GLY-responses was observed. A significant  $21.2 \pm 8.6\%$  reduction ( $P < 0.01$ ) was obtained at  $1 \text{ mM}$  and a  $99.5 \pm 1.0\%$  decrease ( $P < 0.0001$ ) was seen at  $100 \text{ mM}$ .

Application of CGS 9896 ( $1 \mu\text{M}$ ) by diffusion, before application of  $\alpha$ -K- $\delta$ -GVA ( $10 \text{ mM}$ ) by intraperfusion, did not significantly influence the effect of  $\alpha$ -K- $\delta$ -GVA on GABA-responses (Table I).

#### DISCUSSION

Several convulsants have been shown previously

TABLE I

CGS 9896, a benzodiazepine receptor antagonist, did not influence the effect of  $\alpha$ -K- $\delta$ -GVA on GABA-responses on mouse spinal cord neurons

	Number of neurons studied	GABA-responses % of control (mean $\pm$ S.D.)
CGS 9896 $1 \mu\text{M}$	9	100.2 $\pm$ 4.9
$\alpha$ -K- $\delta$ -GVA $10 \text{ mM}$	5	18.0 $\pm$ 5.2
$\alpha$ -K- $\delta$ -GVA $10 \text{ mM}$ + CGS 9896 $1 \mu\text{M}$	3	18.5 $\pm$ 10.0

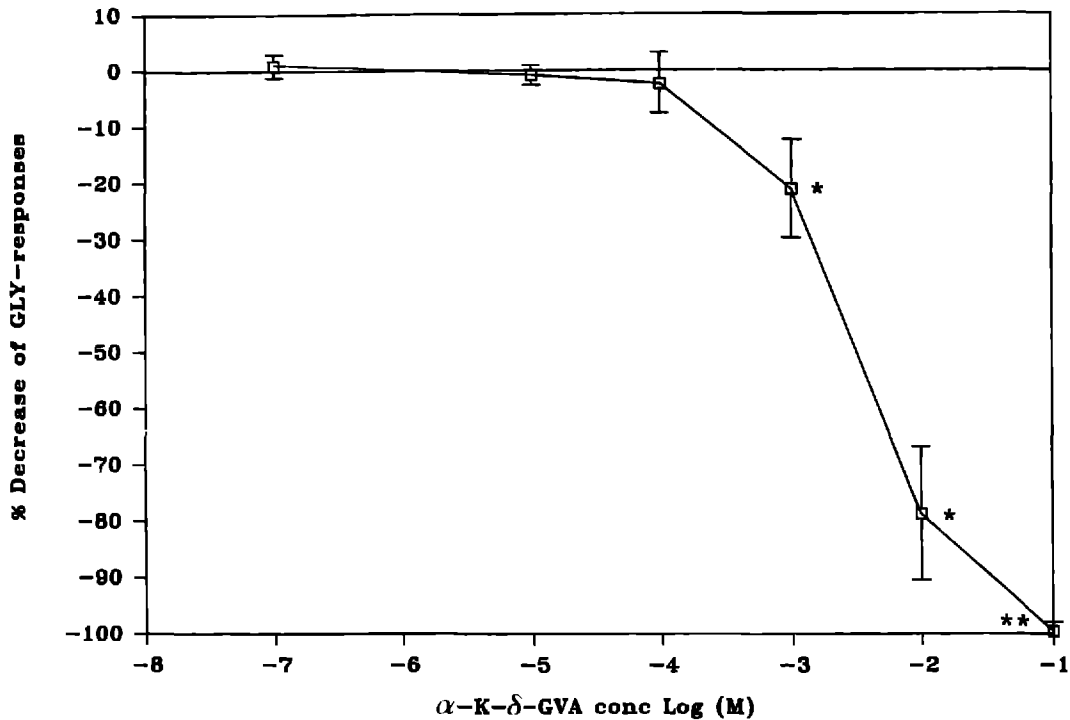


Fig. 3. Concentration-dependent decrease of GLY-responses on spinal cord neurons by  $\alpha$ -K- $\delta$ -GVA. Effects are expressed in percentage decrease of the original GLY-response. Data shown are means and standard deviations. Three to six cells have been studied for each concentration. \* $P < 0.01$ , \*\* $P < 0.001$  from control GLY-responses.  $\alpha$ -K- $\delta$ -GVA concentrations on the abscissa are logarithm molar.

to inhibit GABAergic neurotransmission<sup>12,23,29,30</sup> through interaction with a supramolecular receptor complex<sup>33</sup>. This protein complex, located on the postsynaptic membrane, consists of a functionally coupled benzodiazepine receptor, GABA-receptor and chloride channel. A variety of convulsants reduced responses to iontophoretically applied GABA on mouse neurons in cell culture. DMCM, a convulsant  $\beta$ -carboline, reduced GABA-responses through an interaction with the benzodiazepine receptor<sup>9,30</sup>. Another benzodiazepine receptor ligand with proconvulsant effect, CGS 8216, also inhibited GABA-responses<sup>6</sup>. Furthermore, the convulsants bicuculline, picrotoxin, pentylentetrazol, and penicillin were previously reported to be selective antagonists of GABA-mediated postsynaptic inhibition in cultured mammalian neurons<sup>12,23,29</sup>.

In this study, we evaluated the effects of  $\alpha$ -K- $\delta$ -GVA (an experimentally proven convulsant<sup>16</sup>) on responses to iontophoretically applied inhibitory

neurotransmitters (GABA and GLY) on mouse neurons in primary dissociated cell culture.

Our findings demonstrated that  $\alpha$ -K- $\delta$ -GVA reversibly decreased GABA- and GLY-responses on mouse spinal cord neurons in culture in a concentration-dependent manner. The results furthermore indicated that  $\alpha$ -K- $\delta$ -GVA did not exert its effects through interaction with the benzodiazepine receptor. Indeed, CGS 9896, a benzodiazepine receptor antagonist, did not antagonize the inhibitory effect of  $\alpha$ -K- $\delta$ -GVA on GABA-responses. Earlier studies indicated that the inhibitory neurotransmitters GABA and GLY act through different receptors on the surface membranes of these cultured neurons<sup>13,21,26</sup>. Furthermore, activation of chloride conductance has been demonstrated to underlie the GABA- and GLY-receptor-coupled events<sup>1</sup>. Since GABA and GLY exert their effects by activation of chloride conductance through interaction with different receptors, our observations suggest that  $\alpha$ -K- $\delta$ -GVA,

shown here not to be a benzodiazepine receptor ligand, may inhibit inhibitory amino acid-responses by blocking chloride channels.

One must question the pathophysiological importance of the observed inhibitory effects of  $\alpha$ -K- $\delta$ -GVA on inhibitory neurotransmitter-responses. Indeed, the  $\alpha$ -K- $\delta$ -GVA-induced decrease of GABA- and GLY-responses was obtained only at concentrations higher than those hitherto found in cerebrospinal fluid of hyperargininemic patients. However, determinations of  $\alpha$ -K- $\delta$ -GVA levels have only been performed on cerebrospinal fluid of treated hyperargininemic patients. Higher levels are to be expected in untreated patients. Moreover, when evaluating the possible neurotoxicity of a given compound, one should take its possible accumulation in brain tissue into account. Finally, different guanidino compounds might have an additive epileptogenic effect. A summative toxic effect has, for example, been demonstrated for other guanidino compounds in a paradigm

testing autohemolysis<sup>8</sup>.

In conclusion,  $\alpha$ -K- $\delta$ -GVA, a guanidino compound found to be increased in the biological fluids of hyperargininemic patients, inhibited responses to the inhibitory neurotransmitters GABA and GLY on mouse neurons in cell culture. The underlying mechanism is suggested to be the blocking of chloride channels. This effect might explain the in vivo epileptogenicity of this compound in rabbit. The possible pathophysiological importance of this neurotoxin in hyperargininemic patients is still uncertain.

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