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Stimulated Release of Exogenous GABA and Glutamate from Cerebral Cortical Synaptosomes and Brain Slices by Bis(acetato)tetrakis(imidazole) Copper(II) Complex

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ABSTRACT

In these experiments we have tested the effect of bis(acetato)tetrakis (imidazole) copper(II) on the release and uptake of ^{14}C-GABA and ^{3}H-glutamate from brain slices and brain cortical synaptosomes. $Cu(OAc)_2(Im)_4$ in concentrations ranging from 1 to 100 μ*M* has increased the release of GABA and glutamate from brain slices and synaptosomal preparations in a dose-related manner when the effect on GABA release is two-fold greater than glutamate and 10-fold greater than alanine. Pretreatment with a GABA uptake inhibitor such as 1–2 m*M* nipecotic acid has no effect on ^{14}C-GABA release, whereas hydroxy aspartate, the glutamate uptake inhibitor, has elevated the stimulated release of glutamate. Copper(II) chloride, the inorganic form of copper, had no significant effect either on GABA release or on glutamate release. The stimulated release of exoge-

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nous GABA and glutamate was Ca^{2+}-dependent, because it was inhibited by EGTA, and neuronal, because it was blocked by tetrodotoxin. The recent results can explain the anticonvulsant activity of $Cu(OAc)_2(Im)_4$ against strychnine-induced seizures by increasing the net release of GABA from cortical neurons.

Index Entries: Copper(II) complexes; GABA release; glutamate; brain slices; synaptosomes; tetrodotoxin.

INTRODUCTION

Copper complexes are well known for their various pharmacological activities, which include anti-inflammatory *(1,2),* antiulcer *(3),* antitumor *(4),* antidiabetic *(5,6),* and anticonvulsant activities *(4,5,7).*

The copper(II) complex with 3,5-diisopropylsalicylate was found to have anticonvulsant activity, by preventing metrazol and maximal electroshock-induced seizures *(8,9).* Other copper(II) salicylate derivatives, including aspirinate complexes, were effective in preventing maximal electroshock-induced seizures without having any effect on metrazol-induced seizures *(10,11),* Copper(II) acetate-imidazole (CAI) was reported to have a hypoglycemic activity *(6)* and a protective action against strychnine- and thiosemicarbazide-induced seizures *(12)*

In all cases of antidiabetic, anti-inflammatory, or anticonvulsant activities, the copper complexes were found to be more active and effective than their parent ligands or copper(II) inorganic forms *(6,10,13,14).*

The present study was undertaken to elucidate the mechanism of action of the bis(acetato)tetrakis(imidazole) copper(II) complex, as an anticonvulsant agent, by measuring its effect on the release of GABA and glutamate from synaptosomal preparations and brain slices.

MATERIALS AND METHODS

Drugs and Chemicals

Copper(II) chloride was purchased from Aldrich (Milwaukee, WI). Sucrose and other chemicals were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO). The copper(II) acetate complex was synthesized as previously described *(15,16).* Radioactive 4-amino-*n*-[U-^{14}C]-butyric acid, [U-^{3}H]D-glutamate and ^{3}H-alanine were purchased from Amersham Radio Chemicals (Amersham, Buckinghamshire, UK). Scintillation fluid, Omni-Szintisol cocktail scintillation grade, which contains (methanol, Triton X-100, xylene [mixture of isomers]), was purchased from Merck (Germany).

Preparation of Synaptosomes

Whole cerebral cortex tissue was rapidly dissected from female Albino rats (200–250 g body wt) following death by exsanguination and was placed in ice-cold 0.32 *M* sucrose. Synaptosomes were subsequently prepared from rat cerebral cortex as previously described *(17,18).* The synaptosomal pellet was preincubated for 20 min at 37°C in Krebs-phosphate medium of the following composition (m*M*) 124 NaCl, 5 KCl, 20 Na_2HPO_4, 1.3 $MgSO_4$, 1.2 KH_2PO_4, 0.75 $CaCl_2$, and 10 glucose, and at pH 7.4, the medium was thoroughly gassed with O_2/CO_2 (95/5% v/v) prior to resuspension.

Preparation of Brain Slices

Brain slices were rapidly prepared from each cerebral hemisphere using a McIlwain recessed guide and a razor blade. Two slices (100 µm thick) were incubated in 1 mL of Krebs-Tris incubation medium maintained at 37°C and bubbled with oxygen. The composition of the Krebs-Tris incubation medium was as follows (m*M*): 138 NaCl, 5 KCl, 1 $MgSO_4$, 1.2 $CaCl_2$, 1.2 NaH_2PO_4, 20 Tris, and 10 glucose, pH 7.4.

Release Studies

Synaptosomal preparations or brain slices were preincubated with Krebs phosphate buffer (KPB) for 20 min before preloading with [^{3}H]D-glutamate or 4-amino-*n*-[U-^{14}C]butyric acid for 5 min. After centrifugation for 2 min at 14,000*g,* the supernatant was discharged and the pellet was resuspended in fresh oxygenated KPB. The CAI was added to the treated samples in concentrations of 1, 10, 20, 50, and 100 µ*M* and incubated for 5 min in a shaking water bath. After centrifugation, the supernatant was collected as the released fraction, and the pellet was dissolved in 1 mL trichloroacetic acid (TCA) 10%, mixed well, and centrifuged once again; the supernatant is the amount of radioactivity that remained in the cells and is essential for the calculation of the released fraction. The 100-µL samples of the first and second supernatants were added to 3 mL of Packard Ultragold fluor and counted in a Packard Tricarb 1900-β Counter. The released fraction is the ratio between the counts per minute (cpm) in the first supernatant divided by the cpm in first plus second supernatants.

In other experiments, the CAI was added 1 min after addition of tetrodotoxin (TTX; 1 µ*M*) or EGTA (2 m*M*) or the uptake inhibitor for GABA, nipecotic acid (0.8 m*M*), or the uptake inhibitor for glutamate, hydroxyl aspartate (1 m*M*)

Values were expressed as mean ± standard error of the mean (SEM) for the number of experiments indicated in parentheses, and the statistical analysis was performed using the Student's *t*-test.

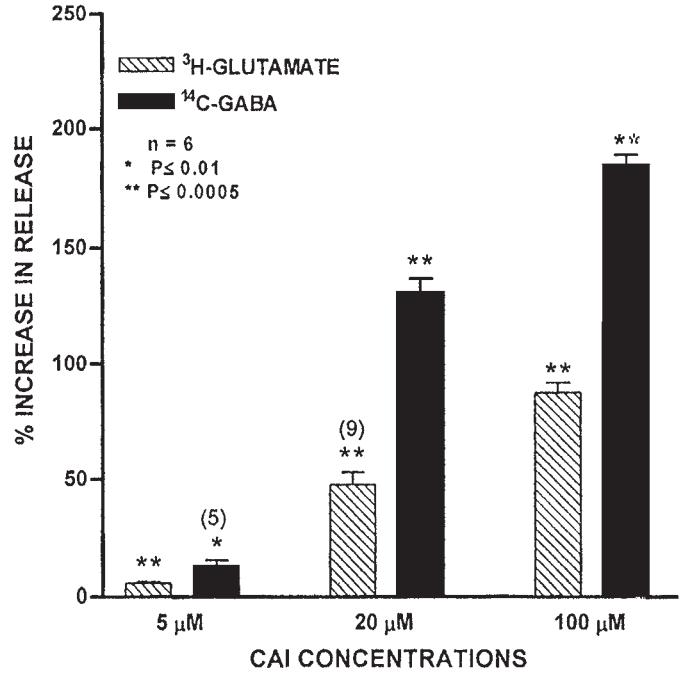

Fig. 1. Brain cerebral synaptosomes were incubated for 20 min at 37°C in the presence of [U-^{14}C]GABA or [^{3}H]D-glutamate (final specific activity 0.5 μCi/mL) and were then centrifuged, washed, and resuspended in fresh medium, following a further 15-min incubation period. Bars represent the percentage increase in [U-^{14}C]-GABA or [^{3}H]D-glutamate release from brain cerebral synaptosomes following treatment with CAI in concentrations ranging from 5 to 100 μ*M*. Values represent the mean ± SEM for the number of experiments indicated in the parentheses.

RESULTS AND DISCUSSION

Effect of Copper(II) Complex on the Release of GABA and Glutamate from Synaptosomal Preparations

Figure 1 shows that CAI (5 μ*M*) has increased the release of preloaded [U-^{14}C]GABA from cerebral cortical synaptosomes by 12.80 ± 2.71% (5) ($p \leq 0.01$). Twenty micromolars of the complex has increased the release by 130.81 ± 6.23% (6) ($p \leq 0.0005$) and 100 μ*M* CAI has increased the release of ^{14}C-GABA by 185.33 ± 4.02% (6) ($p \leq 0.0005$). The same concentrations of CAI have increased the release of ^{3}H-glutamate by lower levels of 5.83 ± 0.63% (6) ($p \leq 0.0005$), 47.63 ± 5.33% (9) ($p \leq 0.0005$), and 87.53 ± 4.41% (6) ($p \leq 0.0005$), respectively.

It is clear from our results that CAI has increased significantly and in a dose-dependent manner the exogenous release of ^{14}C-GABA and ^{3}H-glutamate from cerebral cortical synaptosomal preparations. The effect of CAI on the release of the main inhibitory neurotransmitter GABA was twofold greater than the release of glutamate.

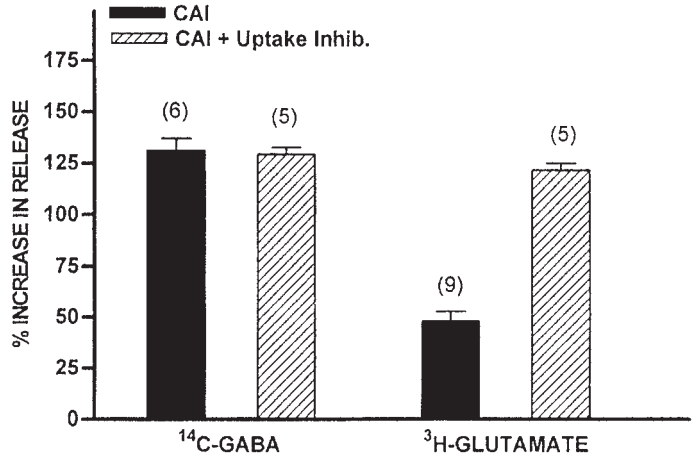

Fig. 2. For experimental details, see Fig. 1. CAI, 20 μ*M*, was added 1 min after the addition of the GABA uptake inhibitor (0.8 m*M* nipecotic acid) or the glutamate uptake inhibitor (1 m*M* hydroxyl aspartate). Data represent the mean ± SEM for the number of experiments indicated in parentheses.

Pretreatment of the synaptosomes with nipecotic acid (0.8 m*M*), a GABA uptake blocker, 1 min before the injection of CAI has no effect on the synaptosomal release of GABA, whereas pretreatment with hydroxyl-aspartate (1 m*M*), a glutamate uptake inhibitor, has increased the release of ^{3}H-glutamate by 155% from 47.63 ± 5.33 (9) to 121.46 ± 3.58 (5) ($p \leq 0.005$) and in levels similar to that of ^{14}C-GABA (Fig. 2), which indicates that the synaptosomal release of GABA from synaptosomes is partially the result of the inhibition of its uptake, and this is the difference between the effect of CAI on GABA and glutamate release.

Effect of Copper(II) Complex on the Release of GABA and Glutamate from Brain Slices

In brain slices, CAI (1 μ*M*) was found to be effective in increasing the release of ^{14}C-GABA by 7.56 ± 3.69% (14) ($p \leq 0.05$) without having any significant effect on the release of ^{3}H-glutamate and ^{3}H-alanine. CAI (10, 20, and 100 μ*M*) has increased significantly the release of ^{14}C-GABA from brain slices by 10.98 ± 1.30% (13) ($p \leq 0.001$), 20.58 ± 4.20% (11) ($p \leq 0.001$), and 33.21 ± 2.26% (16) ($p \leq 0.0005$), respectively. The same concentrations of the copper complex has increased the release of ^{3}H-glutamate in a concentration-dependent manner and in levels similar to that of GABA. The effect on ^{3}H-alanine release was significant but by 70% less than glutamate and GABA (Fig. 3).

Our results show that GABA and glutamate are released from brain slices to medium in large proportions, whereas with alanine, which is not a neurotransmittory amino acid, a smaller order of change was observed.

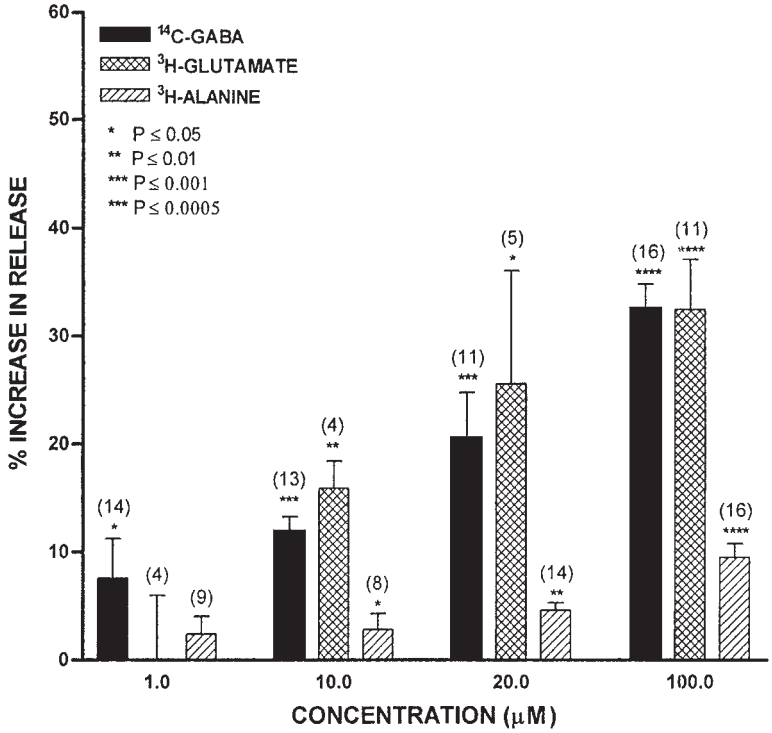

Fig. 3. Brain slices were incubated for 20 min at 37°C in the presence of [U-^{14}C]GABA, [^{3}H]D-glutamate, or [^{3}H]alanine (final specific activity = 0.5 μCi/mL) and were then centrifuged, washed, and resuspended in fresh medium, following a further 15-min incubation period. Bars represents the percentage increase in [U-^{14}C]GABA, [^{3}H]D-glutamate, or [^{3}H]alanine release from brain slices following treatment with CAI in concentrations ranging from 1 to 100 μ*M*. Values represent the mean ± SEM for the number of experiments indicated in parentheses.

Figure 4 shows that the inorganic copper compound $CuCl_2$ has no effect on the release of ^{14}C-GABA or ^{3}H-glutamate from brain slices even with high doses of CAI (100 μ*M*), which indicates that the copper complex is the active form and not the inorganic form of copper. Similar results showing the biological activity of CAI were reported in vivo with anti-seizure activity *(12)* and with hypoglycemic activity *(6)*, suggesting that copper(II) complexes are often more active, less toxic, and more desirable drugs than the parent ligands themselves *(14,19,20)*. The copper complexes increase the bioavailability and the activation of the copper-dependent enzymes, which are essential for normal physiological processes and the prevention of tissue damage.

Other forms of copper complexes have shown anticonvulsant activity; copper salicylate complexes were effective against maximal electroshock-induced seizures and metrazol-induced seizures *(21)*, whereas copper aspirinate complexes were effective anticonvulsant model against seizure without having any effect against metrazol-induced seizures *(11)*.

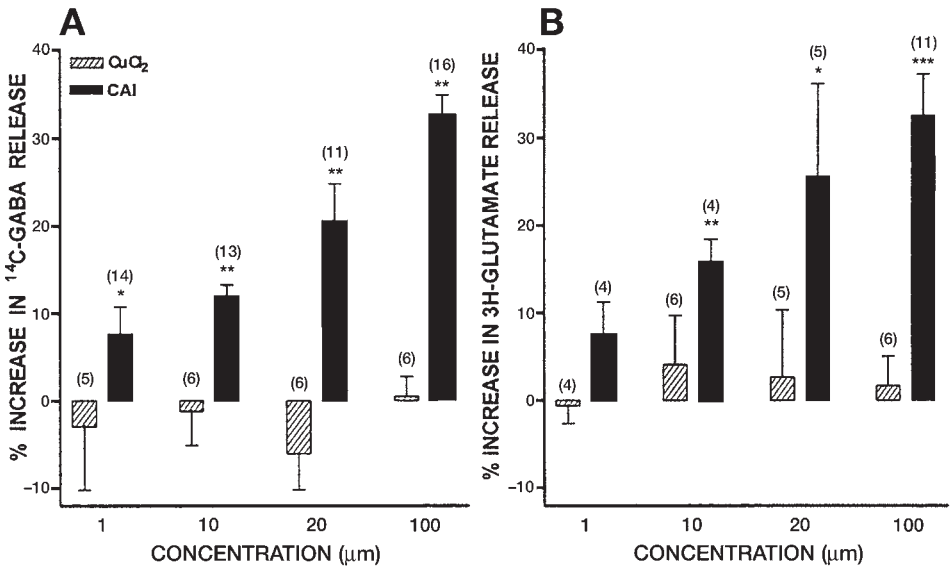

Fig. 4. For experimental details, see Fig. 3. Bars represent the percentage increase in **(A)** [U-^{14}C]GABA or **(B)** [^{3}H]glutamate release from brain slices following treatment with CAI or copper chloride ($CuCl_2$) in concentrations of 1, 10, 20, and 100 μ*M*. **p* ≤ 0.05; ***p* ≤ 0.001, ****p* ≤ 0.0005.

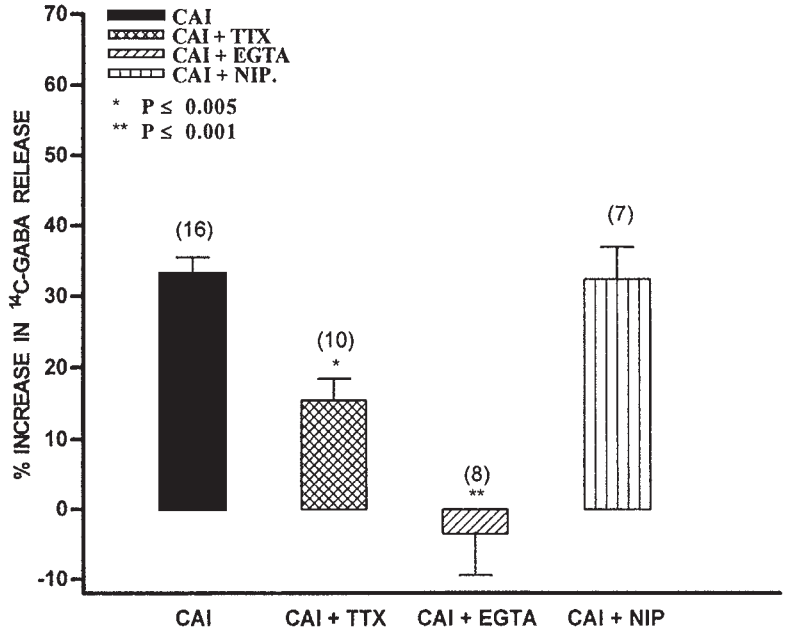

Fig. 5. Brain slices were incubated in Krebs phosphate medium at 37°C for 20 min with 30 μCi of [U-^{14}C]GABA and were then centrifuged, washed, and resuspended in fresh medium. Following a further 15-min incubation period, CAI (100 μ*M*) was added 1 min after the addition of EGTA (2 m*M*), tetrodotoxin (1 μ*M*), nipecotic acid (0.8 m*M*). Data represent the mean ± SEM for the number of experiments indicated in parentheses.

Effect of Pretreatment with Tetrodotoxin, EGTA, and Uptake Inhibitors

Figure 5 shows that CAI (100 μ*M*) has increased the release of ^{14}C-GABA from brain slices by 33% and this effect was blocked partially (53%) from 33.21 ± 2.26 (16) to 15.51 ± 2.99 (10) ($p \leq 0.005$) by the sodium channel blocker tetrodotoxin (TTX, 1 μ*M*) and completely blocked by calcium chelating agent EGTA (2 m*M*). However, pretreatment with a GABA uptake blocker like nipecotic acid (0.8 m*M*) has no effect on GABA release from brain slices by the copper complex.

CONCLUSION

From our results, it is clear that CAI in vitro causes a significant increase in the release of ^{14}C-GABA and ^{3}H-glutamate from synaptosomal preparations and brain slices; the effect is concentration dependent in concentrations ranging from 1 to 100 μ*M.* The effect on synaptosomal release is fivefold greater than the release from brain slices, which indicates that the effect on release is more neuronal and not glial, because brain slices include neurons and glial cells, whereas synaptosomes include neuronal nerve endings.

The effect of CAI on the release of the inhibitory neurotransmitter GABA is much higher (by twofold) than the release of the excitatory neurotransmitter glutamate, which could explain the anticonvulsant activity of the copper complex. Previous publications in our laboratory have shown in vivo the effect of the same copper complex in delaying the onset of strychnine- and thiosemicarbazide-induced convulsions *(12).*

Our results provide evidence for the effect of copper complexes on the release of GABA, the main inhibitory neurotransmitter in the motor cortex. The effect is neuronal and stimulated release, because it was magnified in synaptosomes, blocked by tetrodotoxin, and diminished by EGTA, a calcium chelating agent.

It is well known that substances that elevate GABA content in the extracellular fluid of the brain are considered anticonvulsants *(22).* Sodium dipropyl acetate raises brain GABA content by up to twofold by inhibiting the enzyme succinic semialdehyde dehydrogenase *(23,24).* μ-Vinyl and μ-acetylenic GABA, which inhibited GABA transaminase, lead to a rise in GABA levels in vivo and in vitro and in correlating anticonvulsant action *(25–27).*

On the contrary the depolarizing agent scorpion venom toxin was reported to enhance the release of preloaded [U-^{14}C]glutamate and [U-^{14}C]GABA from rat cortical synaptosomes when the effect on glutamate release was twofold greater than the release of GABA *(28,29).*

These results, emphasize the importance of the ratio between GABA and glutamate release. Compounds that decrease the ratio are considered

convulsants, and compounds that increase the ratio, like CAI, are considered anticonvulsants.

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