Coenzyme Q10 Inhibits the Release of Glutamate in Rat Cerebrocortical Nerve Terminals by Suppression of Voltage-Dependent Calcium Influx and Mitogen-Activated Protein Kinase Signaling Pathway

Yi Chang,[†,‡] Shu-Kuei Huang,[§] and Su-Jane Wang*,[†]

[†]School of Medicine, Fu Jen Catholic University, No. 510 Zhongzheng Road, Xinzhuang District, New Taipei City, Taiwan 24205
[‡]Department of Anesthesiology, Shin Kong Wu Ho-Su Memorial Hospital, 95 Wen-Chang Road, Taipei, Taiwan 111
[§]Department of Anesthesiology, Far-Eastern Memorial Hospital, Pan-Chiao District, New Taipei City, Taiwan 22060

ABSTRACT: This study investigates the effects and possible mechanism of coenzyme Q10 (CoQ10) on endogenous glutamate release in the cerebral cortex nerve terminals of rats. CoQ10 inhibited the release of glutamate evoked by the K^+ channel blocker 4-aminopyridine (4-AP). CoQ10 reduced the depolarization-induced increase in cytosolic $[Ca^{2+}]_c$ but did not alter the 4-AP-mediated depolarization. The effect of CoQ10 on evoked glutamate release was abolished by blocking the $Ca_v2.2$ (N-type) and $Ca_v2.1$ (P/Q-type) Ca^{2+} channels and mitogen-activated protein kinase kinase (MEK). In addition, CoQ10 decreased the 4-AP-induced phosphorylation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) and synaptic vesicle-associated protein synapsin I, a major presynaptic substrate for ERK. Moreover, the inhibition of glutamate release by CoQ10 was strongly attenuated in mice without synapsin I. These results suggest that CoQ10 inhibits glutamate release from cortical synaptosomes in rats through the suppression of the presynaptic voltage-dependent Ca^{2+} entry and ERK/synapsin I signaling pathway.

KEYWORDS: CoQ10, glutamate release, cerebrocortical nerve terminals, ERK, voltage-dependent Ca^{2+} channels

■ INTRODUCTION

Coenzyme Q10 (CoQ10; 2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-benzoquinone, Figure 1A), a critical component of the mitochondrial respiratory chain complexes, is present in vegetables, fruits, and several other dietary sources.[1] Dietary intake or supplementation of CoQ10 has been suggested to confer beneficial effects on cardiovascular disease and neurodegenerative disease such as Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (ALS).[2,3] These health benefits of CoQ10 are generally thought to be derived from its antioxidant and free radical scavenging properties.[4−6] CoQ10 can penetrate the blood−brain barrier (BBB) and plays a role in numerous neuronal events in the central nervous system (CNS). For example, CoQ10 attenuates glutamate- or amyloid-induced neurotoxicity,[7,8] protects against oxidative stress- or glucose-deprivation-induced brain damage,[7,9] and ameliorates β-Amyloid-induced memory impairment.[10] These studies suggest a neuroprotective role for CoQ10; however, the mechanism whereby CoQ10 exerts this capability is unclear.

Glutamate is a major excitatory neurotransmitter in the brain and plays an important role in functions such as synaptic plasticity, learning, and memory.[11] Excessive release of glutamate induces an increase in the intracellular Ca^{2+} levels. This in turn triggers a cascade of cellular responses, including enhanced oxygen free radical production, disturbed mitochondrial function, and protease activation, ultimately leading to neuronal cell death.[12] The neuronal damage induced by overexcitation is likely involved in a number of neuropathological conditions, ranging from acute insults such as stroke, epileptic seizures, traumatic brain and spinal cord injury

to chronic neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and ALS.[13,14] Consequently, if a compound can attenuate glutamate release from nerve terminals, it may have a neuroprotective effect on the pathological conditions related to excessive glutamate release. Some neuroprotective agents have been revealed to decrease glutamate release in human and rat brain tissues.[15−17] Likewise, CoQ10 has a neuroprotective-like effect and whether or not CoQ10 has an effect on endogenous glutamate release should be evaluated.

To the best of our knowledge, there are no studies addressing whether CoQ10 affects glutamate release at the presynaptic level. Therefore, this study uses isolated nerve terminals (synaptosomes) purified from the rat cerebral cortex as a model to investigate the effects of CoQ10 on glutamate release and characterize its underlying molecular mechanisms. Isolated presynaptic terminals represent a model system for directly investigating the molecular mechanisms underlying presynaptic phenomena. Specifically, this preparation is capable of accumulating, storing, and releasing neurotransmitters and is devoid of functional glial and nerve cell body elements that might obscure the interpretation of findings because of modulatory loci at non-neuronal, postsynaptic, or network levels.[18] The first series of experiments investigated the effects of CoQ10 on the release of endogenous glutamate, the
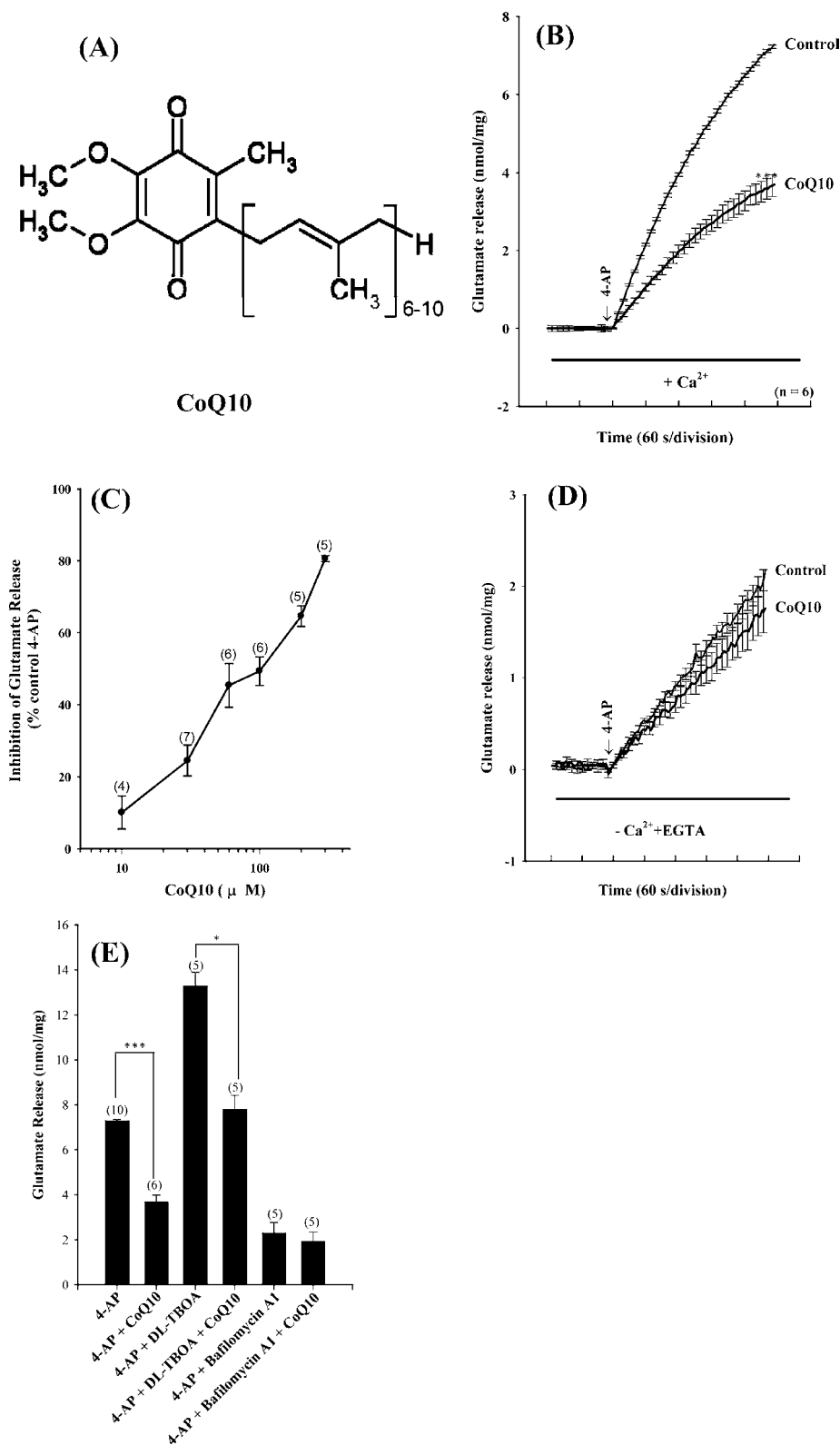
Figure 1. CoQ10 inhibits 4-AP-induced glutamate release from rat cerebrocortical nerve terminals; this effect is due to a decrease in vesicular exocytosis. (A) Chemical structure of CoQ10. Synaptosomes were resuspended in an incubation medium at a final protein concentration of 0.5 mg/mL and incubated for 3 min before the addition of 1 mM $CaCl_2$. 4-AP (1 mM) was added after a further 10 min to effect depolarization (arrow). Ca^{2+}-independent release was assayed by omitting $CaCl_2$ and adding 300 μM EGTA 10 min prior to depolarization. Total glutamate release ($+Ca^{2+}$; B) and Ca^{2+}-independent glutamate release ($-Ca^{2+}$; D) was measured under the control conditions or in the presence of 100 μM CoQ10 added 10 min prior to the addition of 4-AP. (C) Concentration–response curve for CoQ10 inhibition of a 4-AP-evoked glutamate release, showing percentage inhibition compared with the controls. (E) Quantitative comparison of the extent of glutamate release by 1 mM 4-AP in the absence and presence of 100 μM CoQ10 and absence and presence of 10 μM DL-TBOA or 0.1 μM bafilomycin A1. Results are given as the mean ± SEM of 4–10 independent experiments (*** $P < 0.001$, unpaired student's t test; * $P < 0.05$, one-way ANOVA).

synaptosomal plasma membrane potential, and the downstream activation of voltage dependent Ca^{2+} channels (VDCCs). The second series of experiments evaluated to determine if the protein kinase signaling pathway participates in the regulation of glutamate release by CoQ10.

■ MATERIALS AND METHODS

Chemicals. Fura-2-acetoxy-methyl ester (Fura-2-AM) and 3′,3′,3′-dipropylthiadicarbocyanine iodide [DiSC$_3$(5)] were obtained from Invitrogen (Carlsbad, CA). 2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059), dantrolene, bafilomycin A1, DL-threo-β-benzyloxyaspartate (DL-TBOA), 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP37157), N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89), bisin-dolylmaleimide I (GF109203X), and ω-conotoxin MVIIC (ω-CgTX MVIIC) were obtained from Tocris Cookson (Bristol, UK). N-(Cyclopropylmethoxy)-3,4,5-trifluoro-2-[(4-iodo-2-methylphenyl)-amino]-benzamide (PD198306) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibodies directed against ERK1/2 and phospho-ERK1/2 were purchased from Cell Signaling Technology (Beverly, MA). The anti-synapsin I phosphorylation state-specific rabbit polyclonal antibody directed against MAPK/ERK-phosphorylated sites 4, 5 of synapsin I (Ser^{62}/Ser^{67}) was from Millipore. Horseradish peroxidase-conjugated anti-rabbit secondary antibodies were from BioRad (Milan, Italy). Ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), sodium dodecyl sulfate (SDS), and all other reagents were obtained from Sigma-Aldrich Company (St. Louis, MO).

Animals. Two-month old male Sprague−Dawley rats or six-week old male wild-type mice or synapsin I-deficient mutant mice were employed in these studies. All animal procedures were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Fu Jen Catholic University Animal Care and Utilization Committee.

Heterozygous synapsin I knockout (B6; 129S-Syn1^{tm1Sud}/J) female (±) and wild type male (+/y) mice were purchased from the Jackson Laboratory (stock no. 002444, Bar Harbor, ME) and bred to produce an F1 population. Pups were weaned at 3 weeks of age and housed per gender at a maximum of four mice per cage. Due to the gene for synapsin I located on the X chromosome, only male littermates were genotyped for wild-type (+/y) and synapsin I knockout (−/y) mice. All experiments were conducted on mice at 6−8 weeks of age.

For genotyping, shortly after weaning, tail DNA were extracted and analyzed by a modified NEO touchdown (NEOTD) standard polymerase chain reaction (PCR) protocol provided by the Jackson Laboratory. The primers used were as follows: oIMR6916: 5′-CTT GGG TGG AGA GGC TAT TC-3′, oIMR6917: 5′-AGG TGA GAT GAC AGG AGA TC-3′, oIMR8744: 5′-CAA ATG TTG CTT GTC TGG TG-3′, and oIMR8745: 5′-GTC AGT CGA GTG CAC AGT TT-3′. The presence of the 280 bp PCR amplicon derived from the oIMR6916 and oIMR6917 primer combination indicated the knockout allele, whereas the presence of the ~200 bp PCR amplicon from the oIMR8744 and oIMR8745 primers symbolized the wild-type allele.

PCR was carried out with 200 ng template DNA, 1 μM each of oIMR6916, oIMR6917, oIMR8744 and oIMR8745 primers, 0.2 μM dNTP, 2.5 μL 10× buffer, and 1 unit of DNA polymerase (*Takara Ex Taq*, Takara Biotechnology, Shiga, Japan) in a final volume of 25 μL. Following tail DNA extraction, DNA fragments were amplified for 30 cycles of 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 1 min. Then, the mixture of 10 μL of PCR product and 2 μL 6× DNA loading dye (Protech Technology Enterprise Company, Ltd., Taipei, Taiwan) was run on an 1.5% agarose gel stained with ethidium bromide alongside a 1 kb DNA Ladder (Violet Bioscience, Inc., Taipei, Taiwan). Bands were visualized with an ultraviolet light (Tseng Hsiang Life Science Ltd., Taipei, Taiwan) illumination.

Synaptosomal Preparation. Synaptosomes were prepared from the cerebral cortex of 2-month-old male Sprague−Dawley rats as described previously.[19] The final synaptosomal fraction was resuspended in 2 mL HEPES-buffered incubation medium [HBM (mmol/L): NaCl, 140; KCl, 5; NaHCO$_3$, 5; MgCl$_2$·6H$_2$O, 1; Na$_2$HPO$_4$, 1.2; glucose, 10; HEPES, 20; pH 7.4] and the protein concentration was determined using the Bradford assay. Synaptosomes were centrifuged in the final wash to obtain synaptosomal pellets with 0.5 mg of protein. Synaptosomal pellets were stored on ice and used within 2−3 h.

Glutamate Release. Glutamate release was assayed by online fluorimetry as described previously.[19] Synaptosomal pellets (0.5 mg/mL) were resuspended in 2 mL of HBM containing 16 μM bovine serum albumin and incubated in a stirred and thermostatted cuvette maintained at 37 °C in a Perkin-Elmer LS-50B spectrofluorimeter (PerkinElmer Life and Analytical Sciences, Waltham, MA). NADP$^+$ (2 mM), glutamate dehydrogenase (50 units/mL), and CaCl$_2$ (1 mM) were added after 3 min. After a further 5 min of incubation, 4-aminopyridine (4-AP, 1 mM) or KCl (15 mM) was added to stimulate glutamate release. The oxidative decarboxylation of released glutamate leading to the reduction of NADP$^+$ was monitored by measuring the NADPH fluorescence at excitation and emission wavelengths of 340 and 460 nm, respectively. Data were obtained at 2 s intervals. A standard of exogenous glutamate (5 nmol) was added at the end of each experiment, and the fluorescence change produced by the standard administration was used to calculate the released glutamate as nanomoles glutamate per milligram synaptosomal protein (nmol/mg). Values quoted in the text and expressed in bar graphs represent levels of glutamate cumulatively releases after 5 min of depolarization.

Synaptosomal Plasma Membrane Potential. The synaptosomal membrane potential can be monitored by positively charged, membrane-potential sensitive carbocyanine dyes such as DiSC$_3$(5). DiSC$_3$(5) is a positively charged carbocyanine that accumulates in polarized synaptosomes that are negatively charged on the inside. At high concentrations, the dye molecules accumulate, and the fluorescence is quenched. Upon depolarization, the dye moves out, and hence, the fluorescence increases.[20] Synaptosomes were preincubated and resuspended as described for the glutamate release experiments. After 3 min of incubation, 5 μM DiSC$_3$(5) was added and allowed to equilibrate before the addition of CaCl$_2$ (1 mM) after 4 min of incubation. Then, 4-AP (1 mM) was added to depolarize the synaptosomes at 10 min, and DiSC$_3$(5) fluorescence was monitored at the excitation and emission wavelengths of 646 and 674 nm, respectively. Results are expressed in fluorescence units.

Cytosolic-Free Ca^{2+} Concentration, [Ca^{2+}]$_c$. [Ca^{2+}]$_c$ was measured using the Ca^{2+} indicator Fura-2-AM Synaptosomes (0.5 mg/mL) were preincubated in HBM with 16 μM BSA in the presence of 5 μM Fura-2-AM and 0.1 mM CaCl$_2$ for 30 min at 37 °C in a stirred test tube. After Fura-2-AM loading, synaptosomes were centrifuged in a microcentrifuge for 30 s at 3000g (5000 rpm). The synaptosomal pellets were resuspended in HBM with BSA, and the synaptosomal suspension was stirred in a thermostatted cuvette in a Perkin-Elmer LS-50B spectrofluorometer. CaCl$_2$ (1 mM) was added after 3 min, and further additions were made after an additional 10 min. Fluorescence data were accumulated at excitation wavelengths of 340 and 380 nm (emission wavelength of 505 nm) at 7.5 s intervals. Calibration procedures were performed as described previously,[21] using 0.1% SDS to obtain the maximal fluorescence with Fura-2 saturation with Ca^{2+}, followed by 10 mM EGTA (Tris-buffered) to obtain minimum fluorescence in the absence of any Fura-2/Ca^{2+}complex. [Ca^{2+}]$_c$ was calculated using equations described previously.[22]

Western Blotting. Synaptosomes (0.5 mg protein/mL) from control and drug-treated groups were lysed in ice-cold Tris-HCl buffer solution, pH 7.5, that contained 20 mM Tris-HCl, 1% Triton, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM phenylmethanesulfonyl fluoride, 1 mM sodium orthovanadate, and 1 μg/mL leupeptin. The lysates were sonicated for 10 s, and then centrifuged at 13000g at 4 °C for 10 min. Equal amounts of synaptosomal proteins were loaded on an SDS polyacrylamide gel and then transferred electrophoretically to nitrocellulose membranes. The membranes were blocked with Tris-buffered saline (TBS) that contained 5% low-fat milk and incubated with appropriate primary antibodies (antiphospho-ERK1/2, 1:2000;

11911

dx.doi.org/10.1021/jf302875k | *J. Agric. Food Chem.* 2012, 60, 11909−11918
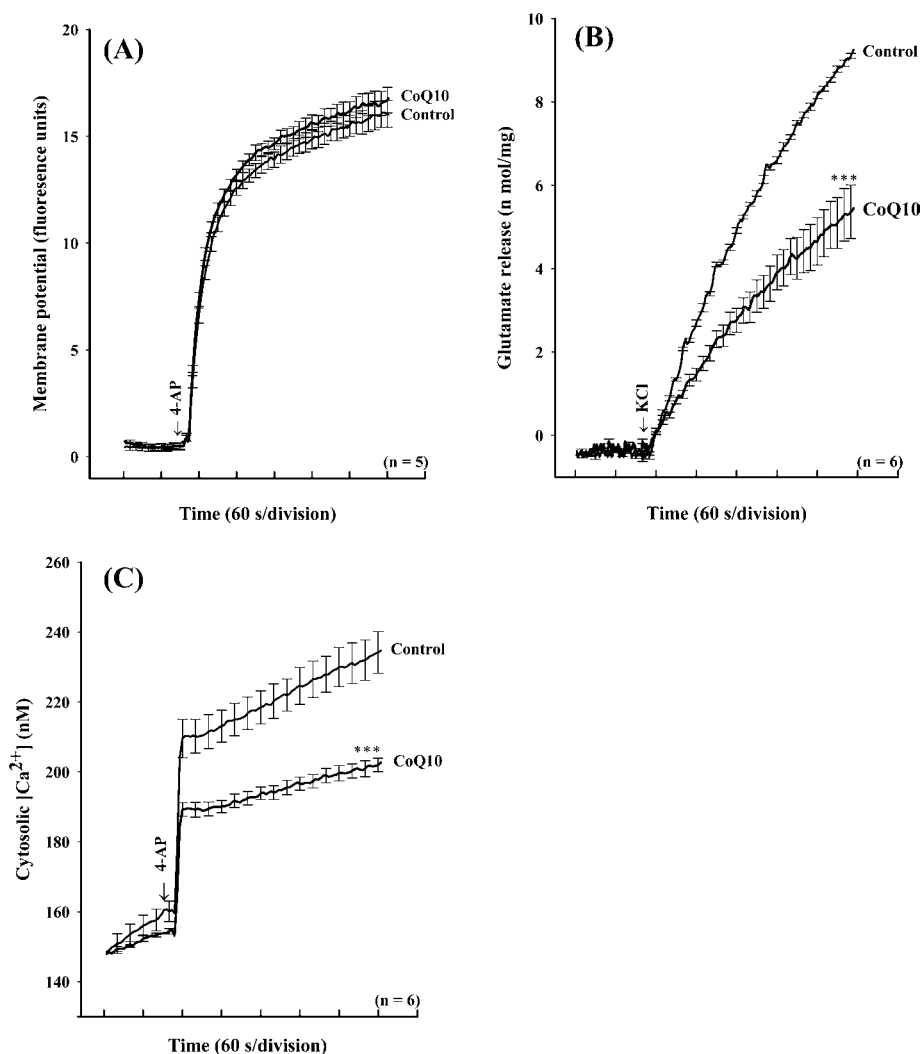
Figure 2. CoQ10 does not change the synaptosomal membrane potential but attenuates the 4-AP-induced increase in cytosolic Ca^{2+} levels. (A) Synaptosomal membrane potential monitored with $DiSC_3(5)$ in the absence (control) and in the presence of 100 μM CoQ10, added 10 min before depolarization with 1 mM 4-AP. (B) Glutamate release was induced by 15 mM KCl in the absence (control) or presence of 100 μM CoQ10, added 10 min before depolarization. (C) Cytosolic free Ca^{2+} concentration (nM) was monitored using Fura-2 in the absence (control) and in the presence of 100 μM CoQ10, added 10 min before depolarization. Results are given as the mean $\pm$ SEM of 6 independent experiments (*** $P < 0.001$, unpaired Student's t test).

anti-ERK1/2, 1:1000; antiphospho-synapsin-I (Ser[62]/Ser[67]), 1:1000; and β-actin, 1:500). Following three washes with TBS, the blots were incubated with the secondary horseradish peroxidase-conjugated antibody (1:3000) at room temperature for 1 h. The blots were washed again for three times by TBS, and the immunoreactive bands were detected by using the enhanced chemiluminescence method. After immunoblotting, films were scanned at 600 dpi in the transmittance mode by using a scanner. The level of phosphorylation was assessed by band density, which was quantified by densitometry.

Data Analysis. Data were analyzed in Lotus 1−2−3 (IBM, White Plains, NY) and MicroCal Origin (OriginLab Corporation, Northampton, MA). Data are expressed as the mean $\pm$ SEM. To test the significance of the effect of a drug versus control, an unpaired Student's t test was used. When an additional comparison was required (such as whether a second treatment influenced the action of CoQ10), a one-way analysis of variance (ANOVA) was used followed by a post hoct LSD comparison. Analysis was completed via software SPSS (17.0; SPSS Inc., Chicago, IL).

RESULTS

CoQ10 Inhibits 4-AP-Evoked Glutamate Release from Rat Cerebrocortical Synaptosomes. The depolarization of nerve terminals with the potassium channel blocker 4-aminopyridine (4-AP) has been shown to open voltage-dependent Ca^{2+} channels (VDCCs) and to induce the release of glutamate.[23] Using an online enzymatic assay for measuring glutamate, 4-AP (1 mM) evoked a glutamate release of 7.3 $\pm$ 0.04 nmol mg^{-1}/5 min from synaptosomes incubated in the presence of 1.2 mM $CaCl_2$. Application of CoQ10 (100 μM) for 10 min reduced the 4-AP-evoked glutamate release (3.7 $\pm$ 0.3 nmol mg^{-1}/5 min; $n = 6$; $P < 0.001$), without altering the basal release of glutamate (Figure 1B). The CoQ10-mediated inhibition of 4-AP-evoked glutamate release was concentration dependent, and the IC_{50} value derived from a concentration−response curve was approximately 60 μM (Figure 1C).
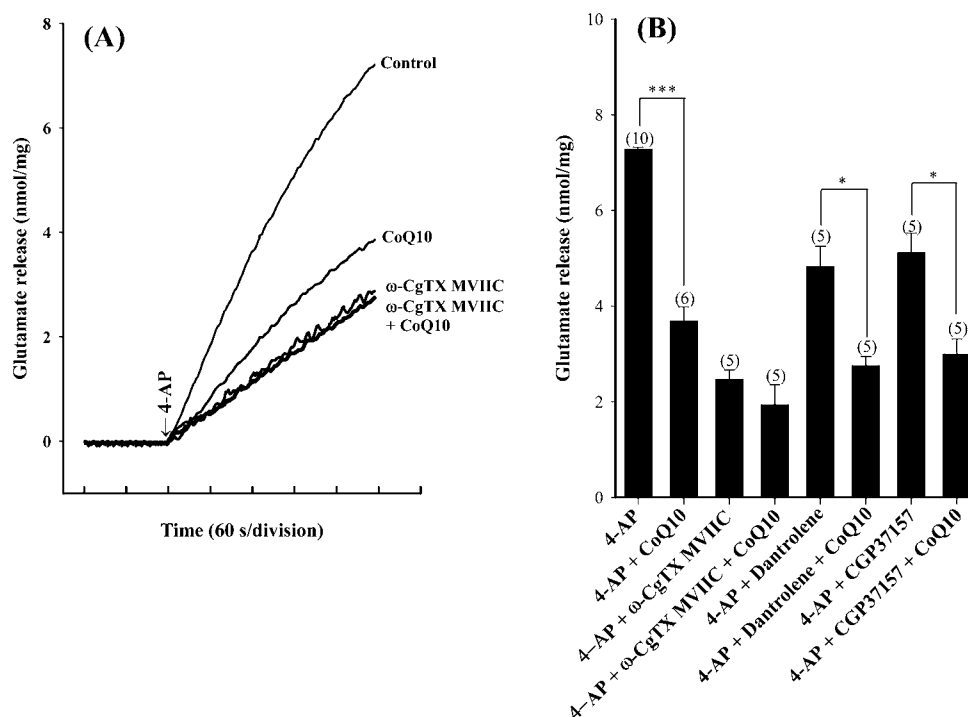
Figure 3. Blockade of $Ca_v2.2$ and $Ca_v2.1$ channels abolishes CoQ10 inhibition of glutamate release in rat cerebrocortical nerve terminals. (A) Glutamate release was evoked by 1 mM 4-AP in the absence (control) or presence of 100 μM CoQ10, 2 μM ω-CgTX MVIIC, 2 μM ω-CgTX MVIIC, and 100 μM CoQ10. (B) Quantitative comparison of the extent of glutamate release by 1 mM 4-AP in the absence or presence of 100 μM CoQ10 and the absence and presence of 2 μM ω-CgTX MVIIC, 10 μM dantrolene, or 100 μM CGP37157. CoQ10 was added 10 min before depolarization, whereas the other drugs were added 30 min before depolarization. Results are given as the mean ± SEM of 5−10 independent experiments (*** $P < 0.001$, unpaired Student's t test; * $P < 0.05$, one-way ANOVA).

Effect of Ca^{2+} Chelation, DL-TBOA, and Bafilomycin A1 on the Inhibition of 4-AP-Evoked Glutamate Release by CoQ10. We next investigated whether the inhibition of the 4-AP-evoked glutamate release by CoQ10 was mediated by an effect on exocytotic vesicular release or on a Ca^{2+}-independent release attributable to cytosolic efflux via reversal of the glutamate transporter.[24] First, the Ca^{2+}-independent glutamate efflux was measured by depolarizing the synaptosomes with 1 mM 4-AP in extracellular Ca^{2+}-free solution that contained 300 μM EGTA. Under these conditions, the release of glutamate evoked by 4-AP (1 mM) was 2.2 ± 0.1 nmol mg^{-1}/5 min. This Ca^{2+}-independent release evoked by 4-AP was, however, not affected by 100 μM CoQ10 (1.8 ± 0.2 nmol mg^{-1}/5 min) ($n = 10$; Figure 1D). Second, we used DL-TBOA, a nonselective inhibitor of all excitatory amino acid transporter (EAAT) subtypes, to examine the effect of CoQ10 on the 4-AP-evoked glutamate release in the normal Ca^{2+} concentration. In Figure 1E, the 4-AP (1 mM)-evoked glutamate release was inhibited by CoQ$_{10}$ (100 μM) to about the same extent both in the absence and presence of DL-TBOA (10 μM). DL-TBOA by itself almost doubled the 4-AP (1 mM)-evoked glutamate release (because of the inhibition of reuptake of the released glutamate) ($n = 5$; $P < 0.001$; Figure 1E). Finally, we examined the action of CoQ10 on the 4-AP-evoked glutamate release in the presence of bafilomycin A1, which causes the depletion of glutamate in synaptic vesicles. Figure 1E shows that bafilomycin A1 (0.1 μM) reduced the release of glutamate evoked by 4-AP (1 mM) (2.3 ± 0.1 nmol mg^{-1}/5 min; $P < 0.001$) and completely prevented the inhibitory effect of CoQ10 on the 4-AP-evoked glutamate release (1.9 ± 0.4 nmol mg^{-1}/5 min; $n = 5$). All these results suggest that the CoQ10-mediated

inhibition of glutamate release is due to a decrease in the Ca^{2+}-dependent, exocytotic component of glutamate release.

Effect of CoQ10 on the Synaptosomal Membrane Potential and the Intrasynaptosomal Ca^{2+} Levels. Because inhibition of Na^+ channels or activation of K^+ channels is known to stabilize membrane excitability and, consequently, cause a reduction in the evoked entry of Ca^{2+} and neurotransmitter release,[25,26] we reasoned that the observed inhibitory effect of CoQ10 on 4-AP-evoked glutamate release could be due to an alteration of nerve terminal excitability. To test this possibility, we examined the effect of CoQ10 on the synaptosomal plasma membrane potential under resting conditions and on depolarization, with the membrane-potential-sensitive dye $DiSC_3(5)$. Figure 2A shows that 4-AP (1 mM) caused an increase in $DiSC_3(5)$ fluorescence by 16.0 ± 0.6 fluorescence units/5 min. Application of CoQ10 (100 μM) for 10 min before 4-AP addition did not alter the resting membrane potential, and produced no significant change in the 4-AP-mediated increase in $DiSC_3(5)$ fluorescence (16.8 ± 0.6 fluorescence units/5 min) ($n = 5$). In addition, we confirmed the CoQ10-mediated inhibition of glutamate release using a alternative secretagogue, high external $[K^+]$ (Figure 2B). Elevated extracellular KCl depolarizes the plasma membrane by shifting the K^+ equilibrium potential above the threshold potential for activation of voltage-dependent ion channels. Whereas Na^+ channels are inactivated under these conditions, VDCCs are activated nonetheless to mediate Ca^{2+} entry, which supports the neurotransmitter release.[27] Addition of 15 mM KCl led to a glutamate release of 9.3 ± 0.03 nmol mg^{-1}/5 min, which was reduced to 5.4 ± 0.6 nmol mg^{-1}/5 min in the presence of CoQ10 (100 μM) ($n = 6$; $P < 0.01$; Figure 2B).
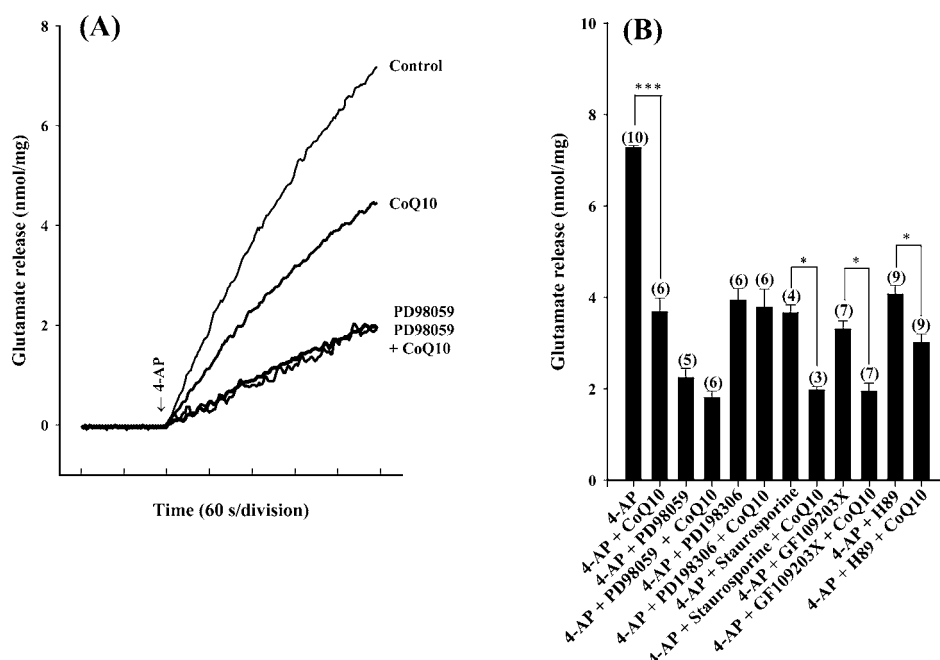
Figure 4. MEK (ERK) inhibitors occlude CoQ10-mediated inhibition of glutamate release from rat cerebrocortical nerve terminals. (A) Glutamate release was induced by 1 mM 4-AP in the absence (control) or presence of 100 μM CoQ10, 50 μM PD98059, or 50 μM PD98059 and 100 μM CoQ10. (B) Quantitative comparison of the extent of glutamate release by 1 mM 4-AP in the absence or presence of 100 μM CoQ10 and absence and presence of 50 μM PD98059, 50 μM PD198306, 2 μM staurosporine, 10 μM GF109203X, or 100 μM H89. PD98059, PD198306, staurosporine, GF109203X, or H89 was added 40 min before depolarization, whereas CoQ10 was added 10 min before depolarization. Results are given as the mean ± SEM of 5−10 independent experiments (*** $P < 0.001$, unpaired Student's t test; * $P < 0.05$, one-way ANOVA).

This indicates that the observed inhibition of evoked glutamate release by CoQ10 is unlikely to have been caused by attenuation of synaptosomal excitability.

Downstream of membrane depolarization, presynaptic inhibition of neurotransmitter release can be mediated by a reduction of intraterminal Ca^{2+} levels. To investigate whether a reduction in $[Ca^{2+}]_c$ was responsible for the CoQ10-mediated inhibition of release, we used a Ca^{2+} indicator Fura-2 to monitor intraterminal Ca^{2+} levels directly. In Figure 2C, after addition of 4-AP (1 mM), the $[Ca^{2+}]_c$ in synaptosomes was elevated to a plateau level of 234.7 ± 5.9 nM. This 4-AP-evoked rise in $[Ca^{2+}]c$ was decreased by the application of CoQ10 (100 μM) to 202.6 ± 1.9 nM ($n = 6$; $P < 0.001$).

Effect of ω-CgTX MVIIC, Dantrolene, and CGP37157 on the Inhibition of 4-AP-Evoked Glutamate Release by CoQ10. In the adult rat cerebrocortical synaptosomes, the release of glutamate evoked by depolarization is reported to be caused by Ca^{2+} influx through $Ca_v2.2$ (N-type) and $Ca_v2.1$ (P/Q-type) channels and Ca^{2+} release from internal stores.[28,29] Therefore, we next sought to establish which part of the Ca^{2+} source was involved in the effect of CoQ10 on the 4-AP-evoked glutamate release. ω-conotoxin MVIIC (ω-CgTX MVIIC), a wide-spectrum blocker of $Ca_v2.2$ and $Ca_v2.1$ channels, was used to assess the role of $Ca_v2.2$ and $Ca_v2.1$ channels. In Figure 3A, glutamate release evoked by 1 mM 4-AP under the control conditions was significantly decreased in the presence of 2 μM ω-CgTX MVIIC alone or 100 μM CoQ10 alone ($P < 0.001$). When ω-CgTX MVIIC (2 μM) and CoQ10 (100 μM) were applied simultaneously, the inhibition of glutamate release following 4-AP depolarization was not significantly different from the effect of ω-CgTX MVIIC alone ($n = 5$; Figure 3B). Dantrolene is known to inhibit Ca^{2+} release from intracellular stores by acting on ryanodine receptors on the endoplasmic

reticulum.[30] Treatment with dantrolene (10 μM) caused a reduction in glutamate release evoked by the same stimulus ($P < 0.01$; Figure 3B). In the presence of dantrolene, however, CoQ10 (100 μM) could still effectively inhibit 4-AP-evoked glutamate release ($n = 5$; $P < 0.05$; Figure 3B). Similar to dantrolene, CGP37157 (100 μM), a membrane-permeable blocker of mitochondrial Na^+/Ca^{2+} exchange, decreased the release of glutamate evoked by 4-AP (1 mM) ($P < 0.01$), but it had no effect on the CoQ10-mediated inhibition of the 4-AP-evoked glutamate release ($n = 5$; Figure 3B).

Involvement of a MAPK/ERK Pathway in the CoQ10-Mediated Inhibition of Glutamate Release. Because various kinases including mitogen-activated protein kinase (MAPK), protein kinase C (PKC), and protein kinase A (PKA) have been shown to regulate glutamate release at the presynaptic level,[31,32] we sought to establish which type of protein kinase signaling pathway participated in the inhibition of 4-AP-evoked glutamate release mediated by CoQ10. First, we examined the effect of PD98059, an inhibitor of mitogen-activated/extracellular signal-regulated kinase kinase (MEK), the immediate upstream regulator of MAPK. Figure 4A shows that control glutamate release evoked by 1 mM 4-AP was reduced by 50 μM PD98059 ($n = 5$; $P < 0.001$). Although CoQ10 (100 μM) reduced the 4-AP-evoked glutamate release ($n = 6$; $P < 0.001$), this effect was abolished by the pretreatment with PD98059, with the release measured in the presence of PD98059 and CoQ10 being similar to that obtained in the presence of PD98059 alone. Similar results were observed with another MEK inhibitor, PD198306. ($n = 6$; Figure 4B). In contrast, the PKC inhibitor GF109203X (10 μM) and the PKA inhibitor H89 (100 μM) reduced control of the 4-AP (1 mM)-evoked glutamate release ($P < 0.001$), but they had no effect on the CoQ10-mediated inhibition of the 4-AP-evoked glutamate
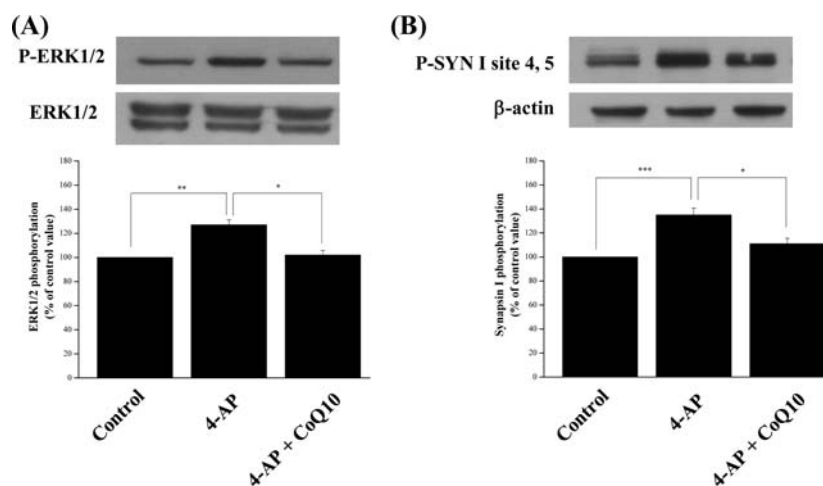
Figure 5. CoQ10 decreases the 4-AP-induced phosphorylation of ERK1/2 and synapsin I in rat cerebrocortical nerve terminals. Phosphorylation of (A) ERK1/2 and (B) synapsin I at MAPK-specific sites 4, 5 (P-SYN I site 4, 5) was detected in synaptosomal lysates by Western blotting using phosphorylation state-specific antibodies. Purified synaptosomes were incubated at 37 °C for 2 min in HBM that contained 1.2 mM $CaCl_2$ in the absence (control) or presence of 1 mM 4-AP, 1 mM 4-AP, and 100 μM CoQ10. CoQ10 was added 10 min before depolarization. Data are expressed as a percentage of the phosphorylation obtained in the controls in the absence of 4-AP stimulation. Results are given as the mean ± SEM of 3−5 independent experiments (*** $P < 0.001$, unpaired Student's t test; * $P < 0.05$, one-way ANOVA).
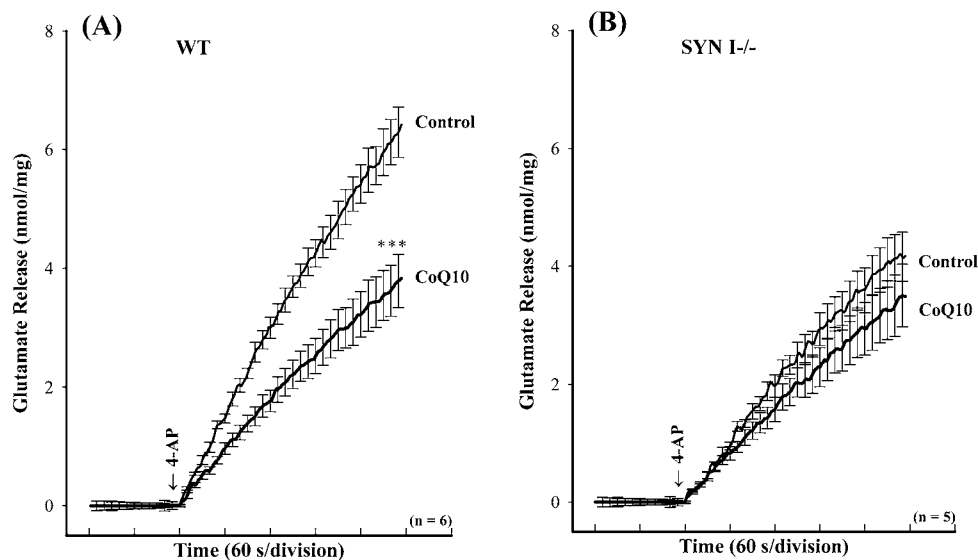
Figure 6. CoQ10-mediated inhibition of evoked glutamate release is attenuated in synaptosomes from synapsin I-deficient mice. Glutamate release was evoked by 1 mM 4-AP. Synaptosomes from wild-type (WT) and synapsin I-deficient (SYN I−/−) mice were preincubated for 10 min under standard conditions in the absence (control) or presence of CoQ10 (100 μM) followed by the addition of 4-AP (1 mM). Results are given as the mean ± SEM of 5−6 independent experiments (*** $P < 0.001$, unpaired Student's t test).

release (n = 7 or 9; Figure 4B). Similar results were observed in the presence of staurosporine (2 μM), an inhibitor of PKC and PKA[33] (n = 4; Figure 4B).

Figure 5A shows that depolarization of synaptosomes with 4-AP (1 mM) markedly increased the phosphorylation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) ($P < 0.01$). When synaptosomes were pretreated with CoQ10 (100 μM) for 10 min before depolarization with 4-AP, a significant decrease in the 4-AP (1 mM)-induced ERK1/2 phosphorylation was observed (n = 5; $P < 0.05$; Figure 5A). Similar results were obtained from the analysis of phosphorylation of synaptic vesicle-associated protein synapsin I, which is the major presynaptic substrate for ERK[34] (n = 3; Figure 5B).

CoQ10-Mediated Inhibition of Evoked Glutamate Release is Attenuated in Synapsin I-Deficient Mice. To further authenticate the role of synapsin I in the observed inhibition of glutamate release by CoQ10, we compared synaptosomes derived from wild-type and synapsin I-deficient mice. We tested the effect of deleting the synapsin I gene on the evoked release of glutamate, in the absence or presence of CoQ10 (Figure 6). In the absence of CoQ10, 4-AP-evoked glutamate release was attenuated in synapsin I-deficient mice (Figure 6). In wild-type mice, CoQ10 caused an inhibition of the 4-AP-evoked glutamate release of 3.2 ± 0.6 nmol mg^{-1}/5 min (41 ± 7%; n = 6; Figure 6A). In contrast, CoQ10 caused no significant inhibition of the 4-AP-evoked glutamate release in synapsin I-deficient mice (Figure 6B). These data support

the idea that synapsin I is involved in the inhibition of glutamate release by CoQ10.

■ DISCUSSION

Using a preparation of nerve terminals from a rat cerebral cortex, this study provides the novel finding that CoQ10 inhibits the 4-AP-evoked release of glutamate. To the best of our knowledge, this study presents the first examination of the effect of CoQ10 on endogenous glutamate release at the presynaptic level. Several possible mechanisms for the CoQ10-mediated inhibition of glutamate release are discussed as follows.

Neurotransmitter release is a complex phenomenon and can be modulated at several putative sites in the nerve terminal, including Na^+ channels, K^+ channels, Ca^{2+} channels, and the release process itself.[11,35] Therefore, when addressing the mechanism responsible for the CoQ10-mediated inhibition of Ca^{2+}-dependent glutamate release, this study considered two scenarios that might be involved: (1) alteration of the synaptosomal plasma membrane potential and downstream modulation of Ca^{2+} influx into the terminal and (2) direct regulation of VDCCs affecting Ca^{2+} entry. The first possibility is unlikely, based on several reasons. First, 4-AP- versus KCl-evoked glutamate release are significantly inhibited by CoQ10. Because 4-AP-evoked glutamate release involves Na^+ and Ca^{2+} channels, whereas KCl-evoked glutamate release involves only Ca^{2+} channels,[23,27] it is unlikely that Na^+ channels modulate glutamate release by CoQ10. Second, no significant effect of CoQ10 on synaptosomal plasma membrane potential appears either in the resting condition or on depolarization with 4-AP (indicating a lack of effect on K^+ conductance). Third, CoQ10 did not affect the 4-AP-evoked Ca^{2+}-independent glutamate release, which depends only on the membrane potential.[36] This indicates that CoQ10 does not affect glutamate release by reversing the direction of the plasma membrane glutamate transporter. The vesicular transporter inhibitor bafilomycin A1 (but not the glutamate transporter inhibitor DL-TBOA) completely abolished the inhibitory effect of CoQ10 on 4-AP-evoked glutamate release, supporting this suggestion. These results clearly suggest that the CoQ10-mediated inhibition of 4-AP-evoked glutamate release is mediated by a decrease in the Ca^{2+}-dependent exocytotic component of glutamate release. Moreover, this phenomenon is not because of a reduction in synaptosomal excitability caused by the modulation of Na^+ or K^+ ion channels.

Using Fura-2, this study demonstrated that CoQ10 significantly reduced the 4-AP-evoked increase in $[Ca^{2+}]_c$, indicating the inhibitory effect of CoQ10 on the glutamate release by decreasing intracellular Ca^{2+} levels. In synaptic terminals, extracellular Ca^{2+} influx through VDCCs and intracellular store Ca^{2+} release mediates a depolarization-induced increase in $[Ca^{2+}]_c$, coupled with glutamate release.[28,29] In the present study, the inhibition of glutamate release by CoQ10 was abolished under conditions in which all release-coupled $Ca_v2.2$ and $Ca_v2.1$ channels had been blocked, suggesting the involvement of $Ca_v2.2$ and $Ca_v2.1$ channels. Conversely, the reduced release of stored Ca^{2+} from the ER ryanodine receptors and mitochondria during the CoQ10-mediated inhibition of glutamate release could be excluded from participating. This is because the inhibitory effect of CoQ10 on 4-AP-evoked glutamate release was insensitive to both the ER ryanodine receptor inhibitor dantrolene and the mitochondrial Na^+/Ca^{2+} exchange inhibitor CGP37157.

Although there is no direct evidence that CoQ10 acts on presynaptic Ca^{2+} channels, these data implicate that a reduction in Ca^{2+} influx mediated by the $Ca_v2.2$ and $Ca_v2.1$ channels is associated with the inhibition of glutamate release by CoQ10. However, the blockade of $Ca_v2.2$ and $Ca_v2.1$ channel activity did not completely eliminate the inhibitory effect of CoQ10 on the 4-AP-evoked glutamate release (approximately 6% of the activity remained), raising the possibility that the $Ca_v2.2$ and $Ca_v2.1$-resistant Ca^{2+} channel types are involved in the action of CoQ10.

Some protein kinases, such as MAPK, PKC, and PKA, have been shown to be involved in the regulation of presynaptic VDCC function and glutamate release.[31,32] This study suggests a role for the MAPK/ERK pathway in the CoQ10-mediated inhibition of glutamate release based on the following results: (1) the MEK (MAP kinase kinase) inhibitors abolished the inhibitory effects of CoQ10 on the 4-AP-evoked glutamate release; (2) neither the PKC inhibitor GF109203X nor the PKA inhibitor H89 altered release inhibition; and (3) CoQ10 decreased 4-AP-induced phosphorylation of ERK1/2 and synapsin I at MAPK-specific sites 4 and 5. MAPK/ERK is a vital intracellular signaling system that is present at the presynaptic level and plays a crucial role in neurotransmitter exocytosis.[31,34,37] The depolarization-stimulated Ca^{2+} entry has been proven to lead to MAPK/ERK activation and to phosphorylation of synapsin I at sites 4 and 5. This phosphorylation reaction promotes the dissociation of synaptic vesicles from the actin cytoskeleton. This in turn makes more vesicles available at the active zone for neurotransmitter exocytosis, resulting in an increased glutamate release.[34,38] Accordingly, the results imply that the suppression of MAPK/ERK-dependent synapsin I phosphorylation and the consequent decreased availability of synaptic vesicles are involved in the observed CoQ10-mediated inhibition of glutamate release. Such a pathway is further supported by the observation that the inhibition of glutamate release by CoQ10 was strongly attenuated in mice lacking synapsin I. However, apart from synapsin I, the possible involvement of other synaptic proteins should be considered. Synapsin II and synapsin III, for example, are reported to be phosphorylated by MAPK.[34,39]

CoQ10 can cross the blood−brain barrier and accumulate in the brain.[40] Although the exact mechanisms responsible for the neuroprotective effect of CoQ10 remain to be elucidated, the possible involvement of scavenging free radicals or antioxidant properties has been reported.[5,6] In this work, the ability of CoQ10 to decrease glutamate release from nerve terminals may explain, in part, its neuroprotective mechanism. This is because excessive glutamate release is likely involved in the pathophysiology of several neurological states, including ischemic brain damage and neurodegenerative diseases.[13,14] Indeed, in patients with amyotrophic lateral sclerosis (ALS), a significant decrease in serum glutamate level is observed after treatment of riluzole, a clinically used neuroprotectant.[16] Furthermore, riluzole decreases depolarization-evoked glutamate release from rat cerebral cortex terminals.[17] In addition, in the present study CoQ10-inhibited glutamate release at a relatively high concentration of 100 μM. Consistent with this, several studies have reported that 100 μM of CoQ10 reduces neurotoxin-induced neurotoxicity in different experimental preparations.[8] However, the neural protective effects of CoQ10 were also reported at a lower concentration range (i.e., 10−30 μM).[7,9,41] The discrepancy is not clear but may be due to different

experimental models used; they used a cell culture model, while we used a nerve terminal (synaptosomal) model. Although the concentration of CoQ10 (100 μM) used in this study to produce the effect was high, the action of CoQ10 was specific. The observation supporting this statement revealed the following: (1) the effect of CoQ10 on the evoked glutamate release was prevented by chelating the extracellular Ca^{2+} ions and by the vesicular transporter inhibitor but was insensitive to the glutamate transporter inhibitor; (2) CoQ10 decreased the depolarization-induced increase in $[Ca^{2+}]_C$, whereas it did not alter the 4-AP-mediated depolarization; (3) CoQ10-mediated inhibition of glutamate release was abolished by the N-, P-, and Q-type Ca^{2+} channel blocker but not by the ryanodine receptor blocker or the mitochondrial Na^+/Ca^{2+} exchanger blocker; (4) MEK inhibitors blocked the effect of CoQ10, but the PKA or PKC inhibitor did not have such an effect; and (5) the effect of CoQ10 was significantly attenuated in mice without synapsin I.

In conclusion, the results of this study demonstrate that CoQ10 inhibits glutamate release from rat cerebrocortical synaptosomes by suppressing presynaptic $Ca_v2.2$ and $Ca_v2.1$ channels and ERK activity. The relevance of our finding to in vivo clinical situations remains to be determined. However, this finding may provide further understanding of the mode of the CoQ10 action in the brain, thereby emphasizing the therapeutic potential of this compound in the treatment of a wide range of neurological and neurodegenerative diseases.

■ AUTHOR INFORMATION

Corresponding Author

* E-mail: med0003@mail.fju.edu.tw. Tel: 886-2-29053465. Fax: 886-2-29052096.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

4-AP, 4-aminopyridine; BBB, blood−brain barrier; $[Ca^{2+}]_C$, cytosolic free Ca^{2+} concentration; CNS, central nervous system; CoQ10, coenzyme Q10; DiSC3(5), 3′,3′,3′-dipropylthiadicarbocyanine iodide; VDCCs, voltage-dependent Ca^{2+} channels; $Ca_v2.2$ and $Ca_v2.1$ channels, $Ca_v2.2$ (N-type) and $Ca_v2.1$ (P/Q-type) channels; DL-TBOA, DL-threo-β-benzyloxy aspartate; Fura-2-AM, fura-2-acetoxymethyl ester; GDH, glutamate dehydrogenase; HBM, HEPES buffer medium; BSA, bovine serum albumin; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N,N-tetraacetic acid; H89, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; SDS, sodium dodecyl sulfate; CGP37157, 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one; ω-CgTX MVIIC, ω-conotoxin MVIIC; ERK1/2, extracellular signal-regulated protein kinase 1 and 2; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; PKC, protein kinase C; PCR, polymerase chain reaction; PD98059, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one); GF109203X, bisindolylmaleimide I; TBS, Tris-buffered saline; PD198306, N-(cyclopropylmethoxy)-3,4,5-trifluoro-2-[(4-iodo-2-methylphenyl)amino]benzamide; H89, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride

■ REFERENCES

(1) Littarru, G. P.; Tiano, L. Bioenergetic and antioxidant properties of coenzyme Q10: Recent developments. *Mol. Biotechnol.* **2007**, *37*, 31−37.

(2) Beal, M. F. Coenzyme Q10 as a possible treatment for neurodegenerative diseases. *Free Radical Res.* **2002**, *36*, 455−460.

(3) Lekli, I.; Das, S.; Das, S.; Mukherjee, S.; Bak, I.; Juhasz, B.; Bagchi, D.; Trimurtulu, G.; Krishnaraju, A. V.; Sengupta, K.; Tosaki, A.; Das, D. K. Coenzyme Q9 provides cardioprotection after converting into coenzyme Q10. *J. Agric. Food Chem.* **2008**, *56*, 5331−5337.

(4) Lambelet, P.; Loliger, J.; Saucy, F.; Bracco, U. Antioxidant properties of coenzyme Q10 in food systems. *J. Agric. Food Chem.* **1992**, *40*, 581−584.

(5) Favit, A.; Nicoletti, F.; Scapagnini, U.; Canonico, P. L. Ubiquinone protects cultured neurons against spontaneous and excitotoxin-induced degeneration. *J. Cereb. Blood Flow Metab.* **1992**, *12*, 638−645.

(6) Schulz, J. B.; Henshaw, D. R.; Matthews, R. T.; Beal, M. F. Coenzyme Q10 and nicotinamide and a free radical spin trap protect against MPTP neurotoxicity. *Exp. Neurol.* **1995**, *132*, 279−83.

(7) Li, G.; Zou, L. Y.; Cao, C. M.; Yang, E. S. Coenzyme Q10 protects SHSY5Y neuronal cells from beta amyloid toxicity and oxygen-glucose deprivation by inhibiting the opening of the mitochondrial permeability transition pore. *Biofactors* **2005**, *25*, 97−107.

(8) Zeron, M. M.; Fernandes, H. B.; Krebs, C.; Shehadeh, J.; Wellington, C. L.; Leavitt, B. R.; Baimbridge, K. G.; Hayden, M. R.; Raymond, L. A. Potentiation of NMDA receptor-mediated excitotoxicity linked with intrinsic apoptotic pathway in YAC transgenic mouse model of Huntington's disease. *Mol. Cell. Neurosci.* **2004**, *25*, 469−479.

(9) Somayajulu, M.; McCarthy, S.; Hung, M.; Sikorska, M.; Borowy-Borowski, H.; Pandey, S. Role of mitochondria in neuronal cell death induced by oxidative stress; neuroprotection by Coenzyme Q10. *Neurobiol. Dis.* **2005**, *18*, 618−627.

(10) Dumont, M.; Kipiani, K.; Yu, F.; Wille, E.; Katz, M.; Calingasan, N. Y.; Gouras, G. K.; Lin, M. T.; Beal, M. F. Coenzyme Q10 decreases amyloid pathology and improves behavior in a transgenic mouse model of Alzheimer's disease. *J. Alzheimer's Dis.* **2011**, *27*, 211−223.

(11) Greenamyre, J. T.; Porter, R. H. Anatomy and physiology of glutamate in the CNS. *Neurology* **1994**, *44*, S7−S13.

(12) Coyle, J. T.; Puttfarcken, P. Oxidative stress, glutamate, and neurodegenerative disorders. *Science* **1993**, *262*, 689−695.

(13) Meldrum, B. S. Glutamate as a neurotransmitter in the brain: review of physiology and pathology. *J. Nutr.* **2000**, *130*, 1007S−1015S.

(14) Raiteri, L.; Stigliani, S.; Zappettini, S.; Mercuri, N. B.; Raiteri, M.; Bonanno, G. Excessive and precocious glutamate release in a mouse model of amyotrophic lateral sclerosis. *Neuropharmacology* **2004**, *46*, 782−792.

(15) Klein, J.; Mdzinarishvili, A.; Sambria, R. K.; Lang, D. Ginkgo extract EGb761 confers neuroprotection by reduction of glutamate release in ischemic brain. *J. Pharm. Pharm. Sci.* **2012**, *15*, 94−102.

(16) Niebroj-Dobosz, I.; Janik, P.; Kwieciński, H. Effect of Riluzole on serum amino acids in patients with amyotrophic lateral sclerosis. *Acta Neurol. Scand.* **2002**, *106*, 39−43.

(17) Wang, S. J.; Wang, K. Y.; Wang, W. C. Mechanisms underlying the riluzole inhibition of glutamate release from rat cerebral cortex nerve terminals (synaptosomes). *Neuroscience* **2004**, *125*, 191−201.

(18) Dunkley, P. R.; Jarvie, P. E.; Heath, J. W.; Kidd, G. J.; Rostas, J. A. A rapid method for isolation of synaptosomes on Percoll gradients. *Brain Res.* **1986**, *372*, 115−129.

(19) Lin, T. Y.; Lu, C. W.; Wang, S. J. Astaxanthin inhibits glutamate release in rat cerebral cortex nerve terminals via suppression of voltage-dependent Ca^{2+} entry and mitogen-activated protein kinase signaling pathway. *J. Agric. Food Chem.* **2010**, *58*, 8271−8278.

(20) Akerman, K. E.; Scott, I. G.; Heikkila, J. E.; Heinonen, E. Ionic dependence of membrane potential and glutamate receptor-linked responses in synaptoneurosomes as measured with a cyanine dye, DiSC3(5). *J. Neurochem.* **1987**, *48*, 552−559.

(21) Sihra, T. S.; Bogonez, E.; Nicholls, D. G. Localized Ca^{2+} entry preferentially effects protein dephosphorylation, phosphorylation, and glutamate release. *J. Biol. Chem.* **1992**, *267*, 1983−1989.

(22) Grynkiewicz, G.; Poenie, M.; Tsien, R. Y. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **1985**, *260*, 3440−3450.

(23) Nicholls, D. G. Presynaptic modulation of glutamate release. *Prog. Brain Res.* **1998**, *116*, 15−22.

(24) Nicholls, D. G.; Sihra, T. S.; Sanchez-Prieto, J. Calcium-dependent and -independent release of glutamate from synaptosomes monitored by continuous fluorometry. *J. Neurochem.* **1987**, *49*, 50−57.

(25) Li, M.; West, J. W.; Numann, R.; Murphy, B. J.; Scheuer, T.; Catterall, W. A. Convergent regulation of sodium channels by protein kinase C and cAMP-dependent protein kinase. *Science* **1993**, *261*, 1439−1442.

(26) Pongs, O.; Leicher, T.; Berger, M.; Roeper, J.; Bahring, R.; Wray, D.; Giese, K. P.; Silva, A. J.; Storm, J. F. Functional and molecular aspects of voltage-gated K^+ channel β subunits. *Ann. N.Y. Acad. Sci.* **1999**, *868*, 344−355.

(27) Barrie, A. P.; Nicholls, D. G.; Sanchez-Prieto, J.; Sihra, T. S. An ion channel locus for the protein kinase C potentiation of transmitter glutamate release from guinea pig cerebrocortical synaptosomes. *J. Neurochem.* **1991**, *57*, 1398−1404.

(28) Millan, C.; Sanchez-Prieto, J. Differential coupling of N and P/Q type calcium channels to glutamate exocytosis in the rat cerebral cortex. *Neurosci. Lett.* **2002**, *330*, 29−32.

(29) Vazquez, E.; Sanchez-Prieto, J. Presynaptic modulation of glutamate release targets different calcium channels in rat cerebrocortical nerve terminals. *Eur. J. Neurosci.* **1997**, *9*, 2009−2018.

(30) Zucchi, R.; Ronca-Testoni, S. The sarcoplasmic reticulum Ca^{2+} channel/ryanodine receptor: Modulation by endogenous effectors, drugs and disease states. *Pharmacol. Rev.* **1997**, *49*, 1−51.

(31) Pereira, D. B.; Carvalho, A. P.; Duarte, C. B. Non-specific effects of the MEK inhibitors PD98059 and U0126 on glutamate release from hippocampal synaptosomes. *Neuropharmacology* **2002**, *42*, 9−19.

(32) Millan, C.; Torres, M.; Sanchez-Prieto, J. Co-activation of PKA and PKC in cerebrocortical nerve terminals synergistically facilitates glutamate release. *J. Neurochem.* **2003**, *87*, 1101−1111.

(33) Wilkinson, S. E.; Hallam, T. J. Protein kinase C: Is its pivotal role in cellular activation over-stated? *Trends Pharmacol. Sci.* **1994**, *15*, 53−57.

(34) Lin, T. Y.; Lu, C. W.; Chang, C. C.; Huang, S. K.; Wang, S. J. Luteolin inhibits the release of glutamate in rat cerebrocortical nerve terminals. *J. Agric. Food Chem.* **2011**, *59*, 8458−8466.

(35) Sihra, T. S.; Nichols, R. A. Mechanisms in the regulation of neurotransmitter release from brain nerve terminals: current hypotheses. *Neurochem. Res.* **1993**, *18*, 47−58.

(36) Attwell, D.; Barbour, B.; Szatkowski, M. Nonvesicular release of neurotransmitter. *Neuron* **1993**, *375*, 645−653.

(37) Jovanovic, J. N.; Benfenati, F.; Siow, Y. L.; Sihra, T. S.; Sanghera, J. S.; Pelech, S. L. Neurotrophins stimulate phosphorylation of synapsin I by MAP kinase and regulate synapsin I-actin interactions. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 3679−3683.

(38) Chi, P.; Greengard, P.; Ryan, T. A. Synaptic vesicle mobilization is regulated by distinct synapsin I phosphorylation pathways at different frequencies. *Neuron* **2003**, *38*, 69−78.

(39) Fdez, E.; Hilfiker, S. Vesicle pools and synapsins: new insights into old enigmas. *Brain Cell Biol.* **2006**, *35*, 107−115.

(40) Matthews, R. T.; Yang, L.; Browne, S.; Baik, M.; Beal, M. F. Coenzyme Q_{10} administration increases brain mitochondrial concentrations and exerts neuroprotective effects. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 8892−8897.

(41) McCarthy, S.; Somayajulu, M.; Sikorska, M.; Borowy-Borowski, H.; Pandey, S. Paraquat induces oxidative stress and neuronal cell death; neuroprotection by water-soluble Coenzyme Q10. *Toxicol. Appl. Pharmacol.* **2004**, *201*, 21−31.