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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

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The purpose of this study was to examine the effect and mechanism of astaxanthin, a natural carotenoid, on endogenous glutamate release in nerve terminals of rat cerebral cortex (synaptosomes). Results showed that astaxanthin exhibited a dose-dependent inhibition of 4-aminopyridine (4-AP)-evoked release of glutamate. The effect of astaxanthin on the evoked glutamate release was prevented by chelating the intrasynaptosomal Ca^{2+} ions and by the vesicular transporter inhibitor, but was insensitive to the glutamate transporter inhibitor. Astaxanthin decreased depolarization-induced increase in $[Ca^{2+}]_C$, whereas it did not alter the resting synaptosomal membrane potential or 4-AP-mediated depolarization. The effect of astaxanthin on evoked glutamate release was abolished by the N-, P- and Q-type Ca^{2+} channel blockers, but not by the ryanodine receptor blocker or the mitochondrial Na^+/Ca^{2+} exchanger blocker. In addition, the inhibitory effect of astaxanthin on evoked glutamate release was prevented by the mitogen-activated protein kinase (MAPK) inhibitors PD98059 and U0126. Western blot analyses showed that astaxanthin significantly decreased the 4-AP-induced phosphorylation of MAPK, and this effect was blocked by PD98059. On the basis of these results, it was concluded that astaxanthin inhibits glutamate release from rat cortical synaptosomes through the suppression of presynaptic voltage-dependent Ca^{2+} entry and MAPK signaling cascade.

KEYWORDS: Astaxanthin; glutamate release; presynaptic Ca^{2+} channels; MAPK; cerebrocortical nerve terminals

INTRODUCTION

Dietary intake or supplementation of natural products has been suggested to confer numerous health benefits such as cardiovascular disease, cancer, and neurodegenerative disease prevention, as well as immune system boosting. Astaxanthin (**Figure 1**) is a naturally occurring carotenoid widely distributed in various plants, algae, and seafoods and is a more powerful antioxidant than other carotenoids, including zeaxanthin, lutein, canthaxanthin, and β-carotene (*1*−*3*). Several studies have demonstrated that astaxanthin possesses many biological activities including anti-inflammatory, antitumor, antidiabetic, and immunomodulatory effects (*4*−*7*). In addition to these, the neuroprotective effects of astaxanthin also have been reported by several in vitro and in vivo studies. It has been shown, for example, that astaxanthin protects against oxidative stress- or neurotoxin-induced neurotoxicity in cultured retinal ganglion cells, human neuroblastoma SH-SY5Y cells, and PC12 cells (*3*, *8*−*11*), as well as attenuates ischemia-induced brain injury and memory impairment in experimental animals (*12*, *13*). On the basis of these reports, astaxanthin might be a potential candidate for a natural neuroprotective agent. The mechanisms responsible for the neuroprotective effects of astaxanthin are, however, not fully clarified, although it has been reported that this beneficial effect is related to its antioxidant and anti-inflammatory effects (*7*, *11*, *13*).

In the mammalian central nervous system (CNS), glutamate is the major excitatory neurotransmitter and plays an important role in many functions such as cognition, movement, learning, and memory (*14*, *15*). However, besides its physiological role, excessive release of glutamate may induce an increase in intracellular Ca^{2+} levels, which in turn triggers a cascade of cellular responses, including enhanced oxygen free radical production, disturbed mitochondrial function, and protease activation, that ultimately kill the neurons (*16*, *17*). This type of overexcitation-induced neuronal damage is thought to be 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 amyotrophic lateral sclerosis (*18*−*20*). Therefore, a reduction in glutamate release may have important consequence and may be a potential mechanism for neuroprotective actions. Although astaxanthin has been shown to reduce ischemia-induced glutamate overflow in rat cerebral cortex (*13*), direct evidence for astaxanthin actions on the presynaptic level is lacking.

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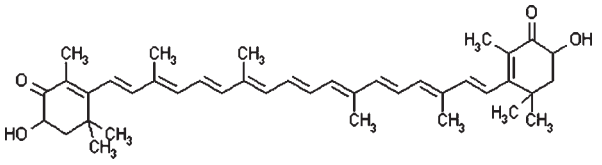

Figure 1. Chemical structure of astaxanthin.

The purpose of the current study was to use isolated nerve terminals (synaptosomes) purified from the rat cerebral cortex to investigate the effect of astaxanthin on the levels of endogenous released glutamate and to characterize the underlying mechanisms of glutamate release regulation by astaxanthin. The isolated presynaptic terminal represents a model system for investigating directly 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 interpretation because of modulatory loci at non-neuronal, postsynaptic, or network levels (*21*). The experiments were performed with synaptosomes by monitoring the effects of astaxanthin on the release of endogenous glutamate, the synaptosomal plasma membrane potential, and the downstream activation of voltage-dependent Ca^{2+} channels (VDCCs). In addition, in view of the demonstrated role of various kinases, including protein kinase C (PKC), protein kinase A (PKA), and mitogen-activated protein kinase (MAPK), in presynaptic modulation (*22*−*24*), this study also examined which type of protein kinase signaling pathway was involved in astaxanthin-regulated glutamate release.

MATERIALS AND METHODS

Chemicals. 3′,3′,3′-Dipropylthiadicarbocyanine iodide [$DiSC_3(5)$], and fura-2-acetoxymethyl ester (Fura-2-AM) were purchased from Invitrogen (Carlsbad, CA). Bafilomycin A1, ω-conotoxin GVIA (ω-CgTX GVIA), ω-conotoxin MVIIC (ω-CgTX MVIIC), ω-agatoxin IVA (ω-AgTX IVA), dantrolene, DL-*threo*-β-benzyloxyaspartate (DL-TBOA), 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD98059), 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126), and bisindolylmaleimide I (GF109203X) were purchased from Tocris Cookson (Bristol, U.K.). Astaxanthin, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester (BAPTA-AM), SDS, and all other reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Animals. Adult male Sprague−Dawley rats (150−200 g) 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 Institutional Animal Care and Utilization Committee.

Synaptosomal Preparation. Synaptosomes were prepared as described previously (*25*). Briefly, the cerebral cortex from male Sprague−Dawley rats was isolated and homogenized in a medium that contained 320 mM sucrose, pH 7.4. The homogenate was spun for 2 min at 3000*g* (5000 rpm in a JA 25.5 rotor; Beckman Coulter, Inc.) at 4 °C, and the supernatant was centrifuged again at 14500*g* (11000 rpm in a JA 25.5 rotor) for 12 min. The pellet was gently resuspended in 8 mL of 320 mM sucrose, pH 7.4. Two milliliters of this synaptosomal suspension was added to 3 mL of Percoll discontinuous gradient that contained 320 mM sucrose, 1 mM EDTA, 0.25 mM DL-dithiothreitol, and 3, 10, and 23% Percoll, pH 7.4. The gradients were centrifuged at 32500*g* (16500 rpm in a JA 20.5 rotor) for 7 min at 4 °C. Synaptosomes placed between the 10 and 23% Percoll bands were collected and diluted in a final volume of 30 mL of HEPES buffer medium (HBM) that consisted of 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1 mM $MgCl_2 \cdot 6H_2O$, 1.2 mM Na_2HPO_4, 10 mM glucose, and 10 mM HEPES, pH 7.4, before centrifugation at 27000*g* (15000 rpm in a JA 25.5) for 10 min. The pellets thus formed were resuspended in 3 mL of HBM, and the protein content was determined using a Bradford Protein Assay Kit (Bio-Rad, Hercules, CA), based on the method of Bradford (*26*), with BSA as a standard. Half a milligram of synaptosomal suspension was diluted in 10 mL of HBM and spun at 3000*g* (5000 rpm in a JA 20.1 rotor) for 10 min. The supernatants were discarded, and the synaptosomal pellets were stored on ice and used within 4−6 h.

Glutamate Release. Glutamate release from purified cerebrocortical synaptosomes was monitored online, with an assay that employed exogenous glutamate dehydrogenase (GDH) and $NADP^+$ to couple the oxidative deamination of the released glutamate to the generation of NADPH detected fluorometrically (*25*). Synaptosomal pellets were resuspended in HBM that contained 16 μM BSA and incubated in a stirred and thermostated cuvette maintained at 37 °C in a Perkin-Elmer LS-50B spectrofluorometer. $NADP^+$ (2 mM), GDH (50 units/mL), and $CaCl_2$ (1 mM) were added after 3 min. In experiments that investigated Ca^{2+}-independent efflux of glutamate, EGTA (200 μM) was added in place of $CaCl_2$. Other additions before depolarization were made as described in the figure captions. After a further 10 min of incubation, 4-AP (1 mM) or KCl (15 mM) was added to stimulate glutamate release. Glutamate release was monitored by measuring the increase of fluorescence (excitation and emission wavelengths of 340 and 460 nm, respectively) caused by NADPH being produced by oxidative deamination of released glutamate by GDH. Data were accumulated at 2 s intervals. A standard of exogenous glutamate (5 nmol) was added at the end of each experiment, and the fluorescence response used to calculate released glutamate was expressed as nanomoles of glutamate per milligram of synaptosomal protein (nmol/mg). Values quoted in the text and expressed in bar graphs represent levels of glutamate cumulatively released 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 (*27*). 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. 4-AP (1 mM) was added to depolarize the synaptosomes at 10 min, and $DiSC_3(5)$ fluorescence was monitored at excitation and emission wavelengths of 646 and 674 nm, respectively.

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 3000*g* (5000 rpm). The synaptosomal pellets were resuspended in HBM with BSA, and the synaptosomal suspension was stirred in a thermostated 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 (*28*), 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 (*29*).

Western Blotting. Synaptosomes (0.5 mg protein/mL) from control and astaxanthin-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 13000*g* at 4 °C for 10 min. Equal amounts of synaptosomal proteins were loaded on a SDS−polyacrylamide gel and transferred electrophoretically to nitrocellulose membranes. The membranes were blocked with Tris-buffered saline that contained 5% low-fat milk and incubated with monoclonal antibody (phospho-p44/42 MAPK; 1:2000; Cell Signaling Technology, Inc., Beverly, MA) or polyclonal antibody (p44/42 MAPK; 1:1000; Cell Signaling Technology) in appropriate dilutions. After incubation with appropriate secondary antibodies (1:3000), protein bands were visualized with the enhanced chemiluminescence detection system (Amersham, Buckinghamshire, U.K.). Films were scanned using a scanner, and the level of phosphorylation was assessed by band density, which was quantified by densitometry.

Data Analysis. Cumulative data were analyzed in Lotus 1-2-3 and MicroCal Origin. Data are expressed as mean ± SEM. To test the significance of the effect of a drug versus control, a two-tailed Student's *t* test was used. When an additional comparison was required (such as whether a second treatment influenced the action of astaxanthin), a one-way repeated-measures analysis of variance (ANOVA) was computed. $P < 0.05$ was considered to represent a significant difference.

RESULTS

Astaxanthin Decreases 4-AP-Evoked Glutamate Release from Cerebrocortical Nerve Terminals. To examine the influence of raloxifene on glutamate release, isolated nerve terminals were depolarized with the K^+-channel blocker 4-aminopyridine (4-AP). 4-AP destabilizes the membrane potential and is thought to cause repetitive spontaneous Na^+-channel-dependent depolarization that closely approximates in vivo depolarization of the synaptic terminal, leading to the activation of voltage-dependent Ca^{2+} channels (VDCCs) and neurotransmitter release (*30*). Under control conditions, 4-AP (1 mM) evoked a glutamate release of 7.3 ± 0.1 nmol/mg/5 min from synaptosomes incubated in the presence of 1 mM $CaCl_2$. Treatment with astaxanthin (50 μM) for 10 min significantly reduced 4-AP-evoked glutamate release to 4.0 ± 0.2 nmol/mg/5 min ($n = 7$; $P < 0.01$), without altering the basal release of glutamate (**Figure 2A**). This effect of astaxanthin was concentration dependent, and the IC_{50} value derived from a dose−response curve was approximately 52 μM (**Figure 2B**). Given the robust repression of evoked glutamate release seen with 50 μM astaxanthin, this concentration was used in subsequent experiments to evaluate the mechanisms that underlie the ability of astaxanthin to reduce glutamate release.

Astaxanthin-Mediated Inhibition of 4-AP-Evoked Glutamate Release Is Mediated by a Reduction in the Ca^{2+}-Dependent Component of Glutamate Release. The 4-AP-evoked release of glutamate from synaptosomes can be sustained by different mechanisms, including exocytosis (Ca^{2+}-dependent release) and reversal of the transporter (Ca^{2+}-independent release) (*31*). To discriminate the effect of astaxanthin on these two components of endogenous glutamate release evoked by 4-AP, we performed a series of experiments as follows. First, we examined the effect of astaxanthin on the Ca^{2+}-independent glutamate efflux. The Ca^{2+}-independent glutamate efflux was measured by depolarizing the synaptosomes with 4-AP (1 mM) in extracellular Ca^{2+}-free solution that contained 50 μM BAPTA, a cell-permeable Ca^{2+} chelator. Under these conditions, the release of glutamate evoked by 4-AP was not affected by 50 μM astaxanthin (**Figure 3**). Second, we used DL-TBOA, a nonselective inhibitor of all excitatory amino acid transporter (EAAT) subtypes, to examine the effect of astaxanthin on 4-AP-evoked glutamate release. In the presence of DL-TBOA (10 μM), although 4-AP (1 mM)-evoked glutamate release was increased by the inhibitor (because of inhibition of reuptake of released glutamate) ($P < 0.01$), application of astaxanthin (50 μM) still significantly reduced the 4-AP (1 mM)-induced release of glutamate (**Figure 2**). Third, the effect of astaxanthin on 4-AP-evoked glutamate release was examined in the presence of bafilomycin A1, which causes the depletion of glutamate in synaptic vesicles. In contrast to DL-TBOA, bafilomycin A1 (0.1 μM) reduced control 4-AP (1 mM)-evoked glutamate release ($P < 0.01$) and significantly blocked the inhibitory effect of astaxanthin (50 μM) on 4-AP (1 mM)-evoked glutamate release (**Figure 3**).

Astaxanthin Does Not Alter the Plasma Membrane Potential. To further understand the potential mechanisms underlying the astaxanthin-mediated inhibition of glutamate release, we used a membrane potential-sensitive dye, $DiSC_3(5)$, to determine the effect of astaxanthin on synaptosomal plasma membrane potential under resting conditions and on depolarization. As shown in **Figure 4A**, 4-AP (1 mM) caused an increase in $DiSC_3(5)$ fluorescence by 11.3 ± 0.3 fluorescence units/5 min. Preincubation with astaxanthin (50 μM) for 10 min before 4-AP addition did not alter the resting membrane potential and had no significant effect on the 4-AP-mediated increase in $DiSC_3(5)$ fluorescence (11.1 ± 0.4 fluorescence units/5 min; $P > 0.05$). This result indicates that the effect of astaxanthin on evoked glutamate release is unlikely to be due to a hyperpolarizing effect of the drug on the synaptosomal plasma membrane potential or due to an attenuation of depolarization produced by 4-AP. Confirmation that the astaxanthin effect did not impinge on synaptosomal excitability was obtained with experiments using high external $[K^+]$-mediated

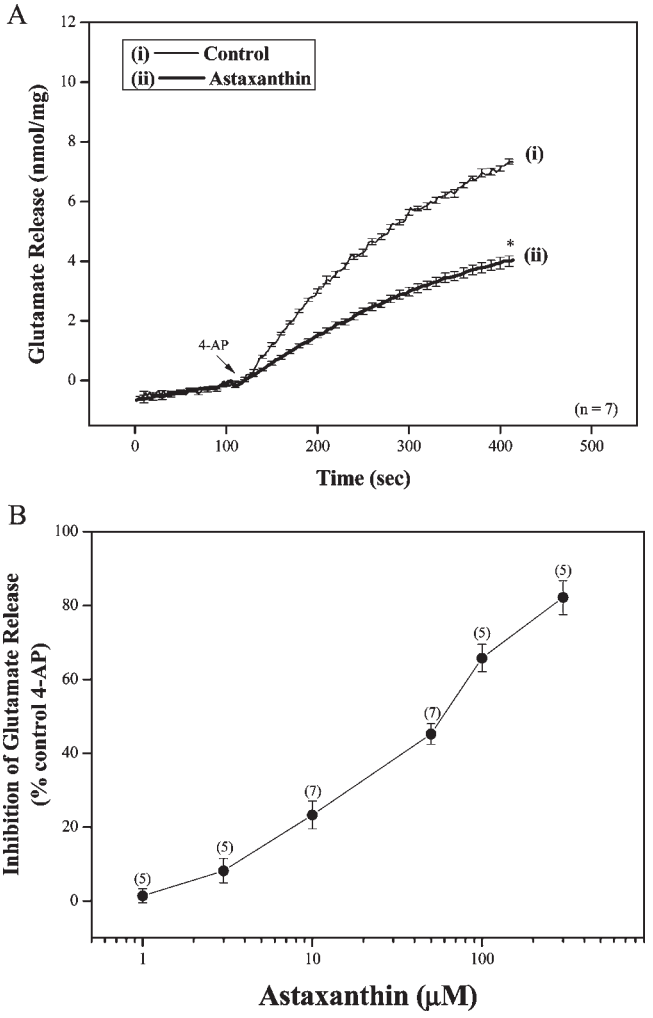

Figure 2. Astaxanthin inhibits 4-AP-induced glutamate release from rat cerebrocortical nerve terminals. (**A**) Representative online measurement of glutamate release. The assay for measurement of glutamate release was performed as described under Materials and Methods. Synaptosomes were resuspended in incubation medium at a final protein concentration of 0.5 mg/mL and incubated for 3 min before the addition of 1 mM $CaCl_2$. To effect depolarization, 1 mM 4-AP was added after 10 min (arrow). Glutamate release was measured in the absence (control conditions) or presence of 50 μM astaxanthin added 10 min before the addition of 4-AP. (**B**) Concentration−effect relationship of astaxanthin on 4-AP-evoked glutamate release. The numbers in parentheses indicate the number of experiments performed using independent synaptosomal preparations. The results are calculated as mean ± SEM. ∗, differences in glutamate release after 1 mM 4-AP depolarization were significant in the absence and presence of astaxanthin ($P < 0.01$, two-tailed Student's *t* test).

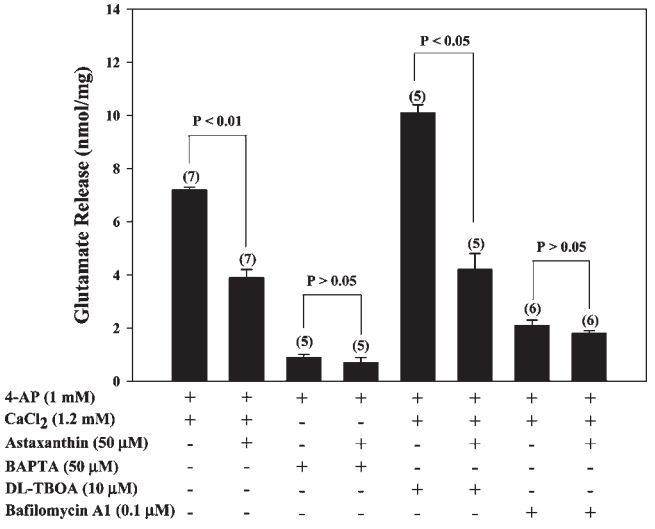

Figure 3. Effects of Ca^{2+} deprivation, DL-TBOA, and bafilomycin A1 on the astaxanthin-mediated inhibition of 4-AP-evoked glutamate release. Glutamate release was induced by 1 mM 4-AP in the absence (control) or presence of 50 μM astaxanthin, 50 μM BAPTA (without $CaCl_2$), 50 μM BAPTA (without $CaCl_2$) and 50 μM astaxanthin, 10 μM DL-TBOA, 10 μM DL-TBOA and 50 μM astaxanthin, 0.1 μM bafilomycin A1, or 0.1 μM bafilomycin A1 and 50 μM astaxanthin. BAPTA, DL-TBOA, or bafilomycin A1 was added 20 min before depolarization, whereas astaxanthin was added 10 min before depolarization. The numbers in parentheses indicate the number of experiments performed using independent synaptosomal preparations. The results are calculated as mean ± SEM ($P < 0.05$, ANOVA followed by two-tailed Student's *t* test).

depolarization. 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 neurotransmitter release (*32*). Addition of 15 mM KCl evoked controlled glutamate release of 9.2 ± 0.2 nmol/mg/5 min, which was reduced to 5.1 ± 0.4 nmol/mg/5 min in the presence of 50 μM astaxanthin ($P < 0.01$; **Figure 4B**).

Astaxanthin Reduces Depolarization-Induced Increase in $[Ca^{2+}]_C$. Glutamate release is triggered by a localized increase in $[Ca^{2+}]_C$ in the active zone. To investigate whether a reduction in $[Ca^{2+}]_C$ is responsible for the astaxanthin-mediated inhibition of release, we determined changes in the cytoplasmic free Ca^{2+} concentration, $[Ca^{2+}]_C$, in nerve terminals loaded with the Ca^{2+} indicator Fura-2. As shown in **Figure 5A**, 4-AP (1 mM) caused a rise in $[Ca^{2+}]_C$ to a plateau level of 212.8 ± 3.5 nM. Astaxanthin (50 μM) did not significantly affect basal Ca^{2+} levels, but caused a 24% decrease in the 4-AP-evoked rise in $[Ca^{2+}]_C$ (162.2 ± 2.8 nM; $P < 0.01$). The inhibitory effect of astaxanthin was also evident with KCl (15 mM)-evoked rise in $[Ca^{2+}]_C$, which decreased 23% from 251.3 ± 5.3 nM in control conditions to 194.2 ± 3.6 nM in the presence of astaxanthin (50 μM) ($P < 0.01$; **Figure 5B**).

Reduction of Ca^{2+} Influx Mediated by N- and P/Q-Type Ca^{2+} Channels Contributes to the Effect of Astaxanthin. In the adult rat cerebrocortical nerve terminal preparation, the release of glutamate evoked by depolarization is expected to cause Ca^{2+} influx through N- and P/Q-type Ca^{2+} channels and Ca^{2+} release from internal stores (*33*, *34*). For this reason, we sought to examine which part of the Ca^{2+} source was involved in the effect of astaxanthin on 4-AP-evoked glutamate release. In **Figure 6**, control 4-AP (1 mM)-evoked glutamate release was significantly reduced after the combined application of 2 μM ω-conotoxin GVIA

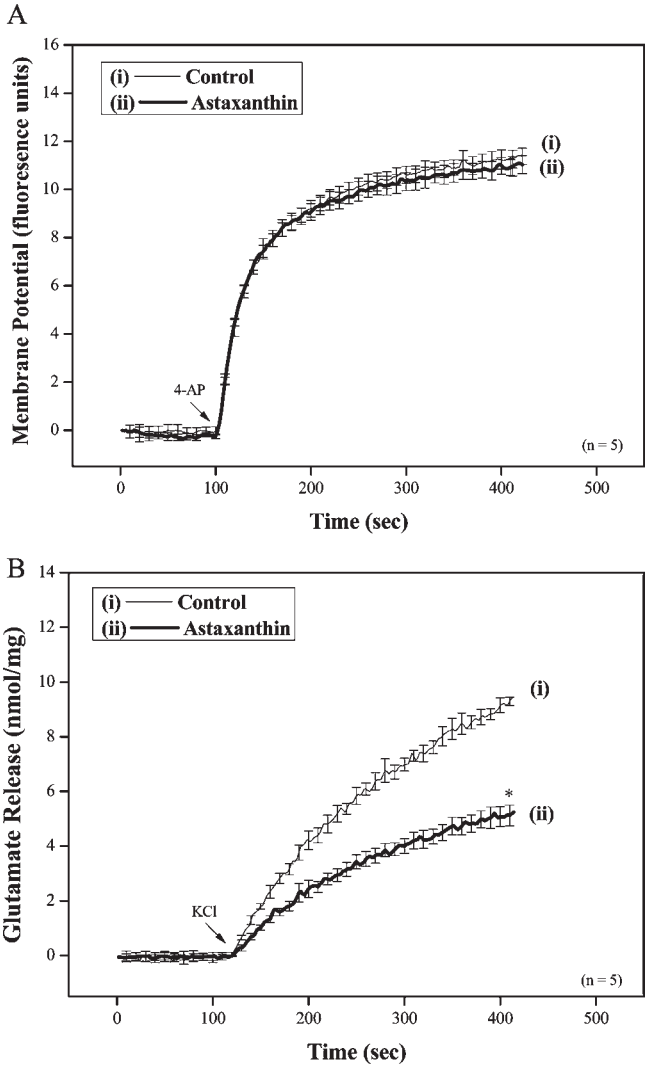

Figure 4. Effect of astaxanthin on synaptosomal membrane potential. (**A**) Synaptosomal membrane potential was monitored with 5 μM $DiSC_3(5)$ on depolarization with 1 mM 4-AP, in the absence (control) or presence of 50 μM astaxanthin added 10 min before depolarization. (**B**) Glutamate release was induced by 15 mM KCl in the absence (control) or presence of 50 μM astaxanthin, added 10 min before depolarization. The numbers in parentheses indicate the number of experiments performed using independent synaptosomal preparations. The results are calculated as mean ± SEM. *, differences in glutamate release after 15 mM KCl depolarization were significant in the absence and presence of astaxanthin ($P < 0.01$, two-tailed Student's *t* test).

(ω-CgTX GVIA) and 1 μM ω-agatoxin IVA (ω-AgTX IVA), which selectively blocks N- and P/Q-type Ca^{2+} channels, respectively (*35*, *36*) ($P < 0.01$). In the presence of both toxins, the inhibitory effect of astaxanthin (50 μM) on 4-AP (1 mM)-evoked glutamate release was largely blocked. Similarly, we found that ω-CgTX MVIIC (2 μM), a wide-spectrum blocker of N-, P-, and Q-type Ca^{2+} channels, reduced control 4-AP (1 mM)-evoked glutamate release and severely abolished the astaxanthin (50 μM)-mediated inhibition of glutamate release evoked by 4-AP (1 mM) (**Figure 6**). In addition, dantrolene is known to inhibit Ca^{2+} release from intracellular stores by acting on ryanodine receptors on the endoplasmic reticulum (*37*). Treatment with dantrolene (50 μM) caused a reduction in glutamate release evoked by 4-AP (1 mM) ($P < 0.01$). In the presence of dantrolene, however, astaxanthin (50 μM) could still effectively inhibit 4-AP (1 mM)-evoked glutamate release (**Figure 6**). Similar to dantrolene,

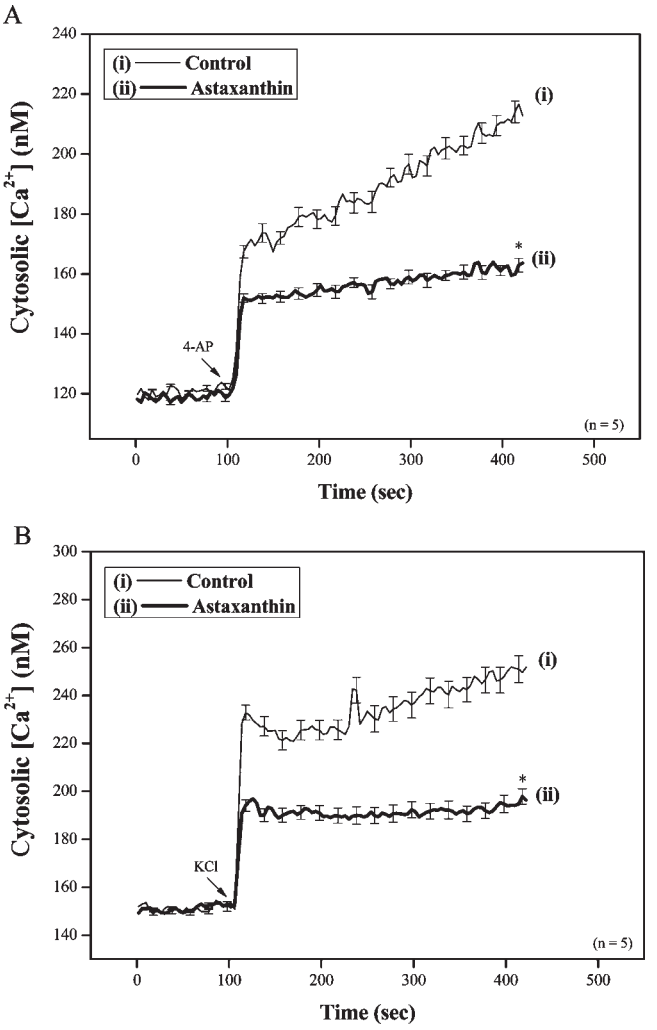

Figure 5. Astaxanthin reduces the 4-AP- and KCl-induced increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_C$). $[Ca^{2+}]_C$ was monitored using Fura-2. The synaptosomes were stimulated using 1 mM 4-AP (**A**) or 15 mM KCl (**B**) in the absence (control) or presence of 50 μM astaxanthin, which was added 10 min before stimulation. The numbers in parentheses indicate the number of experiments performed using independent synaptosomal preparations. The results are calculated as mean ± SEM. *, differences in $[Ca^{2+}]_C$ levels after 1 mM 4-AP or 15 mM KCl depolarization were significant in the absence and presence of astaxanthin ($P < 0.01$, two-tailed Student's *t* test). Differences in basal $[Ca^{2+}]_C$ levels in the absence and presence of astaxanthin were not significant ($P > 0.4$).

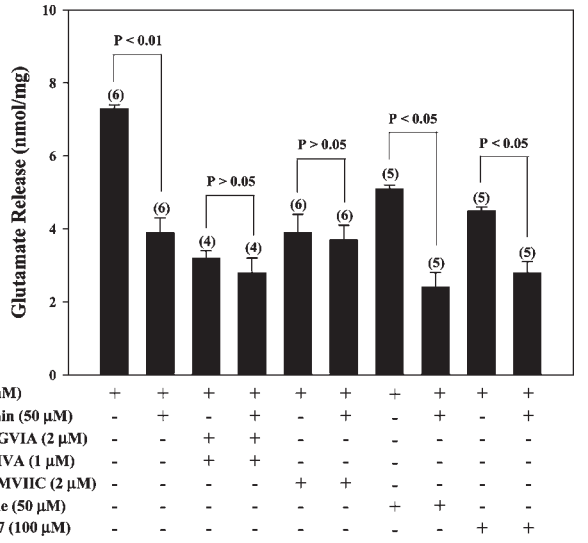

Figure 6. Effects of ω-CgTX GVIA + ω-AgTX IVA, ω-CgTX MVIIC, dantrolene, and CGP37157 on the astaxanthin-mediated inhibition of 4-AP-evoked glutamate release. Glutamate release was induced by 1 mM 4-AP, in the absence (control) or presence of 50 μM astaxanthin, 2 μM ω-CgTX GVIA and 0.5 μM ω-AgTX IVA, 2 μM ω-CgTX GVIA, 0.5 μM ω-AgTX IVA and 50 μM astaxanthin, 1 μM ω-CgTX MVIIC, 1 μM ω-CgTX MVIIC, and 50 μM astaxanthin, 50 μM dantrolene, 50 μM dantrolene and 50 μM astaxanthin, 100 μM CGP37157, or 100 μM CGP37157 and 50 μM astaxanthin. Astaxanthin was added 10 min before depolarization, whereas the other drugs were added 30 min before depolarization. The numbers in parentheses indicate the number of experiments performed using independent synaptosomal preparations. The results are calculated as mean ± SEM ($P < 0.05$, ANOVA followed by two-tailed Student's *t* test).

CGP37157 (100 μM), a membrane-permeable blocker of mitochondrial Na^+/Ca^{2+} exchange, decreased 4-AP (1 mM)-evoked glutamate release ($P < 0.01$), but had a nonsignificant effect on the astaxanthin-mediated inhibition of 4-AP (1 mM)-evoked glutamate release (**Figure 6**).

Suppression of MAPK-Dependent Pathway Involves the Astaxanthin-Mediated Inhibition of Glutamate Release. Because glutamate release is regulated at the presynaptic level by the activation of protein kinases including MAPK, PKC, and PKA (*22*−*24*), we sought to establish which type of protein kinase signaling pathway participated in the astaxanthin-mediated inhibition of evoked glutamate release. To this aim, we performed occlusion experiments with protein kinase inhibitors. **Figure 7** shows that the MAPK inhibitor PD98059 (50 μM) itself reduced control 4-AP (1 mM)-evoked glutamate release ($P < 0.01$) and largely blocked the ability of astaxanthin to depress glutamate release. In the presence of PD98059, therefore, astaxanthin (50 μM) induced a statistically insignificant inhibition of glutamate release of 6% compared with the 45% inhibition produced by astaxanthin alone. This occlusion of the effect of astaxanthin by MAPK inhibition with PD98059 was also observed with another MAPK inhibitor, U0126. As with PD98059, although U0126 (50 μM) attenuated control 4-AP (1 mM)-evoked glutamate release ($P < 0.01$), it also occluded the inhibitory effect of astaxanthin on 4-AP (1 mM)-evoked glutamate release (**Figure 7**). In addition, the inhibitory action of astaxanthin on 4-AP-evoked glutamate release was insensitive to staurosporine at concentrations (1 μM) that inhibit PKC and PKA activities (*38*). Similar results were observed with the PKC inhibitor GF109203X (10 μM) and the PKA inhibitor H89 (100 μM). The application of staurosporine (1 μM), GF109203X (10 μM), or H89 (10 μM) alone significantly reduced the 4-AP (1 mM)-evoked glutamate release ($P < 0.01$; **Figure 7**).

To further confirm that the MAPK signaling pathway was suppressed by astaxanthin during its inhibition of 4-AP-evoked glutamate release, we performed Western blotting to determine the effect of astaxanthin on the phosphorylation of MAPK. **Figure 8** shows the results of analysis performed on extracts of synaptosomes depolarized with 4-AP in the presence of external Ca^{2+} and treated with astaxanthin. The results showed that depolarization of synaptosomes with 4-AP (1 mM) markedly increased MAPK phosphorylation levels. When synaptosomes were pretreated with astaxanthin (50 μM) for 10 min before depolarization with 4-AP, a significant decrease in the 4-AP-induced MAPK phosphorylation was observed ($P < 0.05$; **Figure 8**). In addition, the MAPK inhibitor PD98059 (50 μM) significantly reduced 4-AP (1 mM)-induced MAPK phosphorylation ($P < 0.05$). In the presence of PD98059, the action of astaxanthin on 4-AP (1 mM)-induced MAPK phosphorylation was also prevented (**Figure 8**).

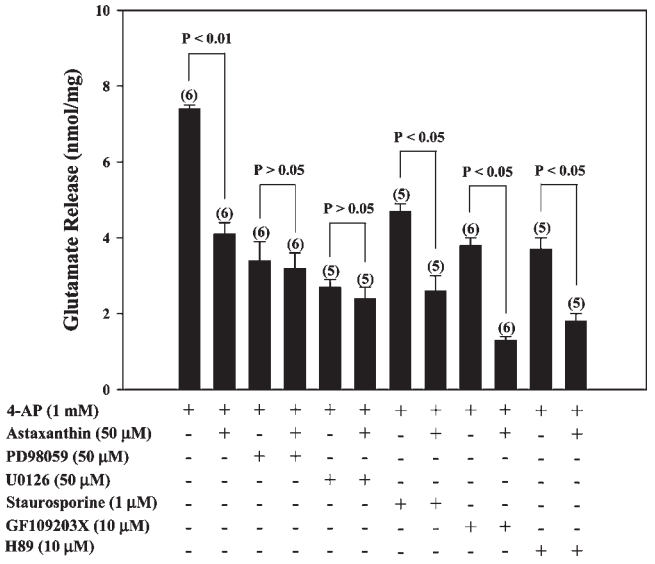

Figure 7. Effects of PD98059, U0126, staurosporine, GF109203X, and H89 on the astaxanthin-mediated inhibition of 4-AP-evoked glutamate release. Glutamate release was induced by 1 mM 4-AP, in the absence (control) or presence of 50 μM astaxanthin, 50 μM PD98059, 50 μM PD98059 and 50 μM astaxanthin, 50 μM U0126, 50 μM U0126, and 50 μM astaxanthin, 1 μM staurosporine, 1 μM staurosporine and 50 μM astaxanthin, 10 μM GF109203X, 10 μM GF109203X and 50 μM astaxanthin, 10 μM H89, or 10 μM H89 and 50 μM astaxanthin. PD98059, U0126, staurosporine, GF109203X, or LY294002 was added 40 min before depolarization, whereas astaxanthin was added 10 min before depolarization. The numbers in parentheses indicate the number of experiments performed using independent synaptosomal preparations. The results are calculated as mean ± SEM ($P < 0.05$, ANOVA followed by two-tailed Student's *t* test).

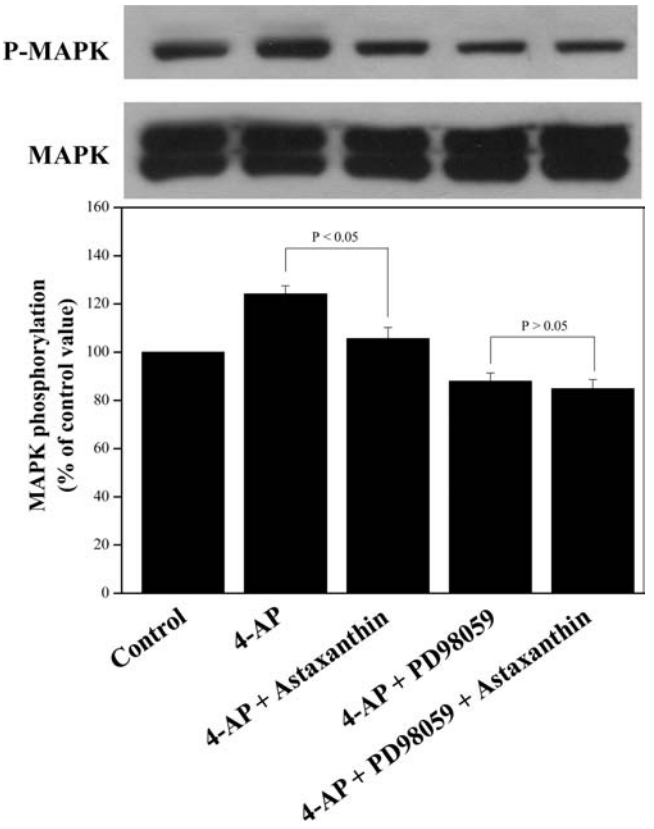

Figure 8. Astaxanthin significantly decreases 4-AP-induced phosphorylation of MAPK, and this effect is prevented by the MAPK inhibitor PD98059. Phosphorylation of MAPK was detected in synaptosomal lysates by Western blotting using antiphospho-MAPK antibody. Purified synaptosomes were incubated at 37 °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 50 μM astaxanthin, 1 mM 4-AP and 50 μM PD98059, or 1 mM 4-AP, 50 μM PD98059, and 50 μM astaxanthin. PD98059 was added 40 min before 4-AP addition, whereas astaxanthin 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. The numbers in parentheses indicate the number of experiments performed using independent synaptosomal preparations. The results are calculated as mean ± SEM. Significant differences in the absence and presence of astaxanthin were as indicated (using ANOVA followed by two-tailed Student's *t* test).

DISCUSSION

Excessive release of glutamate is known to be an underlying cause of neuronal damage in a variety of CNS diseases including cerebral ischemia, epilepsy, and neurodegenerative disorders (*19*, *39*). One of the feasible ways to prevent and treat this overexcitation-induced neuronal damage is to reduce the amount of glutamate in the synaptic clef. Recently, growing evidence points to a neuroprotective role of astaxanthin in various in vitro and in vivo models of neurotoxicity (*7*−*13*); however, the detailed mechanism at the basis of this phenomenon remains unresolved. The main aim of this study was to obtain better knowledge about the mechanism responsible for the neuroprotective effect of astaxanthin in response to excitotoxic insults. By using a preparation of nerve terminals from rat cerebral cortex and by examining the release of endogenous glutamate, we first demonstrated that astaxanthin rapidly and dose-dependently inhibits the release of glutamate evoked by depolarization. Several possible mechanisms for inhibition of glutamate release have been investigated in this study and are discussed in more detail below.

The glutamate release induced by depolarization is known to have two components. The first is a physiologically relevant Ca^{2+}-dependent component, which is produced by exocytosis of glutamate-containing synaptic vesicles. The second is a Ca^{2+}-independent component that results from prolonged depolarization that causes a membrane-potential-mediated shift of the glutamate transporter steady state toward the outward direction, to effect cytosolic glutamate efflux (*31*). In this study, we found that, in the presence of Ca^{2+}-free medium that contained BAPTA, astaxanthin failed to produce any effect on 4-AP-evoked glutamate release (Ca^{2+}-independent release). Additionally, the inhibitory effect of astaxanthin on 4-AP-evoked glutamate release was effectively abolished by bafilomycin A1, which depletes the glutamate content of synaptic vesicles, but not by DL-TBOA, a nonselective inhibitor of all EAAT subtypes. These results suggest that the inhibition of 4-AP-evoked glutamate release, attributable to the astaxanthin treatment, was accounted for only by a reduction in the Ca^{2+}-dependent, exocytotic component of glutamate release.

In principle, neurotransmitter release is a complex phenomenon and can be modulated at several putative sites in the nerve terminal, including Na^+ channels, K^+ channels, and Ca^{2+} channels, as well as the release process itself. Activation of Na^+ channels or inhibition of K^+ channels is known to regulate membrane excitability and consequently the amount of transmitter release (*40*−*42*). Thus, addressing the mechanism responsible for astaxanthin-mediated inhibition of glutamate release, we considered two possibilities that might be involved: (1) an alteration of plasma membrane potential and/or (2) a direct inhibition of the exocytosis-coupled Ca^{2+} channel. The first possibility

appears to be unlikely on the basis of the following observations. First, no significant effect of astaxanthin on synaptosomal plasma membrane potential, measured with a membrane-potential-sensitive dye, $DiSC_3(5)$, was observed either in the resting condition or on depolarization with 4-AP (indicating a lack of effect on K^+ conductance). Second, astaxanthin significantly inhibited the release of glutamate evoked by 4-AP and KCl. This indicates that a direct effect on the function of voltage-dependent Ca^{2+} channel seems to be involved in the inhibitory effect of astaxanthin on glutamate release. This is because 4-AP-evoked glutamate release involves the action of Na^+ and Ca^{2+} channels and 15 mM external KCl-evoked glutamate release involves only Ca^{2+} channels (*30*, *32*). Third, astaxanthin did not affect the 4-AP-evoked Ca^{2+}-independent glutamate release, a component of glutamate release that is dependent only on membrane potential (*43*). These results indicate that the decrease in evoked glutamate release seen with astaxanthin is not due to a reduction of synaptosomal excitability resulting from ion channel (e.g., Na^+ channel or K^+ channel) modulation.

If it is not the modulation of synaptosomal excitability, then the locus of action of astaxanthin must lie further downstream in the stimulus−exocytosis coupling cascade. Using Fura-2, we have demonstrated here that astaxanthin significantly reduces 4-AP- and KCl-evoked increase in $[Ca^{2+}]_C$. This alleviates the inhibitory effect of astaxanthin on glutamate release by decreasing intracellular Ca^{2+} levels. In synaptic terminals, a depolarization-induced increase in $[Ca^{2+}]_C$, coupled to glutamate release, is mediated by extracellular Ca^{2+} influx through N- and P/Q-type VDCCs and Ca^{2+} release from intracellular stores such as endoplasmic reticulum and mitochondria (*33*, *34*). In the present study, the inhibition of Ca^{2+} release from the endoplasmic reticulum and mitochondria could be excluded because the inhibitory effects of astaxanthin on the 4-AP-evoked glutamate release were not affected by dantrolene, an inhibitor of intracellular Ca^{2+} release from the endoplasmic reticulum, and by CGP37157, a membrane-permeable blocker of mitochondrial Na^+/Ca^{2+} exchange. In contrast, the inhibitory effect of astaxanthin on 4-AP-evoked glutamate release was prevented completely under conditions in which the release-coupled N- and P/Q-type VDCCs were blocked. Despite the drawback that direct evidence for astaxanthin action on presynaptic Ca^{2+} channels is lacking, these data indicate the suppression of Ca^{2+} influx through N- and P/Q-type VDCCs participates in the inhibition of glutamate release by astaxanthin.

Various kinases, including PKC, PKA, and MAPK, have been shown to be present at the presynaptic level, at whiche they enhance glutamate release (*22*−*24*). In the current study, we found that MAPK inhibitors PD98059 or U0126 could successfully antagonize the inhibitory effect of astaxanthin on 4-AP-evoked glutamate release, whereas the PKC inhibitor GF109203X or PKA inhibitor H89 was without effect, which demonstrated some specificity for astaxanthin action on the MAPK pathway. Furthermore, astaxanthin significantly decreased 4-AP-induced phosphorylation of MAPK, and this effect was prevented by PD98059. These data confirm the involvement of MAPK signaling in astaxanthin-mediated inhibition of evoked glutamate release. Here, a question arises how suppression of MAPK might be involved in the astaxanthin-mediated inhibition of glutamate release. Previous studies have shown that MAPK is capable of altering the phosphorylation state of synapsin I, a synaptic vesicle protein involved in the synaptic vesicle trafficking/recruitment and exocytosis. This phosphorylation reaction promotes dissociation of synaptic vesicles from the actin cytoskeleton, thereby making more vesicles available at the active zone for neurotransmitter exocytosis resulting in an increased glutamate release

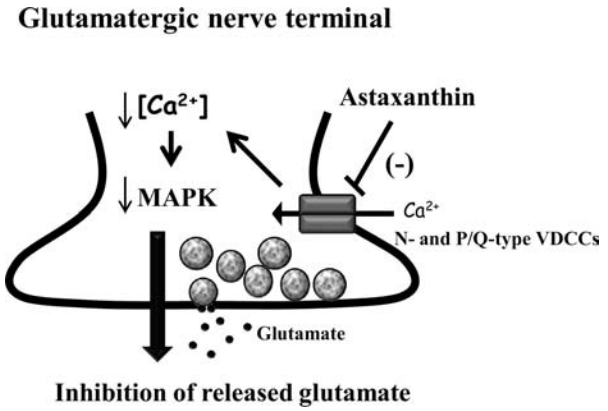

Figure 9. Proposed schema of the mechanism by which astaxanthin inhibited evoked glutamate release in rat cerebrocortical nerve terminals. In synaptic terminals, the depolarization of neurons induces Ca^{2+} entry through the N- and P/Q-type VDCCs. Ca^{2+} influx consequently activates protein kinases and triggers the release of glutamate. In rat cerebrocortical nerve terminals, astaxanthin effects a decrease in the Ca^{2+} influx through N- and P/Q-type Ca^{2+} channels, which subsequently reduces MAPK activity to cause a decrease in evoked glutamate release.

(*44*, *45*). Thus, the possible involvement of the suppression of MAPK-dependent synapsin I phosphorylation should be considered.

On the basis of our present data, we suggest a model, shown in **Figure 9**, for explanation of the inhibition mechanism of astaxanthin on glutamate release from cortical nerve terminals. Briefly, astaxanthin markedly inhibits evoked glutamate release in rat cerebrocortical nerve terminals, presumably through reducing Ca^{2+} influx through N- and P/Q-type Ca^{2+} channels, which subsequently reduces the MAPK activity (**Figure 9**). This suggests that reduction in released glutamate presents an additional explanation for the neuroprotective effect of astaxanthin besides antioxidant and anti-inflammatory properties. Although the relevance of our finding to in vivo clinical situations remains to be determined, this investigation may provide further understanding of the mode of astaxanthin action in the brain and highlights the therapeutic potential of this compound in the prevention and treatment of a wide range of neurological and neurodegenerative disorders.

ABBREVIATIONS USED

4-AP, 4-aminopyridine; $[Ca^{2+}]_C$, cytosolic free Ca^{2+} concentration; $DiSC_3(5)$, 3,3′-dipropylthiadicarbocyanine iodide;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; BAPTA-AM, 1,2-bis(2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester; ω-CgTX GVIA, ω-conotoxin GVIA; ω-AgTX IVA, ω-agatoxin IVA; ω-CgTX MVIIC, ω-conotoxin MVIIC; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; PKC, protein kinase C; GF109203X, bisindolylmaleimide I; CGP37157, 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3*H*)-one; PD98059, 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one); U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene; VDCC, voltage-dependent Ca^{2+} channel; CNS, central nervous system.

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