Cyclooxygenase 2 Inhibitor Celecoxib Inhibits Glutamate Release by Attenuating the PGE2/EP2 Pathway in Rat Cerebral Cortex Endings

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ABSTRACT

The excitotoxicity caused by excessive glutamate is a critical element in the neuropathology of acute and chronic brain disorders. Therefore, inhibition of glutamate release is a potentially valuable therapeutic strategy for treating these diseases. In this study, we investigated the effect of celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor that reduces the level of prostaglandin E2 (PGE2), on endogenous glutamate release in rat cerebral cortex nerve terminals (synaptosomes). Celecoxib substantially inhibited the release of glutamate induced by the K+ channel blocker 4-aminopyridine (4-AP), and this phenomenon was prevented by chelating the extracellular Ca2+ ions and by the vesicular transporter inhibitor bafilomycin A1. Celecoxib inhibited a 4-AP-induced increase in cytosolic-free Ca2+ concentration, and the celecoxib-mediated inhibition of glutamate release was prevented by the Ca2,2.2 (N-type) and Ca2,2.1 (P/Q-type) channel blocker ω-conotoxin MVIIIC. However, celecoxib did not alter 4-AP-mediated depolarization and Na+ influx. In addition, this glutamate release–inhibiting effect of celecoxib was mediated through the PGE2 subtype 2 receptor (EP2) because it was not observed in the presence of butaprost (an EP2 agonist) or PFD4418948 [1-(4-fluorobenzy1)-3-[3-hydroxy-2-naphthalenyl[methyl]-3-azetidinocarboxylic acid; an EP2 antagonist]. The celecoxib effect on 4-AP–induced glutamate release was prevented by the inhibition or activation of protein kinase A (PKA), and celecoxib decreased the 4-AP–induced phosphorylation of PKA. We also determined that COX-2 and the EP2 receptor are present in presynaptic terminals because they are colocalized with synaptophysin, a presynaptic marker. These results collectively indicate that celecoxib inhibits glutamate release from nerve terminals by reducing voltage-dependent Ca2+ entry through a signaling cascade involving EP2 and PKA.

Introduction

Cyclooxygenase-2 (COX-2) is an essential enzyme that converts arachidonic acid into prostaglandin. In the central nervous system (CNS), COX-2 is constitutively expressed, mainly in hippocampal and cortical pyramidal neurons where it plays a role in synaptic transmission and plasticity (Murray and O’Connor, 2003; Cowley et al., 2008). In addition, COX-2 is a crucial mediator of neuroinflammation, and its overexpression in neurons is implicated in numerous brain injuries (Nogawa et al., 1997; Minghetti, 2004). COX-2 inhibition has also been reported to confer neuroprotective effects in various animal models with neurologic disorders, including cerebral ischemia, epilepsy, Alzheimer’s disease, and Parkinson’s disease (Nakayama et al., 1998; Hunter et al., 2007; Trepanier and Milgram, 2010; Serrano et al., 2011; Akram et al., 2013). Consequently, COX-2 inhibitors have been suggested to be a potential therapeutic application for treating these CNS disorders. Celecoxib is a selective COX-2 inhibitor, and its neuroprotective action has been observed in several in vitro and in vivo experimental studies. For example, celecoxib attenuates oxygen and glucose deprivation–induced neuronal cell death (López-Villodres et al., 2012), protects against ischemia or lipopolysaccharide-induced brain damage (Abd El-Aal et al., 2013; Fan et al., 2013; Kaizaki et al., 2013), improves kainic acid–induced cognitive impairment (Gobbo and O’Mara, 2004), and delays the progress of brain degeneration (Drachman et al., 2002; Small et al., 2008). Celecoxib is known to possess

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ABBREVIATIONS: 4-AP, 4-aminopyridine; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’’-tetraacetic acid-acetoxymethyl ester; [Ca2+]c, cytosolic free Ca2+ concentration; CGP37157, 7-chloro-5-(2-chlorophenyl)-1,3-dihydro-4,1-benzothiazepin-2(3H)-one; ω-GTX MVIIIC, ω-conotoxin MVIIIC; CNS, central nervous system; COX-2, cyclooxygenase-2; DSCGb, 3’,3’-dipropylthiadicarbocyanine iodide; DL-TBOA, α,α,α’,α’-tetraacetic acid-acetoxymethyl ester; [Ca2+]v, intracellular Ca2+ concentration; HBM, HEPES buffer medium; KT5720, hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-e环氧-1H-diindololo-benzoazicnic-10-carboxylic acid; PB, phosphate buffer; PFD4418948, 1-[4-fluorobenzy1]-3-[3-hydroxy-2-naphthalenyl[methyl]-3-azetidinocarboxylic acid; PGE2, prostaglandin E2; PKA, protein kinase A; SBD-M, sodium-binding benzo[fluran isophthalate-AM; TCS2510, (5R)-5(3S)-3-hydroxy-4-phenyl-1-buten-1-yl]-1-[6-(2H-tetrazol-5-yl)]hexyl]-2-pyrrolidinone; TTX, tetrodotoxin; VDCC, voltage-dependent Ca2+ channel.
anti-inflammatory activity, and its neuroprotective action is generally assumed to be associated with this property (Hunter et al., 2007; López-Villodres et al., 2012; Kaizaki et al., 2013). However, the pharmacological mechanisms underlying the neuroprotective effects of celecoxib have not been fully clarified.

In the brain, glutamate is a major excitatory neurotransmitter that plays a vital role in numerous brain functions, such as synaptic plasticity, learning, and memory (Fonnum, 1984; Greenamyre and Porter, 1994). However, in addition to the physiologic role of glutamate, excessive glutamate release and activation of the glutamate receptors induces an increase in intracellular Ca2+ levels, subsequently triggering a cascade of cellular responses, including enhanced oxygen free-radical production, disturbed mitochondrial function, and protease activation, which ultimately kills the neurons (Coyle and Puttfarcken, 1993; Schinder et al., 1996; Greenwood and Connolly, 2007). This process has been implicated as a pathophysiological factor in multiple neurologic disorders, both acute (such as stroke and head trauma) and chronic (such as neurodegenerative disorders) (Meldrum and Garthwaite, 1990; Meldrum, 2000). Thus, inhibiting glutamate release might provide a potential target for neuroprotective action. Consistent with this hypothesis, several clinical neuroprotective drugs (e.g., rifuzole, memantine, and minocycline) have been revealed to decrease glutamate release in rat brain tissues (Wang et al., 2004; Gonzalez et al., 2007; Lu et al., 2010).

Because celecoxib has a neuroprotective profile, and the excessive release of glutamate is a critical element in the neuropathology of acute and chronic brain disorders, assessing the effects of celecoxib on glutamate release is warranted. Based on a review of the literature, no study has thus far addressed whether celecoxib directly affects glutamate release at the presynaptic level. Hence, we used isolated nerve terminals (synaptosomes) purified from rat cerebral cortex to investigate the effect of celecoxib on glutamate release and to characterize its underlying molecular mechanisms. The synaptosomes were adopted because it can accumulate, store, and characterize its underlying molecular mechanisms. The synaptosomes were chosen because it can accumulate, store, and characterize its underlying molecular mechanisms.

Glutamate Release Assay. The glutamate release was assayed using on-line fluorimetry, as described previously (Nicholls and Sihra, 1986). The adult rats were sacrificed by decapitation, and the brains were removed at 4°C. The cerebral cortex was rapidly dissected and homogenized in a medium containing 320 mM sucrose at pH 7.4. The homogenate was centrifuged at 10,000 rpm in a JA 25.5 rotor; Beckman Coulter, Inc., Brea, CA) for 10 minutes at 4°C, and the supernatant was centrifuged again at 14,500g (11,000 rpm in a JA 25.5 rotor) for 12 minutes at 4°C. The pellet was gently resuspended in 5 ml of 320 mM sucrose at pH 7.4. Two milliliters of this synaptosomal suspension was placed in 3 ml of Percoll discontinuous gradients containing (in mM) 320 sucrose, 1 EDTA, 0.25 in-dithiothreitol, and 3, 10, and 23% Percoll at pH 7.4. The gradients were centrifuged at 25,500 rpm in a JA 25.5 rotor) for 7 minutes at 4°C. Synaptosomes sedimenting between the 10 and 23% Percoll bands were collected and diluted to a final volume of 30 ml of HEPES buffer medium (HBK) consisting of (in mM) 140 NaCl, 5 KCl, 5 NaHCO3, 1 MgCl2·6H2O, 1.2 Na2HPO4, 10 glucose, and 10 HEPES at pH 7.4. Protein concentration was determined using a Bradford assay. Synaptosomes were centrifuged in a final wash to obtain synaptosomal pellets containing 0.5 mg of protein. The synaptosomal pellets were stored on ice and used within 4–6 hours.

Glutamate Release Assay. The glutamate release was assayed using on-line fluorimetry, as described previously (Nicholls and Sihra, 1986; Wang and Sihra, 2004). Synaptosomal pellets (0.5 mg of protein) were resuspended in HBK containing 16 μM bovine serum albumin and incubated in a stirred and thermostated cuvette at 37°C in a PerkinElmer LS-55 spectrophuorimeter (PerkinElmer Life and Analytical Sciences, Waltham, MA). A filter photometer (PerkinElmer Life and Analytical Sciences, Waltham, MA). A filter photometer (PerkinElmer Life and Analytical Sciences, Waltham, MA). NADP⁺ (2 mM), glutamate dehydrogenase (50 U/ml), and CaCl2 (1.2 mM) were added after 3 minutes. After an additional 10 minutes of incubation, 5-aminopyrine (4-AP, 1 mM) or high external KCl (15 mM) was added to stimulate glutamate release. Glutamate release was monitored by measuring the increase in fluorescence (excitation and emission wavelengths of 340 and 460 nm, respectively) caused by NADPH being produced by the oxidative deamination of released glutamate by glutamate dehydrogenase. Data were accumulated at 2-second 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. Values quoted in the text and depicted in bar graphs represent the levels of glutamate cumulatively released after 5 minutes of depolarization, and are expressed as nmol/mg per 5 minutes. Estimation of the IC50 was based on a one-site model

\[ \text{Inhibition} = (\text{inhibition}_{\text{MAX}} \times \text{celecoxib}) / (\text{IC50} + \text{celecoxib}) \]

and was calculated using the nonlinear curve-fitting function provided in MicroCal Origin (OriginLab Corporation, Northampton, MA). Cumulative data were analyzed using Lotus 1-2-3 (IBM Corporation, Armonk, NY).

Materials and Methods

Chemicals.α-Convotoxin MVIIIC (α-CgTX MVIIIC), dantrolene, CGBP37157 (7-chloro-5-2-chlorophenyl)-1,5-di hydro-4,1-benzothiazepin-2[(3Z)-one], FF0441894 [1-4-(fluorobenzyl)-3-[6-methoxy-2-naphthalenyl) methyl]-3-azetidinonicarboxylic acid], TCS2510 [(5R)-5-(3S)-3-hydroxy-4-phenyl-1-buten-1-yl]-1-[(6-(2H-tetrazol-5-yl)hexyl]-2-pyrrolidinone], H89 (N-[2-(p-bromocinnamylamino)-ethyl]-5-isoquinolinesulfonamide dihydrochloride), PD98059 (2-(2-amino-3-methoxyphenyl)-4-1-benzyopyran-4-one), KT5720 (hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo-benzodiazone-10-carboxylic acid), GF109203X (bisindolylmaleimide I), and DON (6-diaz-o-5-oxo-1-norleucine) were obtained from Tocecs Cookson (Bristol, UK). Fura-2-AM, sodium-binding benzoferan isophthalate-AM (SBFI-AM), and DiSC3(5) (3',3',3'- dipropyldihiacarbocyanine iodide) were purchased from Invitrogen (Carlsbad, CA). PGE2 and sulprostone were purchased from Cayman Chemical (Ann Arbor, MI). Celecoxib, butaprost, EOTA, tetrodotoxin (TTX), and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).
Synaptosomal Plasma Membrane Potential. The plasma membrane potential was determined using a membrane potential-sensitive dye, DiSC3(5). DiSC3(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. On depolarization, the dye moves out, and hence, the fluorescence increases (Akerman et al., 1987). Synaptosomes were resuspended in HBM and incubated in a stirred test tube. After fura-2-AM (Cell Signaling Technology, Beverly, MA) or PGE2 subtype 2 receptor monoclonal antibodies against synaptophysin (1:200; Abcam, Cambridge, UK) was added and allowed to equilibrate before the addition of 1 mM CaCl2 after 4 minutes of incubation. 4-AP was then added to depolarize the synaptosomes for 10 minutes, and DiSC3(5) fluorescence was monitored at excitation and emission wavelengths of 340 and 380 nm, respectively. Cumulative data were analyzed using Lotus 1-2-3 and expressed in fluorescence units.

Cytosolic Free Ca2+ Concentration. Cytosolic free Ca2+ concentration ([Ca2+]C) was measured using the Ca2+ indicator fura-2. Synaptosomes (0.5 mg/ml) were preincubated in HBM with 16 μM bovine serum albumin in the presence of 5 μM fura-2-AM and 0.1 mM CaCl2 for 30 minutes at 37°C in a stirred test tube. After fura-2-AM loading, synaptosomes were centrifuged in a microcentrifuge for 30 seconds at 3000g (5000 rpm). The synaptosomal pellets were resuspended in HBM with bovine serum albumin, and the synaptosomal suspension was stirred in a thermostated cuvette in a PerkinElmer LS-55 spectrophotometer. CaCl2 (1 mM) was added after 3 minutes, and further additions were made after an additional 10 minutes. Fluorescence data were accumulated at excitation wavelengths of 340 and 380 nm (emission wavelength 505 nm) at 7.5-second intervals. [Ca2+]C (in nanomoles) was calculated using calibration procedures (Sihra et al., 1992) and equations described previously (Grynkiewicz et al., 1985). Cumulative data were analyzed using Lotus 1-2-3.

Cytosolic Free Na+ Concentration. Na+ measurements were performed essentially as described for [Ca2+]C determinations, except that synaptosomes were preincubated with 5 μM SBFI-AM instead of fura-2 (Minta and Tsien, 1989). SBFI fluorescence was monitored as in fura-2 experiments. Results are expressed as ratios of fluorescence (emission wavelength 505 nm) at excitation wavelengths of 340 and 380 nm.

Immunocytochemistry. The synaptosomes were allowed to attach to coverslips (diameter 20 mm) precoated with poly-L-lysine for 40 minutes at 4°C before being fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 minutes. After rinsing with PB three times, the synaptosomes were incubated in blocking buffer containing 3% normal goat serum and 0.2% Triton X-100 for 60 minutes. They were then incubated with a mixture of primary mouse monoclonal antibodies against synaptophysin (1:200; Abcam, Cambridge, UK) and rabbit monoclonal antibodies against COX-2 (1:50; Cell Signaling Technology, Beverly, MA) or PGE2 subtype 2 receptor (EP2; 1:100; Abcam) for 90 minutes at room temperature. After rinsing with blocking buffer, the synaptosomes were incubated with a mixture of goat anti-mouse DyLight 549- and goat anti-rabbit fluorescein isothiocyanate-conjugated secondary antibodies (1:200; Jackson ImmunoResearch Inc., West Grove, PA) for 1 hour at room temperature. The synaptosomes were then washed three times with PB and 0.1 M carbonate buffer (pH 9.2) and coverslipped with fluorescence mounting medium (DAKO North America, Inc., Carpinteria, CA). Double immunofluorescence images were observed at a magnification of 400× using upright fluorescence microscopy (Leica DM2000 LED; Leica Microsystems, Wetzlar, Germany), and images were captured using a charge-coupled device camera (SPOT RT3; Diagnostic Instruments, Sterling Heights, MI).

Western Blotting. Synaptosomes (0.5 mg of protein/ml) from control and drug-treated groups were lysed in a pH 7.5 ice-cold Tris-HCl buffer solution containing (in mM) 20 Tris-HCl, 1% Triton X-100, 1 EDTA, 1 EGTA, 150 NaCl, 2.5 sodium pyrophosphate, 1 β-glycerophosphate, 1 phenylmethylsulfonyl fluoride, 1 sodium orthovanadate, and 1 μg/ml leupeptin. The lysates were sonicated for 10 seconds and then centrifuged at 13,000g at 4°C for 10 minutes. Equal amounts of samples were separated through electrophoresis on 7.5% SDS-PAGE, and then transferred to nitrocellulose membranes. The membranes were blocked with Tris-buffered saline that contained 5% low-fat milk and incubated with appropriate primary antibodies [anti-phospho–protein kinase A (PKA), 1:1000; Cell Signaling Technology]. After incubation with appropriate horseradish peroxidase–conjugated donkey anti-rabbit IgG secondary antibodies (1:1000; Cell Signaling Technology), protein bands were detected using the enhanced chemiluminescence system (Amersham, Buckinghamshire, UK). An aliquot of samples was loaded and probed with anti–β-actin antibodies for detecting β-actin as a loading control. Films were scanned using a scanner, and the level of expression or phosphorylation was assessed using band density, which was quantified through densitometry.

Statistical Analysis. All data are expressed as the mean ± S.E. M. Student’s t tests were used for statistical analysis between two groups, whereas an analysis of variance with least significant difference comparisons and post-hoc tests were used to analyze more than two groups. Analysis was completed using SPSS software (version 17.0; SPSS Inc., Chicago, IL). P < 0.05 was considered to represent a significant difference.

Results

Celecoxib Inhibits 4-AP-Induced Glutamate Release in Rat Cerebrocortical Synaptosomes by Reducing Classic External Ca2+-Dependent Exocytosis. To investigate the effect of celecoxib on glutamate release, isolated nerve terminals were depolarized with the potassium channel blocker 4-AP, which has been shown to open VDCCs and induce the release of glutamate (Nicholls, 1998). In synaptosomes incubated in the presence of 1.2 mM CaCl2, 4-AP (1 mM) induced a glutamate release of 7.3 ± 0.1 nmol/mg per 5 minutes, and this release was blocked by the Na+ channel blocker TTX (2 μM) (P = 0.000; Fig. 1A). This is consistent with the observation that 4-AP is able to initiate TTX-sensitive depolarization and Ca2+-dependent glutamate release in synaptosomes (Nicholls, 1998). Application of celecoxib (30 μM) reduced the amount of 4-AP-induced glutamate release to 3.9 ± 0.2 nmol/mg per 5 minutes (P = 0.000) without altering the basal release of glutamate (Fig. 1A). The inhibitory effect of celecoxib on 4-AP–induced glutamate release was concentration-dependent, and produced an IC50 value of approximately 7 μM, which was derived from a dose-response curve (Fig. 1B).

We next investigated whether the inhibition of 4-AP–induced glutamate release by celecoxib was mediated by an effect on exocytotic vesicular release, or on Ca2+-independent release attributable to cytosolic efflux through the reversal of the glutamate transporter (Nicholls et al., 1987). The Ca2+-dependent glutamate efflux was measured by depolarizing the synaptosomes with 1 mM 4-AP in extracellular Ca2+-free solution that contained 300 μM EGTA (Fig. 1C). Under these conditions, the release of glutamate induced by 4-AP was 2.3 ± 0.3 nmol/mg per 5 minutes [F(2,12) = 164.837; P = 0.000; Fig. 1C]. The Ca2+-independent release induced by 4-AP, however, was not affected by 30 μM celecoxib (2.4 ± 0.1 nmol/mg per 5 minutes; P = 0.882; Fig. 1C). Similar results were also obtained using BAIPA [1,2-bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester], a cell-permeable Ca2+ chelator. BAIPA (50 μM) alone reduced the 4-AP–induced glutamate release [F(2,12) = 163.562; P = 0.000; Fig. 1C]. However, the release measured in the presence of both BAIPA and celecoxib was similar to that obtained in the...
presence of BAPTA alone ($P = 0.963$; Fig. 1C). In addition, we examined the action of celecoxib in the presence of DL-threo-β-benzyl-oxy aspartate (DL-TBOA), a nonselective inhibitor of all excitatory amino acid transporter subtypes, or bafilomycin A1, which depletes vesicle content by inhibiting the synaptic vesicle H^+-ATPase that drives glutamate uptake. In the presence of DL-TBOA (10 μM), although the 4-AP–induced glutamate release was increased by the inhibitor (because of inhibition of reuptake of the released glutamate) ($P < 0.001$), celecoxib (30 μM) continued to reduce the 4-AP–induced release of glutamate significantly [$P(2,12) = 71.69; P = 0.000$; Fig. 1C]. In contrast to DL-TBOA, bafilomycin A1 (0.1 μM) reduced the control 4-AP (1 mM)–induced glutamate release [$P(2,12) = 120.558; P = 0.000$; Fig. 1C], and completely prevented the inhibitory effect of celecoxib (30 μM) on the 4-AP–induced glutamate release. Therefore, in the presence of bafilomycin A1, celecoxib (30 μM) induced a statistically non-significant inhibition ($P = 0.972$; Fig. 1C). We also examined the effect of DON, an inhibitor of glutaminase, which catalyzes the conversion of glutamine to glutamate. Figure 1C shows that DON (100 μM) had no effect on 4-AP–induced glutamate release ($P = 0.332$). In the presence of DON, celecoxib (30 μM) still effectively inhibited the 4-AP–induced glutamate release [$P(2,9) = 38.17; P = 0.000$; Fig. 1C].

**Fig. 1.** Celecoxib inhibits the 4-AP–induced release of glutamate in rat cerebrocortical synaptosomes. (A) Glutamate release was induced by the addition of 1 mM 4-AP in the absence (control) or presence of 30 μM celecoxib or 2 μM TTX, added 10 minutes before depolarization. (B) The log dose-response curve for celecoxib inhibition of 4-AP–induced glutamate release, fitted using a logistic function. (C) 4-AP–induced glutamate release under control conditions or in the presence 30 μM celecoxib, 300 μM EGTA (without CaCl₂), 300 μM EGTA (without CaCl₂) and 30 μM celecoxib, 50 μM BAPTA, 50 μM BAPTA and 30 μM celecoxib, 10 μM DL-TBOA, 10 μM DL-TBOA and 30 μM celecoxib, 0.1 μM bafilomycin A1, 0.1 μM bafilomycin A1 and 30 μM celecoxib, 100 μM DON, or 100 μM DON and 30 μM celecoxib. Celecoxib was added 10 minutes before depolarization, and other drugs 10 minutes before this. Results are presented as the mean ± S.E.M. of independent experiments, using synaptosomal preparations from five animals. ***$P < 0.001$ versus the control group; *$P < 0.05$ versus the DL-TBOA–treated group.
Celecoxib Reduces the 4-AP-Induced Increase in $[\text{Ca}^{2+}]_c$ and Glutamate Release by Inhibiting Ca$_{2.2}$ (N-Type) and Ca$_{2.1}$ (P/Q-Type) Channels. Transmitter release can be modulated by regulating the plasma membrane level, and consequently altering the calcium influx. To investigate the potential mechanisms underlying the celecoxib-mediated inhibition of glutamate release, the effect of celecoxib on intrasynaptosomal Ca$^{2+}$ levels was determined using the Ca$^{2+}$ indicator fura-2. As indicated in Fig. 2A, 1 mM 4-AP caused an increase in $[\text{Ca}^{2+}]_c$ from 157.4 ± 2.6 nM to a plateau level of 212.8 ± 5.6 nM ($P = 0.001$). Applying 30 μM celecoxib did not affect basal Ca$^{2+}$ levels (155.6 ± 2.9 nM), but caused an approximate 13% decrease in the 4-AP-induced increase in $[\text{Ca}^{2+}]_c$ (184.6 ± 5.2 nM; $P = 0.01$; Fig. 2A).

In the adult rat cerebrocortical nerve terminals, elevation of the $[\text{Ca}^{2+}]_c$ and glutamate release induced by depolarization was supported by the Ca$^{2+}$ influx through Ca$_{2.2}$ (N-type) and Ca$_{2.1}$ (P/Q-type) channels (Vazquez and Sanchez-Prieto, 1997; Millan and Sanchez-Prieto, 2002). To investigate whether the decrease in Ca$^{2+}$ channel activity was involved in

![Graph A](image)

![Graph B](image)

![Graph C](image)

**Fig. 2.** Celecoxib reduces the 4-AP–induced increase in cytosolic Ca$^{2+}$ levels, and this effect is prevented by blocking the Ca$_{2.2}$ (N-type) and Ca$_{2.1}$ (P/Q-type) channels. (A) Cytosolic-free Ca$^{2+}$ concentration (in nanomoles) was measured in the absence (control) and presence of 30 μM celecoxib, added 10 minutes before depolarization with 1 mM 4-AP. (B and C) The elevation of cytosolic Ca$^{2+}$ levels and glutamate release were induced by 1 mM 4-AP, in the absence (control) or presence of 30 μM celecoxib, 2 μM ω-CgTX MVIIIC, 2 μM ω-CgTX MVIIIC and 30 μM celecoxib, 50 μM dantrolene, 50 μM dantrolene, 30 μM celecoxib, 100 μM CGP37157, or 100 μM CGP37157 and 30 μM celecoxib. Celecoxib was added 10 minutes before depolarization, whereas the other drugs were added 30 minutes before depolarization. Results are presented as the mean ± S.E.M. of independent experiments, using synaptosomal preparations from five animals. ***$P < 0.001$ versus the control group; **$P < 0.01$ versus the control group; *$P < 0.05$ versus the dantrolene- or CGP37157-treated group.
the celecoxib-mediated inhibition of 4-AP–induced increase in [Ca^{2+}]_{i} and glutamate release, we examined the effect of celecoxib in the presence of ω-CgTX MVIC, a wide spectrum blocker of Ca_{2.2} (N-type) and Ca_{2.1} (P/Q-type) channels. In Fig. 2, B and C, 2 μM ω-CgTX MVIC substantially reduced the 1 mM 4-AP–induced increase in [Ca^{2+}]_{i} (F(2,12) = 65.968; P = 0.000) and glutamate release [F(2,12) = 151.722; P = 0.000]. Although the 4-AP–induced increase in [Ca^{2+}]_{i} and glutamate release was considerably reduced in the presence of celecoxib (30 μM) (P = 0.000), this effect was prevented by the presence of ω-CgTX MVIC. The [Ca^{2+}]_{i} and release measured in the presence of both ω-CgTX MVIC and celecoxib was similar to that obtained in the presence of ω-CgTX MVIC alone (P > 0.05) (Fig. 2, B and C). In addition, to test the role of Ca^{2+} release from intracellular stores such as the endoplasmic reticulum (ER) and mitochondria (Berridge, 1998), we examined the effect of dantrolene, an inhibitor of intracellular Ca^{2+} release from the ER, and CGP37157, a membrane-permeant blocker of mitochondrial Na^{+}/Ca^{2+} exchange. As indicated in Fig. 2, B and C, 100 μM dantrolene reduced the 1 mM 4-AP–induced increase in [Ca^{2+}]_{i} (F(2,12) = 55.693; P = 0.000; Fig. 1C) and glutamate release [F(2,12) = 138.982; P = 0.000; Fig. 1C], indicating that Ca^{2+} release from ER ryanodine receptors contributes substantially to the 4-AP–induced increase in [Ca^{2+}]_{i} and glutamate release. In the presence of dantrolene, however, celecoxib (30 μM) still effectively inhibited the 4-AP–induced increase in [Ca^{2+}]_{i} and glutamate release (P < 0.05; Fig. 2, B and C). Similar results were also observed using 100 μM CGP37157 (Fig. 2, B and C).

**Celecoxib Does Not Alter the Synaptosomal Membrane Potential and Depolarization-Induced Na^{+} Influx.** Inhibition of [Ca^{2+}]_{i} elevation by celecoxib might be attributable either to a direct reduction in the amount of Ca^{2+} entering through VDCCs, or to secondary effects caused by, for example, the modulation of potassium channels and the consequently altered plasma membrane potential. To discern between these two possibilities, the effect of celecoxib on the synaptosomal plasma membrane potential under resting conditions and on depolarization was examined using membrane potential–sensitive dye DiSC3(5). Figure 3A shows that 1 mM 4-AP caused an increase in DiSC3(5) fluorescence (11.9 ± 0.5 fluorescence units/5 minutes). Preincubation of the synaptosomes with 30 μM celecoxib for 10 minutes before adding 4-AP did not alter the resting membrane potential, and produced no substantial change in the 4-AP–mediated increase in DiSC3(5) fluorescence (12.1 ± 0.6 units/5 minutes; P = 0.937; Fig. 3A). This suggested that the observed inhibition of 4-AP–induced glutamate release by celecoxib is unlikely to be due to a hyperpolarizing effect of the drug on the synaptosomal plasma membrane potential or an attenuation of depolarization produced by 4-AP. Confirmation that the celecoxib effect did not affect synaptosomal excitability was obtained through experiments using high external [K^{+}]-mediated depolarization. Elevated extracellular KCl depolarized the plasma membrane by shifting the K^{+} equilibrium potential above the threshold for activation of voltage-dependent ion channels. Although Na^{+} channels are inactivated under these conditions, VDCCs are activated nonetheless to mediate Ca^{2+} entry, which supports neurotransmitter release (Barrie et al., 1991). As indicated by the inset in Fig. 3A, 15 mM KCl induced a glutamate release of 6.9 ± 0.4 nmol/mg per 5 minutes, which was reduced to 4.8 ± 0.4 nmol/mg per 5 minutes in the presence of 30 μM celecoxib (P = 0.006). In addition, the Na^{+}-sensitive probe SBFI was used to measure cytosolic Na^{+} levels. Figure 3B indicates that 4-AP (1 mM) caused a clear rise in Na^{+} influx, but celecoxib (30 μM) failed to affect this increase (P = 0.996). The failure of celecoxib to produce an effect on this increase was not caused by an insufficient level of sensitivity of the SBFI probe to alterations in Na^{+} channel activity because in parallel experiments, the Na^{+} channel blocker TTX (2 μM) caused an 86% inhibition of 4-AP–induced Na^{+} influx (P = 0.000; Fig. 3B).

**PGE_{2}/EP2 Is Involved in the Celecoxib-Mediated Inhibition of Glutamate Release.** PGE_{2}, the predominant reaction product of COX-2, has been reported to increase glutamate release (Nishihara et al., 1995). If the effect of celecoxib on glutamate release results from the blocking of PGE_{2} synthesis, exogenous application of PGE_{2} should occlude the celecoxib-mediated inhibition of glutamate release. To test this hypothesis, the effect of celecoxib on 4-AP–induced glutamate release in the absence or presence of PGE_{2} was compared. As shown in Fig. 4A, 1 mM 4-AP induced a glutamate release of 6.7 ± 0.3 nmol/mg per 5 minutes, which was facilitated by 45% (9.7 ± 0.5 nmol/mg per 5 minutes) in the presence of PGE_{2} (5 μM) [F(2,13) = 7.86; P = 0.006]. Celecoxib (30 μM) alone reduced the 4-AP–induced glutamate release to 3.9 ± 0.4 nmol/mg per 5 minutes (P = 0.000), but this inhibition was abolished by pretreatment with PGE_{2}, and no statistical difference was observed between the release after PGE_{2} alone and after the PGE_{2} and celecoxib treatment (9.5 ± 0.6 mM/mg per 5 minutes; P = 0.986; Fig. 4, A and B). Similarly, 5 μM butaprost, an EP2 agonist, enhanced the 4-AP–induced glutamate release [F(2,13) = 10.539; P = 0.003] and completely prevented the inhibition of glutamate release by 30 μM celecoxib (P = 0.492; Fig. 4B). Furthermore, 100 μM PF04418948 (an EP2 antagonist) reduced 4-AP–induced glutamate release [F(2,13) = 45.782; P = 0.000] and completely prevented the effect of celecoxib (P = 0.954; Fig. 4B). By contrast, 5 μM sulprostone, a PGE_{2} receptor 3 (EP3) agonist, exerted no effect on 4-AP–induced glutamate release (P = 1.000). In the presence of sulprostone, 30 μM celecoxib still effectively inhibited 4-AP–induced glutamate release [F(2,12) = 27.516; P = 0.000; Fig. 4B], A similar result was also obtained using TCS2510, a PGE_{2} receptor 4 (EP4) agonist. As reported in Fig. 4B, TCS2510 (100 μM) exerted no effect on either control 4-AP–induced glutamate release (P = 0.603) or inhibition of glutamate release by celecoxib [F(2,12) = 83.813; P = 0.008; Fig. 4B]. These results suggested that the observed effect of celecoxib on glutamate release is mediated by PGE_{2}/EP2 receptors.

**COX-2 and EP2 Are Expressed in Cerebrocortical Nerve Terminals.** To determine whether COX-2 and EP2 are expressed in purified cerebrocortical synaptosomes, we performed a double immunostaining of COX-2 and EP2 using synaptophysin, a presynaptic marker. As shown in Fig. 5, the nerve terminals that contained synaptophysin also contained COX-2 or EP2, confirming that COX-2 and EP2 are present in presynaptic terminals, which is consistent with previous studies (Sang et al., 2005; Zhu et al., 2005).

**The PKA Pathway Is Involved in the Celecoxib-Mediated Inhibition of Glutamate Release.** Because EP2 is linked to the Ga-cAMP/PKA pathway (Sang et al., 2005), we used PKA inhibitors H89 and KT5720 to examine whether the celecoxib-mediated inhibition of glutamate release can be prevented. Figure 6A shows that 100 μM H89
reduced 1 mM 4-AP–induced glutamate release \( F(2,13) = 7.115; P = 0.01 \), which indicated basal PKA activity. Although celecoxib (30 μM) reduced the 4-AP–induced glutamate release \( P < 0.001 \), this effect was prevented by H89; the release measured in the presence of H89 and celecoxib was similar to that obtained in the presence of H89 alone \( P = 0.837 \); Fig. 6, A and B). Similar results were also observed in the presence of KT5720 (1 μM) \( P = 1.0 \); Fig. 6B). We also determined whether direct activation of PKA is capable of preventing the inhibitory effect of celecoxib on glutamate release. The application of PKA activator forskolin (100 μM) facilitated 4-AP–evoked glutamate release \( P < 0.001 \); Fig. 6B). Interestingly, however, when celecoxib (30 μM) was applied together with forskolin, the inhibitory effect of celecoxib on the release of glutamate evoked by 4-AP was prevented \( P = 0.462 \); Fig. 6B). In addition, the mitogen-activated protein kinase inhibitor PD98059 (50 μM) reduced 1 mM 4-AP–induced glutamate release \( P < 0.001 \). In the presence of PD98059, however, 30 μM celecoxib still effectively inhibited 4-AP–induced glutamate release \( F(2,12) = 76.364; P = 0.000 \); Fig. 6B). Similar to PD98059, the protein kinase C inhibitor GF109203X (10 μM) decreased 4-AP–induced glutamate release \( F(2,12) = 68.696; P = 0.000 \), but exerted no effect on the celecoxib-mediated inhibition of 4-AP–induced glutamate release (Fig. 6B).

To further confirm that the PKA signaling pathway was suppressed by celecoxib during its inhibition of 4-AP–induced glutamate release, we determined the effect of celecoxib on the phosphorylation of PKA in cerebrocortical synaptosomes. As indicated in Fig. 6C, depolarization of synaptosomes with 1 mM 4-AP in the presence of 1.2 mM CaCl₂ increased the phosphorylation of PKA \( (126.1 ± 2.1%; P < 0.001) \). When synaptosomes were pretreated with 30 μM celecoxib for 10 minutes before depolarization with 4-AP, a significant decrease
in the 4-AP–induced PKA phosphorylation was observed [106.6 ± 2.5%; F(2,6) = 52.215; P = 0.001; Fig. 6C].

Discussion

Celecoxib, a selective COX-2 inhibitor, has recently received considerable attention because of its beneficial effects on numerous CNS diseases, including cerebral ischemia and neurodegenerative disorders (Drachman et al., 2002; Small et al., 2008; Abd El-Aal et al., 2013). Excessive glutamate release is involved in the pathophysiology of these diseases, and reducing glutamate release might provide a potential strategy for treating these diseases (Meldrum and Garthwaite, 1990; Meldrum, 2000). Therefore, we elucidated the potential role of celecoxib in glutamate release in rat cerebral cortex nerve terminals (synaptosomes). We demonstrated for the first time that celecoxib inhibits depolarization-induced glutamate release. The possible underlying mechanisms for the celecoxib-mediated...
inhibition of glutamate release were investigated in the present study.

The process through which neurotransmitter release occurs is complex, involving Na\(^+\), K\(^+\), and Ca\(^{2+}\) channels (Wu and Saggau, 1997; Nicholls, 1998). The inhibition of Na\(^+\) channels and the activation of K\(^+\) channels stabilizes membrane excitability and, consequently, causes a reduction in the levels of Ca\(^{2+}\) entry and neurotransmitter release (Rehm and Tempel, 1991; Li et al., 1993). According to this concept, the observed inhibitory effect of celecoxib on induced glutamate release might occur through a reduction of nerve terminal excitability, but this is unlikely because of the following
observations. First, celecoxib inhibited the release of glutamate induced by 4-AP and KCl. Because 4-AP–induced glutamate release involves the activation of Na\(^+\) and Ca\(^{2+}\) channels, 15 mM external KCl–induced glutamate release involves only Ca\(^{2+}\) channels (Barrie et al., 1991; Nicholls, 1998); thus, Na\(^+\) channels are not involved in the effect of celecoxib on glutamate release. This was supported by our observation that celecoxib did not affect 4-AP–induced Na\(^+\) influx. Second, celecoxib was not observed to substantially affect the synaptosomal plasma membrane potential, indicating that celecoxib does not affect K\(^+\) conductance. Third, celecoxib did not significantly inhibit 4-AP–induced Ca\(^{2+}\)-independent glutamate release, a component of glutamate release that is exclusively dependent on membrane potential (Nicholls et al., 1987; Attwell et al., 1993). Furthermore, the celecoxib-mediated inhibition of 4-AP–induced glutamate release was prevented by the vesicular transporter inhibitor bafilomycin A1, but was insensitive to the glutamate transporter inhibitor P0,TBOA. This indicated that celecoxib does not affect glutamate release by reversing the direction of the plasma membrane glutamate transporter. These data suggest that the celecoxib-mediated inhibition of 4-AP–induced glutamate release is mediated by a decrease in the exocytotic pool of release. Moreover, this phenomenon is not the result of a reduction in synaptosomal excitability caused by ion channel (e.g., Na\(^+\) or K\(^+\) channels) modulation. This finding is inconsistent with that of previous electrophysiological studies, which have shown that celecoxib modulates Na\(^+\) and K\(^+\) currents in rat retinal and ganglion neurons (Park et al., 2007; Du et al., 2011; Mi et al., 2013). The reasons for the difference in results are unclear, but might be attributable to the distinct experimental models used; previous studies have used a cell culture model, whereas we used a nerve terminal (synaptosomal) model.

Therefore, if the effect is not caused by the modulation of synaptosomal excitability, celecoxib might inhibit glutamate release by decreasing the levels of Ca\(^{2+}\) entry through the Ca\(_{2.2}\) (N-type) and Ca\(_{2.1}\) (P/Q-type) Ca\(^{2+}\) channels that are coupled to glutamate exocytosis in the nerve terminals (Vazquez and Sanchez-Prieto, 1997; Millan and Sanchez-Prieto, 2002). This hypothesis is plausible because the present study demonstrated that celecoxib reduced the 4-AP–induced increase in [Ca\(^{2+}\)]\(_{C}\) and glutamate release, and that this effect was prevented by blocking the Ca\(_{2.2}\) (N-type) and Ca\(_{2.1}\) (P/Q-type) channels. Conversely, the reduced release of stored Ca\(^{2+}\) from the ER ryanodine receptors and mitochondria during the celecoxib-mediated inhibition of glutamate release can be excluded. This is because the inhibitory effect of celecoxib on the 4-AP–induced increase in [Ca\(^{2+}\)]\(_{C}\) and glutamate release was insensitive to the ER ryanodine receptor inhibitor dantrolene and the mitochondrial Na\(^{+}\)/Ca\(^{2+}\) exchange inhibitor CGP37157. Although no direct evidence has indicated that celecoxib acts on presynaptic Ca\(^{2+}\) channels, these results implied that the suppression of Ca\(^{2+}\) influx through Ca\(_{2.2}\) and Ca\(_{2.1}\) channels is involved in the inhibition of glutamate release induced by celecoxib.

Determining how celecoxib inhibits the Ca\(_{2.2}\) and Ca\(_{2.1}\)-channels and glutamate release is critical. Celecoxib is a selective COX-2 inhibitor that inhibits the production of PGE\(_2\). PGE\(_2\) is lipophilic, and diffuses rapidly and activates its specific membrane receptors (EP1–4). These receptors are G-protein–coupled receptors, and their intracellular signaling is distinct. The EP1 receptors couple with the Gq-phospholipase C–inositol triphosphate pathway. The EP2 and EP4 receptors are linked to the Gs-cAMP/PKA pathway and increase cAMP levels. Conversely, activation of the EP3 receptors reduces cAMP production through a pertussis toxin–sensitive Gi-coupled pathway (Breyer et al., 2001; Sugimoto and Narumiya, 2007). PGE\(_2\) has been shown to increase glutamate release through presynaptic EP2 signaling (Nishihara et al., 1995; Sang et al., 2005). Thus, speculating that the inhibitory effect of celecoxib on Ca\(^{2+}\) influx and glutamate release is a consequence of reduced PGE\(_2\) signaling is reasonable. In elucidating this hypothesis, we observed that 1) a PGE\(_2\) or EP2 agonist increased glutamate release and abrogated the inhibitory effect of celecoxib on 4-AP–induced glutamate release; 2) an EP3 or EP4 agonist did not elicit effects on either 4-AP–induced glutamate release or the celecoxib-mediated inhibition of glutamate release.

**Fig. 7.** Potential mechanisms by which celecoxib inhibits glutamate release. In rat cerebrocortical nerve terminals, the inhibition of COX-2 activity by celecoxib induces the reduction of PGE\(_2\) levels, causing a decrease in EP2 activation and PKA activity. This, in turn, reduces the Ca\(^{2+}\) influx through N- and P/Q-type Ca\(^{2+}\) channels to cause a decrease in glutamate release.
glutamate release; 3) the inhibitory effect of celecoxib on 4-AP–induced glutamate release was blocked by an EP2 antagonist; 4) the cerebrocortical nerve terminals expressed COX-2 and EP2 receptors, and these two proteins were co-expressed with synaptophysin, a presynaptic marker, within the same nerve terminals; 5) PKA inhibitors prevented the inhibitory effect of celecoxib on glutamate release, but neither the mitogen-activated protein kinase inhibitor nor the protein kinase C inhibitor exerted any effects; 6) activating PKA prevented the celecoxib-mediated inhibition of glutamate release; and 7) celecoxib significantly reduced the 4-AP–induced phosphorylation of PKA. These results suggested that presynaptic EP2, not EP3 or EP4, is involved in the inhibition of 4-AP–evoked glutamate release produced by celecoxib. Furthermore, these data confirmed the involvement of the PKA pathway in the action of celecoxib. In nerve terminals, PKA has been shown to phosphorylate VDCCs and several synaptic proteins, subsequently increasing glutamate release (Herrero and Sanchez-Prieto, 1996; Chheda et al., 2001). Thus, a reduction of presynaptic Ca2,2.2 and Ca2,2.1 channel phosphorylation should be considered when determining the possible mechanism of celecoxib-mediated presynaptic inhibition. However, this possibility cannot be examined in the current study, because antibodies for the phosphorylation of the N- and P/Q-type calcium channels are not available. Based on our data, Fig. 7 presents a model to explain the mechanism through which celecoxib inhibits glutamate release from cortical nerve terminals. In brief, the inhibition of COX-2 activity by celecoxib induces the reduction of PGE2 levels, causing a decrease in EP2 activation and PKA activity. This, in turn, reduces Ca2+ influx and glutamate release. COX-2–derived prostaglandins other than PGE2, such as prostaglandin D2 and prostaglandin F2α, are among the most abundant prostaglandins in the brain and regulate numerous functions, including pain and sleep (Horting and Seregi, 1989; Urade and Hayashi, 1999). These functions are thought to be associated with the glutamate system (Gilmour et al., 2013; Suto et al., 2014). Therefore, the role of prostaglandin D2 or prostaglandin F2α in the celecoxib-mediated inhibition of glutamate release cannot be ruled out. In addition, the possible involvement of other presynaptic pathways should be considered. For example, GABAA receptors are expressed in the brain and localize both pre- and postsynaptically. At the presynaptic level, GABAA receptors have been shown to inhibit Ca2+ influx and glutamate release (Long et al., 2009). Future studies are needed to determine whether GABAA receptors play any role in the celecoxib-mediated inhibition of glutamate release.

Cerebrocortical 4-AP–induced glutamate release and COX-2 pharmacological effects such as the inhibition of inflammatory processes, stabilization of mitochondrial function, and attenuation of oxidative stress. These effects are likely to be associated with the neuroprotective activity of celecoxib (Hunter et al., 2007; López-Villodres et al., 2012; Kaizaki et al., 2013). We observed that celecoxib reduced glutamate release from nerve terminals, and this effect was dose-dependent and peaked at 30 μM with an IC50 of 7 μM. This suggested that the decrease in released glutamate presents an additional explanation for the neuroprotective effect of celecoxib. This hypothesis was based on the excessive glutamate release and activation of glutamate receptors resulting in neurotoxic cell damage that has been implicated in the neuropathology of acute and chronic brain disorders (Meldrum and Garthwaite, 1990; Meldrum, 2000). The blockade of glutamate neurotransmission, such as glutamate receptor antagonists, has conferred neuroprotection in in vitro and in vivo studies (Schauwecker, 2010; Yeganeh et al., 2013). The reduction of glutamate release is an even earlier phenomenon, and can prevent the postsynaptic toxicity of glutamate at all glutamate receptors.

In conclusion, the current study provides evidence that the COX-2 inhibitor celecoxib exerts an inhibitory effect on glutamate release. This effect might be exerted mainly through the suppression of the presynaptic EP2/PCA pathway. COX-2 expression is significantly increased in patients with Alzheimer’s disease and in experimental stroke or epilepsy, suggesting that COX-2 and its reaction products are involved in several neurologic disorders (Nogawa et al., 1997; Minghetti, 2004). Thus, our finding is valuable because it provides new insight into the mechanism by which COX-2 inhibitors act in the brain.

**References**


