σ-1 Receptor Agonist SKF10047 Inhibits Glutamate Release in Rat Cerebral Cortex Nerve Endings

Cheng-Wei Lu, Tzu-Yu Lin, Chia-Chuan Wang, and Su-Jane Wang

Department of Anesthesiology, Far-Eastern Memorial Hospital, New Taipei, Taiwan (C.-W.L., T.-Y.L.); Department of Mechanical Engineering, Yuan Ze University, New Taipei, Taiwan (T.-Y.L.); and School of Medicine (C.-W., S.-J.W.) and Graduate Institute of Basic Medicine (S.-J.W.), Fu Jen Catholic University, New Taipei, Taiwan

Received December 20, 2011; accepted February 21, 2012

ABSTRACT

σ-1 Receptors are expressed in the brain, and their activation has been shown to prevent neuronal death associated with glutamate toxicity. This study investigates the possible mechanism and effect of [2S-(2α,6α,11R*)]-1,2,3,4,5,6-hexahydro-6,11-dimethyl-3-(2-propenyl)-2,6-methano-3-benzazocin-8-ol (SKF10047), a σ-1 receptor agonist, on endogenous glutamate release in the nerve terminals of rat cerebral cortex. Results show that SKF10047 inhibited the release of glutamate evoked by the K+ channel blocker 4-aminopyridine (4-AP), and the σ-1 receptor antagonist N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(dimethylamino)ethylamine (BD1047) blocked this phenomenon. The effects of SKF10047 on the evoked glutamate release were prevented by the chelating extracellular Ca2+ ions and the vesicular transporter inhibitor bafilomycin A1. However, the glutamate transporter inhibitor α,β-threo-β-benzyl-α-aspartate did not have any effect on the action of SKF10047. SKF10047 decreased the depolarization-induced increase in the cytosolic free Ca2+ concentration ([Ca2+]c), but did not alter 4-AP-mediated depolarization. Furthermore, the effects of SKF10047 on evoked glutamate release were prevented by blocking the Ca2,2.2 (N-type) and Ca2.1 (P/Q-type) channels, but not by blocking the ryanodine receptors or the mitochondrial Na+/Ca2+ exchange. In addition, conventional protein kinase C (PKC) inhibitors abolished the SKF10047 effect on 4-AP-evoked glutamate release. Western blot analyses showed that SKF10047 decreased the 4-AP-induced phosphorylation of PKC and PKCα. These results show that σ-1 receptor activation inhibits glutamate release from rat cortical nerve terminals. This effect is linked to a decrease in [Ca2+]c caused by Ca2+ entry through presynaptic voltage-dependent Ca2+ channels and the suppression of the PKC signaling cascade.

Introduction

σ-1 Receptors are widely distributed in the brain and are involved in learning and memory, drug dependence, analgesia, and psychological disorders (Alonso et al., 2000; Cobos et al., 2006; Maurice and Su, 2009). A number of studies have demonstrated the neuroprotective effects of σ-1 receptor activation. For example, σ-1 receptor agonists attenuate glutamate- or amyloid-induced neurotoxicity (DeCoster et al., 1995; Senda et al., 1998; Marrazzo et al., 2005), protect against ischemia- or glucose deprivation-induced brain damage (Takahashi et al., 1995; Yang et al., 2010), and ameliorate β-amyloid-induced memory impairment (Maurice et al., 1998). However, the mechanisms involved in these neuroprotective effects have yet to be fully elucidated.

Glutamate is a major excitatory neurotransmitter in the brain and plays an important role in functions such as synaptic plasticity, learning, and memory (Greenamyre and Porter, 1994). Excessive release of glutamate induces an increase in intracellular Ca2+ 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.
(Schinder et al., 1996). The neuronal damage induced by overexcitation probably is involved in a number of neuro-pathological conditions, ranging from acute insults such as stroke, epileptic seizures, and traumatic brain and spinal cord injury to chronic neurodegenerative disorders such as Alzheimer’s disease, Parkinson’s disease, and amyotrophic lateral sclerosis (Obrenovitch and Urenjak, 1997; Meldrum, 2000). Consequently, if a drug can attenuate glutamate release from nerve terminals, it may have a neuroprotective effect on the pathological conditions related to excessive glutamate release.

To our knowledge, there are no studies addressing whether α-1 receptor activation directly affects glutamate release at the presynaptic level. Therefore, this study uses isolated nerve terminals (synaptosomes) purified from the rat cerebral cortex to investigate the effects of [2S-(2α,6α,11R)-1,2,3,4,5,6-hexahydro-6,11-dimethyl-3-(2-propenyl)-2,6-methano-3-benzazocin-8-ol (SKF10047), a α-1 receptor agonist, 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 used as a model system for studying presynaptic phenomena. Therefore, this study uses isolated nerve terminals to investigate the effects of SKF10047 on the release of endogenous glutamate, the synaptosomal plasma membrane potential, and the downstream activation of voltage-dependent Ca2+ channels (VDCCs). The second series of experiments evaluated whether the protein kinase signaling pathway participates in α-1 receptor activation directly affects glutamate release at the presynaptic level. Therefore, this study uses isolated nerve terminals (synaptosomes) purified from the rat cerebral cortex to investigate the effects of [2S-(2α,6α,11R)-1,2,3,4,5,6-hexahydro-6,11-dimethyl-3-(2-propenyl)-2,6-methano-3-benzazocin-8-ol (SKF10047), a α-1 receptor agonist, 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 used as a model system for studying presynaptic phenomena. Therefore, this study uses isolated nerve terminals to investigate the effects of SKF10047 on the release of endogenous glutamate, the synaptosomal plasma membrane potential, and the downstream activation of voltage-dependent Ca2+ channels (VDCCs). The second series of experiments evaluated whether the protein kinase signaling pathway participates in α-1 receptor activation directly affects glutamate release at the presynaptic level.

Inhibition of Glutamate Release by α-1 Receptor Activation

Materials and Methods

Materials. 3’,3’-Dipropylthiadicarbocyanine iodide [DiSC3(5)] and fura-2-acetoxyethyl ester (Fura-2-AM) were obtained from Invitrogen (Carlsbad, CA). SKF10047, 2-(4-morpholinethinyl)-1-phenylcylohexancarboxylate (PREE84), baclofen, bathylomycin A1, N-[2,3,4-dichlorophenyl]ethyl-N-ethyl-2-dimethylaminoethylamine (BD1047), 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP37157), α,ω-conotoxin (α-CgTX) MVIIIC, 2-[3-(4-nitrobenzoyloxy)phenyl]ethanesulfonic acid (BAPTA), tetrodotoxin (10 μM TBOA), 5,6,7,13-tetrahydro-13-methyl-5-oxo-12H-indole(2,3-c)pyrrole(3,4-c)carbazole-12-propanenitrile (6G676), rotllerin, and 2-[3-(3-dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl) maleimide (GF109203X) were obtained from Tocris Bioscience (Bristol, UK). The anti-α-1 receptor rabbit polyclonal antibody was bought from Abcam plc (Cambridge, UK). Rabbit polyclonal antibodies directed against phosphor-protein kinase C (PKC) (pan) and phospho-PKCβ were bought from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase-conjugated anti-rabbit secondary antibodies were from Bio-Rad Laboratories (Hercules, CA). Pertussis toxin (PTX) and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Animals. Adult male Sprague-Dawley rats (150–200 g) were used 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 (Institute of Laboratory Animal Resources, 1996) and approved by the Fu Jen Institutional Animal Care and Utilization Committee.

Preparation of Synaptosomes. Animals were killed by decapitation, and the cerebral cortex was rapidly dissected. Purified synaptosomes were prepared by homogenizing the tissue in a medium that contained 320 mM sucrose, pH 7.4. The homogenate was spun for 2 min at 3000g (5000 rpm in a JA 25.5 rotor; Beckman Coulter, Fullerton, CA) at 4°C, and the supernatant was spun again at 14,500g (11,000 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 gradients that consisted of 320 mM sucrose, 1 M EDTA, 0.25 mM EDTA, 0.25 mM MgCl2·6H2O, 1.2 mM Na3HPO4, 10 mM glucose, and 10 mM HEPES, pH 7.4, before centrifugation at 27,000g (15,000 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 by using a Bradford Protein Assay Kit (Bio-Rad Laboratories), based on the method of Bradford (1976), with BSA as a standard. Synaptosomal suspension (0.5 mg) was diluted in 10 ml of HBM and spun at 3000g (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 to 6 h.

Glutamate Release. Glutamate release from purified cerebrocortical synaptosomes was monitored online, with an assay that used exogenous glutamate dehydrogenase (GDH) and NADP+ to couple the oxidative deamination of the released glutamate to the generation of NADPH detected fluorometrically (Nicholls et al., 1987). Synaptosomal pellets were resuspended in HBM that contained 16μM BSA and incubated in a stirred and thermostatted cuvette maintained at 37°C in a PerkinElmer LS-55 spectrophotofluorometer (PerkinElmer Life and Analytical Sciences, Waltham, MA). NADP+ (2 mM), GDH (50 units/ml), and CaCl2 (1 mM) were added after 3 min. In experiments that investigated Ca2+-independent efflux of glutamate, EGTA (200 μM) was added in place of CaCl2. Other additions before depolarization were made as described in the figure legends. After another 10 min of incubation, 4-aminopyridine (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. Values cited 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 plasma membrane potential can be monitored by positively charged, membrane potential-sensitive carbocyanine dyes such as DiSC3(5), DiSC2(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 the fluorescence increases (Akerman et al., 1987). Synaptosomes were preincubated and resuspended as described for the glutamate release experiments. After 3-min incubation, 5 μM DiSC3(5) was added and allowed to equilibrate before the addition of CaCl2 (1 mM) after 4-min incubation. 4-AP (1 mM) was added to depolarize the synaptosomes at 10 min, and DiSC3(5) fluorescence was monitored at excitation and emission wavelengths of 646 and 674 nm, respectively.

Cytosolic Free Ca2+ Concentration. Cytosolic free Ca2+ concentration ([Ca2+]i) was measured by using the Ca2+ indicator Fura-2-AM. Synaptosomes (0.5 mg/ml) were preincubated in HBM with 16μM BSA in the presence of 5 μM Fura-2-AM and 0.1 mM CaCl2 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 PerkinElmer LS-55 spectrophotometer. CaCl₂ (1 mM) was added after 3 min, and further additions were made after another 10 min. Fluorescence data were accumulated at excitation wavelengths of 340 and 380 nm (emission wavelength 505 nm) at 7.5-s intervals. Calibration procedures were performed as described previously (Siha et al., 1992), using 0.1% SDS to obtain the maximal fluorescence with Fura-2 saturation with Ca²⁺, followed by 10 mM EGTA (Tris buffered) to obtain minimum fluorescence in the absence of any Fura-2/Ca²⁺ complex. [Ca²⁺]₀ was calculated by using equations described previously (Grynkiewicz et al., 1985).

Western Blot. Synaptosomes 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 13,000g at 4°C for 10 min. Equal amounts of membrane protein were separated by electrophoresis on 7.5% SDS-polyacrylamide gel electrophoresis, and then transferred to nitrocellulose membranes. The membranes were blocked with Tris-buffered saline that contained 5% low-fat milk and incubated with the following primary polyclonal antibodies: α-1 receptor, 1:200; phospho-PKC (pan) (to detect PKC-α, -β, -δ, -ε, -η, and -δ, phosphorylated at a carboxy-terminal residue homologous to Ser-660 of PKC-β), 1:3000; and phospho-PKCa, 1:4000. After incubation with the appropriate peroxidase-conjugated donkey anti-rabbit IgG secondary antibodies (1:3000), protein bands were detected by using the enhanced chemiluminescence system (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). An aliquot of samples was loaded and probed with anti-β-actin antibody for detection of β-actin as a loading control. Films were scanned by using a scanner, and the level of phosphorylation was assessed by band density, which was quantitated by densitometry.

Data Analysis. Cumulative data were analyzed in Lotus 1-2-3 (IBM, White Plains, NY) and MicroCal Origin (OriginLab Corp., Northampton, MA). Data are expressed as mean ± S.E.M. 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 SKF10047), a one-way repeated-measures analysis of variance (ANOVA) was computed. P < 0.05 was considered to represent a significant difference.

Results

SKF10047 Reduces 4-AP-Evoked Glutamate Release in Cerebrocortical Nerve Terminals. Depolarization of nerve terminals with the K⁻-channel blocker 4-AP opens VDCCs and initiates neurotransmitter release (Nicholls, 1998). Glutamate release after 5-min depolarization of cerebrocortical nerve terminals in the presence of 1 mM CaCl₂ was 7.2 ± 0.1 nmol/mg. This release was reduced with the α-1 receptor agonist SKF10047 (100 μM) (4.1 ± 0.1 nmol/mg; P < 0.001; n = 7; Fig. 1A) or PreE804 (100 μM) (3.9 ± 0.2 nmol/mg; P < 0.001; n = 5; Fig. 1D). The SKF10047-induced inhibition of 4-AP-evoked glutamate release strictly depended on the presence of external Ca²⁺ (n = 5; Fig. 1B). A maximal inhibition of 65% occurred with 200 μM SKF10047 (Fig. 1C). The IC₅₀ value for SKF10047 inhibition of 4-AP-evoked Ca²⁺-dependent glutamate release, derived from a log dose-response curve, was 118 μM (Fig. 1C).

We next investigated whether the inhibition of 4-AP-evoked glutamate release by SKF10047 was mediated by an effect on exocytotic vesicular release or Ca²⁺-independent release attributable to cytosolic efflux via reversal of the glutamate transporter (Nicholls et al., 1987). For this, we examined the action of SKF10047 in the presence of DL-TBOA, a nonselective inhibitor of all excitatory amino acid transporter subtypes, or bafilomycin A1, which causes the depletion of glutamate in synaptic vesicles. As seen in Fig. 1D, the 4-AP (1 mM)-evoked glutamate release was inhibited by SKF10047 (100 μM) to approximately the same extent in both the absence and presence of DL-TBOA (10 μM). DL-TBOA by itself almost doubled the 4-AP (1 mM)-evoked glutamate release (P < 0.001; n = 5). In contrast to DL-TBOA, bafilomycin A1 (0.1 μM) reduced 4-AP (1 mM)-evoked glutamate release (P < 0.001) and completely prevented the inhibitory effect of SKF10047 (100 μM) on 4-AP-evoked glutamate release (n = 5; Fig. 1D). All of these results suggest that the SKF10047-mediated inhibition of 4-AP-evoked glutamate release is mediated by a reduction in the Ca²⁺-independent exocytotic component of glutamate release.

Inhibition by SKF10047 Is Mediated by α₁ Receptors. To determine whether the observed effect of SKF10047 on glutamate release is mediated by α₁ receptors, we first checked the expression of the α₁ receptors in purified cerebrocortical synaptosomes. The result showed that the α₁ receptors were detected as a 25-kDa band in Western blotting (Fig. 2A). Next, we examined the effect of SKF10047 on glutamate release in the presence of the α₁ receptor antagonist BD1047. Figure 2B shows that application of BD1047 (100 μM) alone had no effect on 4-AP-evoked glutamate release (P > 0.05; n = 6). The 4-AP-evoked glutamate release was reduced to 59.7 ± 2.5% of control by 100 μM SKF10047 alone (Fig. 2B). However, as shown (Fig. 2B), pretreatment with BD1047 completely abolished the effect of SKF10047 (96 ± 4.2% of control; n = 6). Thus, these results support first the presence of α₁ receptor on cerebrocortical nerve terminals, with these receptors serving to limit the release of glutamate. Second, these experiments favor the activation of α₁ receptors in the SKF10047-mediated inhibition of glutamate release.

In addition, some action of α₁ receptor is proposed to be associated with pertussis toxin-sensitive G proteins (G₁ or G₂) (Soriano et al., 1999; Cobos et al., 2008). To determine whether the inhibitory G₁/G₂ proteins were involved in the coupling of presynaptic α₁ receptors to the metabolic effects, culminating in the inhibition of evoked release of glutamate, synaptosomes were incubated for 4 h in the absence or presence of PTX (2 μg/ml). PTX, by ADP ribosylation of the α₁ subunit, prevents inhibitory G-protein activation. This interferes with a subunit signaling and blocks the association of G₁/α₁ subunits with their upstream G protein-coupled receptors (Brown and Siha, 2008). Figure 2B shows that PTX treatment of synaptosomes did not significantly alter 4-AP (1 mM)-evoked glutamate release (7.4 ± 0.3 nmol/mg; P > 0.05; n = 5). In the PTX-treated synaptosomes, SKF10047 (100 μM) induced 4-AP-evoked glutamate release to 4.1 ± 0.2 nmol/mg, which was similar with the inhibition produced by SKF10047 alone (4.3 ± 0.1 nmol/mg; n = 5; Fig. 2B). In parallel experiments, PTX prevented inhibition of the release by the GABA_A receptor agonist 50 μM baclofen (58%, n = 5, and 4%, n = 3, of release inhibition, in the absence and presence of PTX, respectively; Fig. 2B). Thus, these results suggest that PTX-sensitive inhibitory G proteins are not involved in the presynaptic mechanism of SKF10047.
Effect of SKF10047 on the Synaptosomal Membrane Potential and Depolarization-Induced Ca^{2+} Influx. The mechanism by which \( \alpha \)-1 receptor activation transduces the inhibition of glutamate release was addressed by assessing the effects of SKF10047 on synaptosomal plasma membrane potential and cytosolic Ca^{2+} levels under resting conditions and on depolarization. Synaptosomal plasma membrane potential was determined with the membrane potential-sensitive dye DiSC3(5). 4-AP (1 mM) caused an increase in DiSC3(5) fluorescence of 10.01 ± 0.22 fluorescence units/5 min. Preincubation of synaptosomes with SKF10047 (100 \( \mu \)M) for 10 min before 4-AP addition did not alter the resting plasma membrane potential and produced no significant change in the 4-AP-mediated increase in DiSC3(5) fluorescence (10.13 ± 0.37 fluorescence units/5 min; \( P > 0.05; n = 6; \) Fig. 3). This indicates that the observed inhibition of evoked glutamate release by SKF10047 is unlikely to have been caused by a hyperpolarizing effect of the drug on the synaptosomal plasma membrane potential or attenuation of the depolarization produced by 4-AP. Confirmation that the SKF10047 effect did not impinge on synaptosomal excitability was obtained with experiments using high external [K\(^+\)]-mediated 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 (Barrie et al., 1991). Addition of 15 mM KCl evoked controlled glutamate release of 9.5 ± 0.2 nmol/mg/5 min, which was reduced to 6.1 ± 0.3 nmol/mg/5 min in the presence of 100 μM SKF10047 ($P < 0.001$; $n = 6$; Fig. 3, inset).

Downstream of membrane depolarization presynaptic inhibition of neurotransmitter release can be mediated by a reduction of Ca$^{2+}$ influx into nerve terminals. To investigate
this possibility, we used the Ca\(^{2+}\) indicator Fura-2 to assess the effect of SKF10047 on the 4-AP-and KCl-induced increase in [Ca\(^{2+}\)]\(_{c}\). As shown in Fig. 4A, after the addition of 4-AP (1 mM), [Ca\(^{2+}\)]\(_{c}\) in synaptosomes was elevated to a plateau level of 234.1 ± 7.3 nM. This 4-AP-evoked rise in [Ca\(^{2+}\)]\(_{c}\) was decreased by 44.8 nM with 100 μM SKF10047 (189.3 ± 4.2 nM; \(n = 6\); \(P < 0.001\)). A similar result was obtained with experiments using high external [K\(^{+}\)]-mediated depolarization. Figure 4B shows that KCl (15 mM) depolarization caused a rise in [Ca\(^{2+}\)]\(_{c}\) to a plateau level of 253.7 ± 2.3 nM. This KCl-evoked rise in [Ca\(^{2+}\)]\(_{c}\) was decreased by 46.8 nM with 100 μM SKF10047 (206.9 ± 3.2 nM) (\(n = 6\); \(P < 0.01\)). It is noteworthy that the reduction of 4-AP and KCl-evoked increase of [Ca\(^{2+}\)]\(_{c}\) by SKF10047 were suppressed by 100 μM BD1047 (\(n = 5\); Fig. 4C). These data indicate that SKF10047 reduces depolarization-evoked changes in [Ca\(^{2+}\)]\(_{c}\) with a pharmacological profile similar to that observed for the reduction of release (Fig. 2B).

**A Reduction of Calcium Influx through Ca\(_{2,2}\) (N-Type) and Ca\(_{2,1}\) (P/Q-Type) Channels Is Involved in the Action of SKF10047 on Glutamate Release.** In the preparation of cerebrocortical nerve terminal from adult rats, the release of glutamate evoked by depolarization is reported to be caused by Ca\(^{2+}\) influx through Ca\(_{2,2}\) (N-type) and Ca\(_{2,1}\) (P/Q-type) channels and Ca\(^{2+}\) release from intracellular stores (Berridge, 1998; Millán and Sanchez-Prieto, 2002). For this reason, we examined which part of Ca\(^{2+}\) source was involved in the effect of SKF10047 on 4-AP-evoked glutamate release. ω-CgTX MVIIIC, a wide-spectrum blocker of Ca\(_{2,2}\) and Ca\(_{2,1}\) channels, was used to assess the role of Ca\(_{2,2}\) and Ca\(_{2,1}\) channels. In Fig. 5A, 4-AP (1 mM)-evoked glutamate release was significantly reduced in

![Fig. 4](https://example.com/fig4.png)

**Fig. 4.** A and B, SKF10047 attenuates 4-AP-induced (A) and KCl-induced (B) cytosolic Ca\(^{2+}\) levels. [Ca\(^{2+}\)]\(_{c}\) was monitored by using Fura-2. Synaptosomes were stimulated with 1 mM 4-AP or 15 mM KCl in the absence or presence of 100 μM SKF10047, 100 μM BD1047, or 100 μM BD1047 + 100 μM SKF10047 added 10 min before stimulation. C, results are the mean ± S.E.M. values of independent experiments, using synaptosomal preparations from five to six animals. \(***\), \(P < 0.001\), two-tailed Student’s \(t\) test.
the presence of 2 μM ω-CgTX MVIIC (P < 0.001). When ω-CgTX MVIIC (2 μM) and SKF10047 (100 μM) were applied simultaneously, the inhibition of glutamate release after 4-AP-depolarization was not significantly different from the effect of ω-CgTX MVIIC alone (P > 0.05; n = 6). In addition to VDCCs, Ca\(^{2+}\) influx induced by depolarization had been found to occur in part through plasmalemmal Na\(^+\)/Ca\(^{2+}\) exchanger working in its reverse mode (Blaustein and Lederer, 1999). Therefore, we used the plasmalemmal Na\(^+\)/Ca\(^{2+}\) exchange inhibitor KB-R7943 to examine the effect of SKF10047. Figure 5B shows 4-AP-evoked glutamate release was slightly, although significantly, reduced by KB-R7943 (10 μM) (P < 0.01). In the presence of KB-R7943, application of SKF10047 (100 μM) still effectively reduced 4-AP-evoked glutamate release (P < 0.05; n = 5).

On the other hand, a potential role of intracellular Ca\(^{2+}\) release in SKF10047-mediated inhibition of glutamate release was tested in the presence of dantrolene, an inhibitor of intracellular Ca\(^{2+}\) release from endoplasmic reticulum, and 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP37157), a membrane-permeant blocker of mitochondrial Na\(^+\)/Ca\(^{2+}\) exchange. Dantrolene (100 μM) reduced control 4-AP-evoked release (P < 0.01; n = 5). In the presence of dantrolene, however, SKF10047 (100 μM) still effectively inhibited 4-AP-evoked glutamate release (P < 0.05; Fig. 5B). Similarly to dantrolene, CGP37157 (100 μM) decreased the release of glutamate evoked by 4-AP (1 mM) (P < 0.01), but it had no effect on the SKF10047-mediated inhibition of 4-AP-evoked glutamate release (n = 5; Fig. 5B).

**Involvement of PKC in the SKF10047-Mediated Inhibition of Glutamate Release.** In view of the demonstrated role of PKC in presynaptic modulation (Coffey et al., 1993; Vaughan et al., 1998; Wang and Sihra, 2004), we examined the effect of inhibition of PKC on the SKF10047-mediated inhibition of glutamate release. In Fig. 6A, the PKC inhibitor GF109203X (10 μM) reduced the 4-AP-evoked glutamate release (P < 0.001) and blocked the ability of SKF10047 to depress glutamate release (n = 6). In the presence of GF109203X, therefore, SKF10047 (100 μM) induced a statistically insignificant inhibition of glutamate release of 3% compared with the 45% inhibition produced by SKF10047 alone. Similar results were also obtained with another conventional PKC inhibitor Go6976. Go6976 (3 μM) by itself reduced the 4-AP-evoked glutamate release (P < 0.001) and occluded the effect of SKF10047 (P > 0.05; n = 5; Fig. 6B). In contrast to the conventional PKC inhibitors, rottlerin (3 μM), a novel PKC inhibitor, failed to influence the ability of SKF10047 to inhibit 4-AP-evoked release of glutamate (n = 5; Fig. 6B). In addition, the mitogen-activated protein kinase inhibitor 2-((2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one) (PD98059) (50 μM) alone reduced the 4-AP-evoked glutamate release (P < 0.001; n = 5). However, in the presence of PD98059, the inhibitory action of SKF10047 on 4-AP-evoked release of glutamate was unaffected (P < 0.05; n = 5; Fig. 6B). This demonstrated some specificity for SKF10047 action on the PKC pathway.

To further authenticate the role of PKC in the observed inhibition of glutamate release by SKF10047, we performed Western blotting to examine the effect of SKF10047 on the phosphorylation of PKC. Figure 7 shows the results of analysis performed on extracts of synaptosomes depolarized with 4-AP in the presence of external Ca\(^{2+}\) and treated with
SKF10047. The results show that depolarization of synaptosomes with 1 mM 4-AP markedly increased the phosphorylation of PKC (118.7 ± 4.6%; P < 0.01; n = 5). When synaptosomes were pretreated with SKF10047 (100 μM) for 10 min before depolarization with 1 mM 4-AP, 4-AP-enhanced phosphorylation of PKC was markedly decreased to 97.2 ± 5.1% (P < 0.05; one-way repeated-measures ANOVA) (Fig. 7). Similar results were obtained from analysis of phosphorylation of PKCα, a PKC isozyme that is found presynaptically in the cerebral cortex (Shearman et al., 1991) (n = 5; Fig. 7).

Discussion

Using a preparation of nerve terminals from a rat cerebral cortex, this study provides the novel finding that SKF10047, a σ-1 receptor agonist, inhibits the 4-AP-evoked release of glutamate. To the best of our knowledge, this study presents the first examination of the effect of σ-1 receptor activation on endogenous glutamate release at the presynaptic level.

Inhibition of Glutamate Release by Presynaptic σ-1 Receptors. σ-1 Receptors are located primarily at postsynaptic sites. However, the low, but consistent, presynaptic immunoreactivity of the σ-1 receptor suggests its involvement in regulating neurotransmitter release (Alonso et al., 2000). In the present study, through Western blotting we confirmed the existence of σ-1 receptors in cerebrocortical nerve terminals. Furthermore, the selective σ-1 receptor antagonist BD1047 completely blocked the inhibitory effect of SKF10047 on 4-AP-evoked glutamate release. These results prove that SKF10047 acts at σ-1 receptors present on cerebrocortical nerve terminals, reducing the evoked glutamate release.

Previous studies have suggested that σ-1 receptor responses are coupled with PTX-sensitive G proteins (Soriana et al., 1999; Cobos et al., 2008). However, other studies have proposed the G protein-independent transduction by σ-1 receptors (Wilke et al., 1999; Zhang and Cuevas, 2005). The data in this study show that PTX treatment failed to abrogate the inhibitory effect of SKF10047 on 4-AP-evoked glutamate release. It is unlikely that the lack of effect with PTX is caused by an incomplete inactivation of the G protein by the toxin, because the highest concentration of PTX (2 μg/ml) used and length of incubation (4 h) with synaptosomes was twice that previously used to obtain inhibition of G-coupled presynaptic receptor responses in rat brain synaptosomes (Herrero et al., 1992). Furthermore, PTX prevented the inhibition of glutamate release by the GABAB receptor agonist baclofen, indicating the inactivation of the G protein by the toxin. Thus, these data suggest that the inhibition of glutamate release by presynaptic σ-1 receptor activation does not involve a PTX-sensitive G protein.

Mechanism of SKF10047-Induced Inhibition of Glutamate Release. Neurotransmitter release can be modulated at several putative sites in the nerve terminal, including Na\(^+\) channels, K\(^+\) channels, Ca\(^{2+}\) channels, and the release process itself (Wu and Saggau, 1997; Nicholls, 1998). Therefore, when addressing the mechanism responsible for the SKF10047-mediated inhibition of glutamate release, this study considers 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 for several reasons. First, 4-AP- versus
KCl-evoked glutamate release is significantly inhibited by SKF10047. 4-AP-mediated depolarization involves upstream K\(^+\) and Na\(^+\)-channel activity, 15 mM external KCl affects experimental clamping of the K\(^+\) electrochemical potential, and hence, the membrane potential (Nicholls, 1998). The latter type of depolarization bypasses regulation at the level of K\(^+\) channels and also effectively removes voltage-dependent Na\(^+\) involvement, in that these channels are desensitized in response to strong and continued depolarization (Barrie et al., 1991). Based on the mechanistic differences between the 4-AP- and KCl-mediated depolarization discussed above, it is unlikely that Na\(^+\) channels modulate glutamate release by SKF10047. Second, no significant effect of SKF10047 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, SKF10047 does not affect the 4-AP-evoked Ca\(^{2+}\)-independent glutamate release, which depends only on the membrane potential (Nicholls et al., 1987). This indicates that SKF10047 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 abolishes the inhibitory effect of SKF10047 on 4-AP-evoked glutamate release, supporting this suggestion. First, these results suggest that the \(\sigma\)-1 receptor-mediated decrease in evoked glutamate release is not because of a reduction in synaptosomal excitability caused by the modulation of Na\(^+\) or K\(^+\) ion channels. This finding disagrees with previous electrophysiological studies, which have shown that \(\sigma\)-1 receptor activation inhibits Na\(^+\) and K\(^+\) currents in several experimental preparations (Soriano et al., 1999; Wilke et al., 1999; Zhang and Cuevas, 2005; Zhang et al., 2009). The reason for this discrepancy between the current and previous in vitro studies is unclear, but may be related to the different experimental models applied. Second, these data confirm the \(\sigma\)-1 receptor-mediated modulation of the exocytotic pool of release, rather than an effect on the glutamate transporter responsible for cytosolic efflux.

Using the Ca\(^{2+}\) indicator Fura-2, this study demonstrates that SKF10047 reduces the 4-AP-evoked increase in [Ca\(^{2+}\)]\(_{c}\). 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 (Berridge, 1998; Millán and Sanz-Medel, 2002). This study shows that the inhibition of glutamate release by SKF10047 is highly sensitive to the suppression of Ca\(_{2.2}\) and Ca\(_{2.1}\) channels, suggesting that Ca\(_{2.2}\) and Ca\(_{2.1}\) channels are involved in the action of SKF10047. In addition to VDCCs, extracellular Ca\(^{2+}\) entry through the plasmalemmal Na\(^+\)/Ca\(^{2+}\) exchanger in reverse mode may be responsible for [Ca\(^{2+}\)]\(_{c}\) increase under depolarization (Blaustein and Lederer, 1999). This possibility was rejected by the observation that KB-R7943, a plasmalemmal Na\(^+\)/Ca\(^{2+}\) exchange inhibitor, fails to influence the ability of SKF10047 to inhibit 4-AP-evoked glutamate release. Conversely, the reduced release of stored Ca\(^{2+}\) from the endoplasmic reticulum ryanodine receptors and mitochondria during the \(\sigma\)-1 receptor-mediated inhibition of glutamate release can be excluded. This is because the inhibitory effect of SKF10047 on 4-AP-evoked glutamate release is insensitive to the endoplasmic reticulum ryanodine receptor inhibitor dantrolene and the mitochondrial Na\(^+\)/Ca\(^{2+}\) exchange inhibitor CUP37157. Although there is no direct evidence that SKF10047 acts on presynaptic Ca\(^{2+}\) channels, these results imply that the inhibition of glutamate release by \(\sigma\)-1 receptor activation occurs primarily through the suppression of Ca\(^{2+}\) influx through Ca\(_{2.2}\) and Ca\(_{2.1}\) channels. This suggestion is consistent with a previous electrophysiological research demonstrating that \(\sigma\)-1 receptors inhibit high-voltage-activated Ca\(^{2+}\) channels in rat sympathetic and parasympathetic neurons (Zhang and Cuevas, 2002). However, it remains unclear how \(\sigma\)-1 receptors modulate Ca\(_{2.2}\) and Ca\(_{2.1}\) channels. Previous research shows that \(\sigma\)-1 receptors directly interact with VDCCs (Tchere et al., 2008). Thus, a direct interaction between \(\sigma\)-1 receptors and presynaptic Ca\(_{2.2}\) and Ca\(_{2.1}\) channels should be considered when determining the possible mechanism of SKF10047-mediated presynaptic inhibition.

PKC is an important intracellular signaling system at the presynaptic level and plays a crucial role in neurotransmitter exocytosis (Vaughan et al., 1998; Wang and Sihra, 2004; Yang and Wang, 2009). Depolarization-stimulated Ca\(^{2+}\) entry enhances PKC-dependent phosphorylation and gluta-
Inhibition of Glutamate Release by α-1 Receptor Activation

541

mate release (Coffey et al., 1994). Thus, the inhibitory effect of SKF10047 on Ca2+ entry observed here may decrease PKC activity and, in turn, glutamate release. The following results support this hypothesis: 1) the PKC inhibitor GF109203X significantly prevented the inhibitory effect of SKF10047 on 4-AP-evoked glutamate release; 2) Go6976, an inhibitor of conventional calcium-dependent PKCs (α, β, I, γ, and δ), occluded the inhibitory effect of SKF10047 on 4-AP-evoked glutamate release; 3) rotterlin, an inhibitor of novel calcium-independent PKCs (δ, ε, η, and θ), had no effect; and 4) SKF10047 significantly decreased 4-AP-induced phosphorylation of PKC and PKCa. These data suggest that part of the SKF10047-mediated inhibition of glutamate release may be attributed to the suppression of Ca2+-dependent PKC subtypes. In fact, PKC is capable of altering the phosphorylation state of a number of proteins involved in synaptic vesicle trafficking, recruitment, and exocytosis, including SNAP-25, synapsin I, and munc18-1. The phosphorylation of these synaptic proteins 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 (Jarvis and Zamponi, 2001; Craig et al., 2003). The present study does not exclude the possibility that α-1 receptor activation inhibits glutamate release by decreasing PKC-dependent phosphorylation of synaptic proteins and the availability of synaptic vesicles.

In the current study, SKF10047 inhibited glutamate release in cerebrocortical synaptosomes at a relatively high concentration of 100 μM. Several studies have reported that 100 to 300 μM SKF10047 inhibits K+ currents in different experimental preparations (Wilke et al., 1999; Zhang and Cuevas, 2005). The present study is consistent with these reports, but reveals the neural protective effects of SKF10047 at a lower concentration range (i.e., 1–10 μM) (Tchreda and Yorio, 2008). The discrepancy is not clear, but may be caused by different experimental models used. Tchreda and Yorio used a cell culture model, whereas we used a nerve terminal (synaptosomal) model.

In conclusion, the results of this study demonstrate that α-1 receptor activation inhibits glutamate release from rat cerebrocortical synaptosomes by suppressing presynaptic Ca2+ and Ca2+1 channels and PKC activity. These findings support the hypothesis that the α-1 receptor is a potential source for brain therapeutics against glutamate-induced neurotoxicity. This is because excessive glutamate release probably is involved in the pathophysiology of several neurological states, including ischemic brain damage and neurodegenerative diseases (Meldrum, 2000). Although the relevance of these findings to in vivo clinical situations remains to be determined, this study may provide a better understanding of the mode of α-1 receptor agonist action in the brain.

Acknowledgments
We thank Sin-Yi Tsai and Yu-Wan Lin for excellent experimental assistance.

Authorship Contributions
 Participated in research design: Lu and S.-J. Wang.
Wrote or contributed to the writing of the manuscript: Lu and S.-J. Wang.

References
DeCoste MA, Klett KL, Kettler ES, and Tortella FC (1995) Sigma receptor-mediated neuroprotection against glutamate toxicity in primary rat neuronal cultures. Brain Res 671:45–53.
Schinder AF, Olson RC, Spitzer NC, and Montal M (1996) Mitochondrial dysfunction is a primary event in glutamate neurotoxicity. J Neurosci 16:1215–1233.


Address correspondence to: Su-Jane Wang, Graduate Institute of Basic Medicine, Fu Jen Catholic University, 510, Chung-Cheng Road, Hsin-Chuang, New Taipei, Taiwan 24205. E-mail: med0003@mail.fju.edu.tw