Neurochemistry International 54 (2009) 526–534

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Inhibitory effect of glutamate release from rat cerebrocortical synaptosomes by dextromethorphan and its metabolite 3-hydroxymorphinan

Tzu-Yu Lin [a], Cheng-Wei Lu [a], Su-Jane Wang [b,*]

[a] Department of Anesthesiology, Far Eastern Memorial Hospital, 21, Section 2, Nan-Ya South Road, Pan-Chiao, Taipei 220, Taiwan
[b] School of Medicine, Fu Jen Catholic University, 510, Chung-Cheng Rd., Hsin-Chuang, Taipei Hsien 24205, Taiwan

ARTICLE INFO

Article history:
Received 18 February 2009
Accepted 19 February 2009
Available online 4 March 2009

Keywords:
Dextromethorphan
Voltage-dependent Ca^{2+} channel
Protein kinase C
Glutamate exocytosis
Cerebrocortical synaptosomes

ABSTRACT

Dextromethorphan (DM), a widely used antitussive, has demonstrated an effective neuroprotective effect. Excessive release of glutamate is considered to be an underlying cause of neuronal damage in several neurological diseases. In the present study, we investigated whether DM or its metabolite 3-hydroxymorphinan (3-HM) could affect glutamate release in rat cerebral cortex nerve terminals (synaptosomes). DM or 3-HM inhibited the Ca^{2+}-dependent release of glutamate that was evoked by exposing synaptosomes to the K^+ channel blocker 4-aminopyridine (4-AP), and this presynaptic inhibition was concentration-dependent. Inhibition of glutamate release by DM or 3-HM was resulted from a reduction of vesicular exocytosis, because the vesicular transporter inhibitor bafilomycin A1 completely blocked DM or 3-HM-mediated inhibition of 4-AP-evoked glutamate release. DM or 3-HM did not alter the resting synaptosomal membrane potential or 4-AP-mediated depolarization, but significantly reduced depolarization-induced increase in $[Ca^{2+}]_C$. DM or 3-HM-mediated inhibition of 4-AP-evoked glutamate release was blocked by ω-conotoxin MVIIC, an antagonist of N- and P/Q-type Ca^{2+} channel, not by dantrolene, an intracellular Ca^{2+} release inhibitor. DM or 3-HM modulation of 4-AP-evoked glutamate release appeared to involve a protein kinase C (PKC) signaling cascade, insofar as pretreatment of synaptosomes with the PKC inhibitors GF109203X or Ro318220 all effectively occluded the inhibitory effect of DM or 3-HM. Furthermore, 4-AP-induced phosphorylation of PKC was reduced by DM or 3-HM. These results suggest that DM or 3-HM inhibits glutamate release from rat cortical synaptosomes through the suppression of presynaptic voltage-dependent Ca^{2+} entry and PKC activity. This may explain the neuroprotective effects of DM against neurotoxicity.

1. Introduction

Dextromethorphan (DM), a non-opioid morphinan derivate, is one of the most widely used antitussive. Many *in vitro* and *in vivo* experimental models of central nervous system (CNS) injury have demonstrated that DM has significant neuroprotective properties. It has been shown, for example, that DM attenuates glutamate or N-methyl-D-aspartate (NMDA)-induced neurotoxicity (Choi, 1987; Choi et al., 1987; Keller et al., 2008), protects against ischemia- and oxidative stress-induced brain damage (George et al., 1988; Bokesch et al., 1994; Liu et al., 2003) as well as reduces the volume of cerebral infarction and improves functional recovery following injury (Lo and Steinberg, 1991). The mechanisms underlying the neuroprotective effects of DM are, however, not fully clarified, although it has been reported that this beneficial effect is associated with NMDA receptor and voltage-dependent Ca^{2+} channel antagonism, or scavenging oxygen-free radicals (Trotella et al., 1999; Liu et al., 2003; Werling et al., 2007; Shin et al., 2008).

Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS) (Greenamyre and Porter, 1994; Danbolt, 2001). It has been shown that excessive release of glutamate is an underlying cause of neuronal damage in acute disorders such as stroke, epileptic seizures, traumatic brain and spinal cord injury, as well as in chronic, neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson disease (PD), and amyotrophic lateral sclerosis (ALS) (Obrenovitch and Urenjak, 1997; Meldrum, 2000; Raiteri et al., 2004; Hazell, 2007). This type of over-excitation-induced neuronal damage is accompanied by calcium influx and the generation of reactive oxygen species (ROS), which in turn result in damage of intracellular membranes and subsequently delayed cell death (Coyle and Puttfarcken, 1993; Tretter and Adam-Vizi, 2002; Malarkey and Parpura, 2008). Thus, inhibition of glutamate release from nerve terminals may have important consequences and may be a potentially mechanism for neuroprotective actions.

* Corresponding author. Tel.: +886 2 29053465; fax: +886 2 29052096.
E-mail address: med0003@mail.fju.edu.tw (S.-J. Wang).

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doi:10.1016/j.neuint.2009.02.012

Considering the proved neuroprotective properties of DM, the aim of this study was to investigate whether DM and its metabolite 3-hydroxymorphinan (3-HM) could influence the amount of released glutamate at the presynaptic level. For this we used isolated nerve terminals (synaptosomes) prepared from the rat cerebral cortex to examine the effect of DM and 3-HM on the release of glutamate and to study the possible mechanism. The synaptosome preparation is a system particularly suited to investigate presynaptic events given that the preparation is devoid of functional glial and nerve cell body elements that might obfuscate interpretation because of modulatory loci at non-neuronal, postsynaptic, or network levels (Nicholls, 1993). By using this model system, we find that both DM and 3-HM inhibits glutamate release from cerebrocortical nerve terminals by suppression of Ca^{2+} influx and PKC activity.

2. Experimental procedures

All animal procedures were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, as approved by the Fu Jen Institutional Animal Care and Utilization Committee.

2.1. Materials

3′,3′,3′-Dipropylthiadicarbocyanine iodide ($DiSC_3(5)$) and Fura-2-acetoxymethyl ester (Fura-2-AM) were obtained from Molecular Probes (Eugene, OR). Bafilomycin A1, ω-conotoxin MVIIC, dantrolene, and bisindolylmaleimide I (GF109203X) were obtained from Tocris Cookson (Bristol, UK). Dextromethorphan (DM), 3-hydroxymorphinan (3-HM), BAPTA-AM, sodium dodecyl sulfate (SDS) and all other reagents were obtained from Sigma (Poole, Dorset, UK).

2.2. Synaptosomal preparation

Synaptosomes were prepared as described previously (Wang et al., 2008; Chang and Wang, 2009). Briefly, the cerebral cortex from 2-month-old male Sprague–Dawley rats was isolated and homogenized in a medium containing 320 mM sucrose, pH 7.4. The homogenate was spun for 2 min at 3000 × *g* (5000 rpm in a JA 25.5 rotor from Beckman) at 4 °C, and the supernatant was spun again at 14,500 × *g* (11,000 rpm in a JA 25.5 rotor from Beckman) 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 placed into 3 ml Percoll discontinuous gradients containing 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 32,500 × *g* (16,500 rpm in a JA 20.5 rotor from Beckman) for 7 min at 4 °C. Synaptosomes placed between the 10% and the 23% percoll bands were collected and diluted in a final volume of 30 ml of HEPES buffer medium (HBM) consisting 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, 10 mM HEPES (pH 7.4) before centrifugation at 27,000 × *g* (15,000 rpm in a JA 25.5 rotor from Beckman) for 10 min. The pellets thus formed were resuspended in 3 ml of HBM, and the protein content was determined using Bradford Protein Assay Kit (Bio-Rad, Hercules, CA) based on the method of Bradford (1976) with bovine serum albumin as a standard. 0.5 mg of synaptosomal suspension was diluted in 10 ml of HBM and spun at 3000 × *g* (5000 rpm in a JA 20.1 rotor from Beckman) for 10 min. The supernatants were discarded, and the synaptosomal pellets were stored on ice and used within 4–6 h.

2.3. Glutamate release assay

Glutamate release from purified cerebrocortical synaptosomes was monitored online, with an assay employing exogenous glutamate dehydrogenase and $NADP^+$ to couple the oxidative deamination of the released glutamate to the generation of NADPH detected fluorometrically (Nicholls, 1993). Synaptosomal pellets were resuspended in HBM containing 16 μM bovine serum albumin (BSA) and incubated in a stirred and thermostated cuvette maintained at 37 °C in a PerkinElmer LS-50B spectrofluorimeter. $NADP^+$ (2 mM), glutamate dehydrogenase (GDH; 50 units/ml) and $CaCl_2$ (1 mM) were added after 3 min. In experiments examining Ca^{2+}-independent efflux of glutamate, EGTA (200 μM) was added in place of $CaCl_2$. Other additions before depolarization were made as detailed in the figure legends. 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 nm and 460 nm, respectively) due to NADPH being produced by the 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 glutamate per milligram synaptosomal protein (nmol/mg). Values quoted in the text and expressed in bar graphs represent levels of glutamate cumulatively release after 5 min of depolarization.

2.4. Styryl dye release assay

Synaptic vesicle fusion with the plasma membrane was measured using release of the fluorescent dye FM1-43 as described previously (Baldwin et al., 2003). In brief, synaptosomes (0.5 mg/ml) were incubated in HEPES buffer medium with 1.2 mM $CaCl_2$ for 2 min at 37 °C in a stirred test tube. FM1-43 (100 μM) was added 1 min before stimulation with 30 mM KCl. After 3 min of stimulation to load FM1-43, synaptosomes were washed twice in HEPES buffer medium containing 1.2 mM $CaCl_2$ and 1 mg/ml bovine serum albumin to remove non-internalized FM1-43. Synaptosomes were then resuspended in 2 ml of HEPES buffer medium (plus 1.2 mM Ca^{2+}) and incubated in a stirred and thermostated cuvette maintained at 37 °C in a PerkinElmer LS-50B spectrofluorimeter. Release of accumulated FM1-43 was induced by the addition of 1 mM 4-AP and measured as the decrease in fluorescence upon release of the dye into solution (excitation 488 nm, emission 540 nm). Data points were obtained at 2.2 s intervals, and data presented as the Ca^{2+}-dependent decrease in FM1-43 fluorescence. Any drugs were added after the dye-loading procedure, and the synaptosomes were preincubation with DM or 3-HM for 10 min before depolarization with 4-AP.

2.5. Determination of synaptosomal plasma membrane potential

The synaptosomal membrane potential can be monitored by positively charged membrane potential-sensitive carbocyanine dyes such as $DiSC_3(5)$. The dye becomes incorporated into the synaptosomal plasma membrane lipid bilayer. Upon depolarization with 1 mM 4-AP, the release of the dye from the membrane bilayer is indicated as an increase in fluorescence. Synaptosomes were pre-incubated and resuspended as described for the glutamate release experiments. After 3 min incubation, 5 μM $DiSC_3(5)$ was added and allowed to equilibrate before the addition of $CaCl_2$ (1 mM) after 4 min incubation. 4-AP was added to depolarize the synaptosomes at 10 min, and $DiSC_3(5)$ fluorescence was monitored at excitation and emission wavelengths of 646 nm and 674 nm, respectively. Results are expressed in fluorescence units.

2.6. Determination of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_C$)

$[Ca^{2+}]_C$ was measured using the Ca^{2+} indicator Fura-2. Synaptosomes (0.5 mg/ml) were preincubated in HBM with 16 μM BSA in the presence of 5 μM Fura-2 and 0.1 mM $CaCl_2$ for 30 min at 37 °C in a stirred test tube. After Fura-2 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 stirred in a thermostatted cuvette in a PerkinElmer LS-50B spectrofluorimeter. $CaCl_2$ (1 mM) was added after 3 min and further additions were made after an additional 10 min, as described in the legends to the figures. Fluorescence data were accumulated at excitation wavelengths of 340 nm and 380 nm (emission wavelength 505 nm) at data accumulated at 7.5 s intervals. Calibration procedures were performed as described by previously (Sihra et al., 1992) using 0.1% sodium dodecyl sulphate to obtain the maximal fluorescence with Fura-2 saturation with Ca^{2+}, followed by the addition of 10 mM EGTA (Tris buffered) to obtain minimum fluorescence in the absence of any Fura-2/Ca^{2+} complex. Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_C$, nM) was calculated using equations described previously (Grynkiewicz et al., 1985).

2.7. Western blotting analysis

Synaptosomes (0.5 mg protein/ml) from control and drug–treated were lysed in ice-cold Tris–HCl buffer solution, pH 7.5, containing 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 (PMSF), 1 mM sodium orthovanadate and 1 (g/ml leupeptin. The lysates were sonicated for 10-s and then centrifuged at 14,000 × *g* at 4 °C for 10 min. Equal amounts of protein (20 (g) were separated by electrophoresis on 7.5% SDS-polyacrylamide gels (SDS-PAGE), and then transferred to nitrocellulose membranes. The membranes were blocked overnight at 4 °C with Tris-buffered saline (TBS) containing 5% low fat milk and then blotted for 2 h at room temperature with polyclonal anti-phospho-PKC (pan) antibody (to detect PKC-α, -β_I, -β_{II}, -ζ, -ε, -η, and -δ, phosphorylated at a carboxy-terminal residue homologous to Ser-660 of PKC-β_{II}) (1:1000; NOVUS Biologials, Inc., Beverly, MA, USA). After several washes with 0.1% Tween 20/Tris-buffered saline (T-TBS), the membranes were incubated with donkey antirabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc., Baltimore Pike, USA) diluted 1:1000 with TBS 5% low fat milk for 2 h at room temperature. They were then washed at least 3 times with T-TBS and visualized using the ECL chemiluminescence system (Amersham, Buckinghamshire, UK).

2.8. Statistical analysis

Cumulative data were analyzed in Lotus 1-2-3 and MicroCal Origin. 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 DM or 3-HM), a one-way repeated-measures analysis of variance (ANOVA) was computed. $P < 0.05$ was considered to represent a significant difference.