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# ACTIVATION OF NEUROPEPTIDE Y Y1 RECEPTORS INHIBITS GLUTAMATE RELEASE THROUGH REDUCTION OF VOLTAGE-DEPENDENT $Ca^{2+}$ ENTRY IN THE RAT CEREBRAL CORTEX NERVE TERMINALS: SUPPRESSION OF THIS INHIBITORY EFFECT BY THE PROTEIN KINASE C-DEPENDENT FACILITATORY PATHWAY

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**Abstract—Neuropeptide Y (NPY) is known to regulate the presynaptic glutamate release and neuronal responses to excitatory neurotransmission. The aim of this study was to investigate the effect of NPY on the release of endogenous glutamate from rat cerebrocortical nerve terminals (synaptosomes). NPY inhibited the $Ca^{2+}$-dependent glutamate release evoked by 4-aminopyridine, and this inhibitory effect was mediated via NPY Y1 receptors, because it was mimicked by the specific NPY Y1 receptor agonist [$Leu^{31}$ $Pro^{34}$] NPY and blocked by the NPY Y1 receptor antagonist GR 231118. The inhibitory action of NPY was not due to it decreasing synaptosomal excitability or directly interfering with the release process at some point subsequent to $Ca^{2+}$ influx, because NPY did not alter the 4-aminopyridine-evoked depolarization of the synaptosomal plasma membrane potential or ionomycin and hypertonic solution-induced glutamate release. Examination of the effect of NPY on the cytosolic [$Ca^{2+}$] revealed that the inhibition of glutamate release could be attributed to a reduction in voltage-dependent $Ca^{2+}$ influx. Consistent with this, the NPY-mediated inhibition of glutamate release was completely abolished in synaptosomes pretreated with N- and P/Q-type $Ca^{2+}$ channel blocker, ω-conotoxin MVIIC. Moreover, NPY-mediated inhibition of 4-aminopyridine-evoked glutamate release was insensitive to KT 5720 and Ro32–0432 but was suppressed when protein kinase C was stimulated with phorbol ester. Together, these results suggest that NPY acting predominantly on NPY Y1 receptors inhibits glutamate release from rat cerebrocortical synaptosomes, likely by a mechanism involving direct coupling of receptors to N- and P/Q-type $Ca^{2+}$ channels, and this coupling is subject to regulation by protein kinase C-dependent pathway. This implies that selective ligand for NPY receptors may be of value for treatment of conditions characterized by excessive glutamate release in the cerebral cortex.**


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E-mail address: med0003@mails.fju.edu.tw (S.-J. Wang).
*Abbreviations:* ω-AgTX IVA, ω-agatoxin IVA; BSA, bovine serum albumin; $[Ca^{2+}]_c$, cytosolic free $Ca^{2+}$ concentration; cAMP, cyclic AMP; ω-CgTX GVIA, ω-conotoxin GVIA; ω-CgTX MVIIC, ω-conotoxin MVIIC; $DiSC_3(5)$, diethyl-thiacarbocyanine iodide; EDTA, ethylenediamine tetraacetic acid; EGTA, ethyleneglycolbis(aminoethyl ether)-tetra-acetate; fura-2-AM, fura-2 acetoxymethyl ester; GDH, glutamate dehydrogenase; HBM, HEPES buffer medium; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; KT 5720, hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo-benzodiazocine-10-carboxylic acid, hexyl ester; NPY, neuropeptide Y; PKA, protein kinase A; PKC, protein kinase C; Ro32-0432, bisindolylmaleimide XI; 4AP, 4-aminopyridine; 4β-PDBu, 4β-phorbol 12,13-dibutyrate; 4α-PDD, 4α-phorbol 12,13-didecanoate.

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Neuropeptide Y (NPY) is one of the most abundant and widely distributed peptides in the mammalian brain (Dumont et al., 1992) where it is involved in various functions including regulation of blood pressure, feeding behavior, memory processing and cognition (Wettstein et al., 1995). These actions are mediated by NPY receptors that belong to the G protein-coupled receptor superfamily. So far, five subtypes (Y1, Y2, Y4, Y5 and Y6) of NPY receptors have been identified, but NPY Y1 and NPY Y2 are the most extensively characterized (Wan and Lau, 1995; Blomqvist and Herzog, 1997). In CNS, besides the regulation of normal physiological functions, NPY may also play an important role in pathological conditions associated with excessive glutamate function such as seizure initiation and propagation (Vezzani et al., 1999). For example, a considerable increase in the expression of both NPY and NPY receptors is observed in the hippocampus and cortex after experimentally induced seizures (Bellmann et al., 1991; Gobbi et al., 1998). Furthermore, NPY knockout mice are prone to seizures or exert an increased susceptibility to pentylenetetrazol and kainic acid-induced seizures, and these effects are antagonized by intracerebral administration of NPY (Baraban et al., 1997). These findings permit a hypothesis that NPY may serve as an endogenous neuromodulator to limit seizure activity. In support of this hypothesis, data from electrophysiological studies have revealed that NPY inhibits spontaneous and stimulation-evoked epileptiform discharges in rat hippocampal and cortical slices *in vitro* (Klapstein and Colmers, 1997; Bijak, 1999, 2000). Concerning the possible mechanisms of antiepileptic effect of NPY, reduction in the release of glutamate from excitatory terminals, suppression of presynaptic voltage-dependent $Ca^{2+}$ channels activity or activation of presynaptic potassium conductances has been reported (McQuiston and Colmers, 1996; McQuiston et al., 1996; Qian et al., 1997; Sun et al., 2001; Silva et al., 2003).

Cerebral cortex plays a crucial role in higher brain functions, and cortical neurodegeneration of this brain region has been attributable to glutamate, the primary excitatory neurotransmitter in the brain (Meldrum and Garthwaite, 1990). Because an excessive release and accumulation of glutamate resulting in neurotoxic cell damage has

been implicated in the etiology of several disease states (Lipton and Rosenberg, 1994), to explore the mechanism of NPY modulation of cerebral glutamate release may be of value for understanding of the role of NPY in pathological conditions occurred in cerebral cortex such as cerebral ischemic brain damage and epilepsy. Despite the fact that NPY and its receptors are particularly well expressed in the cerebral cortex (Larsen et al., 1993; Dumont et al., 1996; Kopp et al., 2002) and that NPY inhibits glutamate release presynaptically in rat cortex slices (Bijak, 1999, 2000), nothing is presently known about their effects on the release of glutamate from nerve terminals purified from the cerebral cortex. Based on the abovementioned facts, the aim of this study was to use synaptosomes isolated from rat cerebral cortex to investigate the effect of NPY on the 4-aminopyridine (4AP)-evoked glutamate release and elucidate the mechanism underlying the NPY modulation of glutamate release by determining the effects of NPY on the synaptosomal plasma membrane potential and downstream activation of voltage-dependent $Ca^{2+}$ channels. In addition, in view of the fact that G protein-coupled receptor-mediated presynaptic inhibition could be suppressed by activation of protein kinase C (PKC) (Swartz et al., 1993), this study further examined whether presynaptic PKC pathway can influence NPY response. The results showed that, in cerebrocortical glutamatergic nerve terminals, NPY-mediated inhibition of glutamate release seems to be due to a reduction of $Ca^{2+}$ influx through N- and P/Q-type $Ca^{2+}$ channels. This mechanism is subject to modulation by PKC.

## EXPERIMENTAL PROCEDURES

### Materials

Fura-2-acetoxymethyl ester and diethyl-thiacarbocyanine iodide ($DiSC_3(5)$) were obtained from Molecular Probes (Eugene, OR, USA). Percoll was obtained from Pharmacia. NPY, NPY13-36, [$Leu^{31}$ $Pro^{34}$] NPY, GR 231118, ω-conotoxin GVIA (ω-CgTX GVIA), KT 5720 and 4β-phorbol 12,13-dibutyrate (4β-PDBu) were obtained from Tocris Cookson (Bristol, UK). Glutamate dehydrogenase and all other reagents were obtained from Sigma (Poole, UK) or Merck (Poole, UK).

### Preparation of cerebrocortical synaptosomes

Synaptosomes were purified as described previously (Sihra, 1997). 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* (50,000 r.p.m. in a JA 25.5 rotor from Beckman Coulter, Inc., USA) at 4 °C, and the supernatant was spun again at 14,500×*g* (11,000 r.p.m. 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 r.p.m. 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 r.p.m. 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 by the Bradford assay. Synaptosomal suspension (0.5 mg) was diluted in 10 ml of HBM and spun at 3000×*g* (5000 r.p.m. 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.

### Measurement of glutamate release

Glutamate release was assayed by on-line fluorimetry as described previously (Nicholls and Sihra, 1986). Synaptosomal pellets were resuspended in HBM containing 16 μM bovine serum albumin (BSA) and incubated in a stirred and thermostatted cuvette maintained at 37 °C in a Perkin-Elmer LS-50B spectrofluorimeter. Nicotinamide adenine dinucleotide phosphate$^+$ (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, 4AP (3 mM), KCl (30 mM) or ionomycin (5 μM) 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 nmol glutamate per mg synaptosomal protein (nmol/mg). Cumulative data were analyzed using Lotus 1-2-3 and MicroCal Origin. Statistical analysis was performed by two-tailed Student's *t*-test.

### Membrane potential measurement using $DiSC_3(5)$

The synaptosomal membrane potential can be monitored by positively charged membrane potential-sensitive carbocyanine dyes such as $DiSC_3(5)$ (Enkvist et al., 1988). The dye becomes incorporated into the synaptosomal plasma membrane lipid bilayer. Upon depolarization with 4AP, the release of the dye from the membrane bilayer is indicated as an increase in fluorescence. Synaptosomes were preincubated 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), NPY or 4AP as described for glutamate release experiments. $DiSC_3(5)$ fluorescence was monitored at excitation and emission wavelengths of 646 nm and 674 nm, respectively, and data accumulated at 2-s intervals. Cumulative data were analyzed using Lotus 1-2-3 and results are expressed in fluorescence units.

### Cytosolic $Ca^{2+}$ measurements using fura-2 acetoxymethyl ester (fura-2)

Cytosolic free $Ca^{2+}$ concentration ($[Ca^{2+}]_c$) was measured using the $Ca^{2+}$ indicator fura-2-AM. Synaptosomes (0.5 mg/ml) were preincubated in HBM with 16 μM BSA in the presence of 5 μM fura-2 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 r.p.m.). The synaptosomal pellets were resuspended (0.5 mg/ml) in HBM with BSA and the synaptosomal suspension stirred in a thermostatted cuvette in a Perkin-Elmer LS-50B spectrofluorometer. $CaCl_2$ (1 mM) was added after 3 min and further additions were made after an additional 5 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-s intervals. Calibration procedures were per-