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Co-expression of Cholecystokinin and Neuropeptide Y by Glutamatergic Stimulation in PC12 Cells

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Abstract : Co-expression of cholecystokinin (CCK) and neuropeptide Y (NPY) by glutamatergic stimulation was examined by double stained immunocytochemistry in the rat PC12 cells. Co-expression of CCK and NPY was inducible by glutamate and *N*-methyl-D-aspartate (NMDA), but not by kainic acid. Pretreatment of the cells with non-competitive NMDA antagonist, MK-801 (10 μM), and competitive NMDA antagonist, AP5 (10 μM), slightly suppressed the elevated levels of glutamate-induced CCK and NPY immunoreactivity, but markedly inhibited those of NMDA-induced CCK and NPY immunoreactivity. These results suggest that CCK and NPY expressions are mainly regulated through the NMDA-induced signaling pathways, and both molecules could be co-expressed *via* NMDA receptors upon glutamatergic neuroexcitation at the synaptic junction.

Key Words : Cholecystokinin, Glutamatergic stimulation, Neuropeptide Y, PC12 cell

Introduction

It has been known that many neuroactive peptides serve as neurotransmitters, thus causing either inhibition or excitation, or both, depending on the target neurons when applied. They coexist with classical neurotransmitters in the central nervous system, however, their physiological function has not yet been fully elucidated. There are nemerous reports supporting that cholecystokinin (CCK) and neuropeptide Y (NPY) act as neurotransmitters [1-3] or neuromodulators [4,5] that could regulate the release of classical neurotransmitters such as glutamate (GLU), γ-aminobutyric acid (GABA), and dopamine. CCK-octapeptide (CCK-8) is highly concentrated in the limbic system and cortex [6]. It is known that CCK plays a role in feeding, anxiety, stress, learning, memory, and psychiatric disorders including schizophrenia [7,8].

NPY is also widely distributed throughout the peripheral and central nervous systems, especially abundant in limbic structures, cerebral cortex, hypothalamus, and striatum [2,9]. NPY is known to have effects on the networks related to feeding,

circadian rhythms, memory, cerebrocortical excitation, neuroendocrine function, and central autonomic control [10,11]. Recently, NPY receptor agonists have been introduced as a possible therapeutic agent in obesity or an anticonvulsant in epilepsy [12,13]. Although both CCK and NPY cause excitation of neurons in various brain regions, they have also been shown to reduce excitatory amino acid-induced depolarization and to counteract excitotoxic damage in neurons [13-15].

In order to examine the expression of CCK and NPY under glutamatergic neuroexcitation and to explore a plausible mechanism underlying it, we investigated the changes in the immunoreactivity of CCK and NPY upon glutamatergic neuroexcitation using GLU, *N*-methyl-D-aspartate (NMDA), and kainic acid (KA) in the rat pheochromocytoma PC12 cells.

Materials & Methods

1. Cell culture

The rat PC12 cells were obtained from Seoul National University Cell Line Bank (Seoul, South Korea), and were cultured as follows: the culture medium was RPMI 1640 medium (Gibco BRL, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 ㎍/ml of streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 ℃. The cells were seeded onto autoclaved cover glasses in 6 well plates, and allowed to differentiate in the presence of nerve growth factor (NGF; 50 ng/ml) for 7-10 days. The medium and NGF were changed every 2 days. Ten days after the NGF treatment, over 90% of cells developed neurites.

2. Drug treatment

All experiments were conducted in the culture medium containing 10% FBS, and medium was replaced with fresh medium before each treatment. GLU (1, 10 ㎛M, and 1 mM for 1 h), NMDA (1, 10 ㎛M, and 1 mM for 1 h), and KA (1, 10 ㎛M, and 1 mM for 1 h) were used to induce neuroexcitation in the PC12 cells. The pretreatment of the cells with MK-801 (10 ㎛M) or AP5 (10 ㎛M) was done 30 min before the above-mentioned glutamatergic stimulation, respectively. GLU, NMDA, KA, and AP5 were obtained from Sigma Chemical Company (St. Louis, USA), MK-801 from RBI (Natick, USA), and NGF was from Promega (Madison, USA). All other reagents were of analytical grade and purchased from various commercial sources.

3. Immunocytochemistry

The PC12 cells were cultured and differentiated onto sterilized cover glasses and fixed with 4% paraformaldehyde for 30 min at room temperature. The cells were washed three times in PBS, and then placed in a 1:5 dilution of appropriate normal serum in PBS for 10 min to block nonspecific secondary antibody staining. Then, the cells were washed thoroughly in Tris-buffered saline containing 0.1% Triton X-100 (TBST buffer), and labeled with a 1:2000 dilution of primary antibodies in PBS containing 1% bovine serum albumin (PBSA). The primary antibodies used were anti-sheep NPY antiserum (Chemicon Int, USA) and anti-rabbit CCK antiserum (Sigma, USA). After an hour of incubation at room temperature, the cells were washed once for 15 min and twice for 10 min in PBS, and then incubated for 1 h in FITC-conjugated rabbit anti-sheep IgG (DAKO, USA) and TRITC-conjugated goat anti-rabbit IgG (Sigma, USA), which were diluted 1:40 in PBSA. Stained sections were washed three times in PBS, air dried, and mounted on slides in DABCO (Sigma, USA). Bound antibodies were visualized under a confocal laser scanning microscope (LSM 401, Carl Zeiss, Germany).

Results

Intense CCK immunoreactivity was observed within the perimeter of soma and in the neurites of the cells, however, NPY immunoreactivity was less prominent than that of CCK. The merged image revealed co-labeled regions in the soma and neurites (Fig. 1). GLU (1, 10 μM, & 1 mM) and NMDA (1, 10 μM, & 1 mM) treatment resulted in marked increases of CCK and NPY immunoreactivity as compared with normal (Fig. 3&4), these increases of CCK and NPY immunoreactivity were not dose-dependent. Contrary to the above, KA (1, 10 μM, & 1 mM) treatment did not induce significant changes in CCK and NPY immunoreactivity (Fig. 2). Pretreatment of the cells with non-competitive NMDA antagonist, MK-801 (10 μM) suppressed slightly the elevated level of GLU-induced CCK immunoreactivity (Fig. 3B), but markedly inhibited that of NPY immunoreactivity (Fig. 3C). As shown in Fig. 4. pretreatment of the cells with both MK-801 and AP5 markedly reversed the NMDA-induced CCK and NPY immunoreactivity (Fig. 4).

Fig. 1. Confocal laser scanning micrographs showing immunolocalization of CCK and NPY in unstimulated PC12 cells (control). Panel D represents the merged images of CCK and NPY immunoreactivity, panel C is the representative image of CCK immunolabeling, and panel N indicates the cells expressing NPY only. Images are representative of at least three independent experiments.

Discussion

In this study, using double-labeled immunocyto-

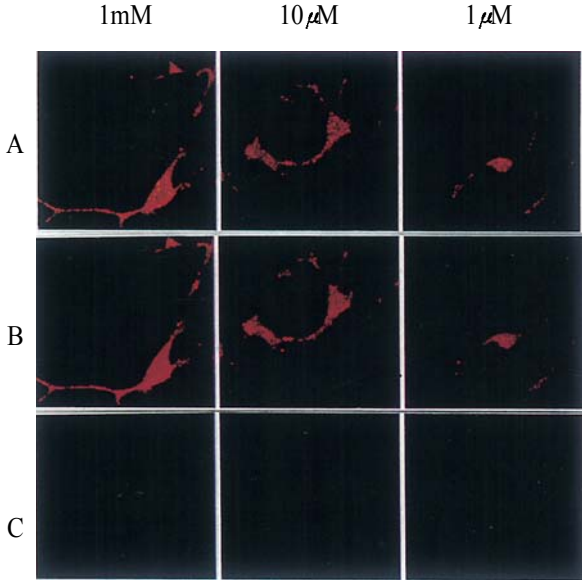

Fig. 2. Confocal laser scanning micrographs showing immunolocalization of CCK and NPY in KA-induced neuroexcitation in the PC12 cells. Shown are a representative series of confocal sections through the KA-stimulated cells at the concentrations indicated. Panel A represents the merged images of CCK and NPY immunoreactivity, panel B is the representative image of CCK immunolabeling, and panel C indicates the cells expressing NPY only. Images are representative of at least three independent experiments.

chemistry, we for the first time demonstrated physiological co-localization of the CCK and NPY in the rat PC12 cells. Furthermore, we examined whether endogenous CCK and NPY expression is regulated by glutamatergic stimulation, and observed that CCK expression was enhanced by GLU and NMDA, but not by KA. GLU-induced enhancement of CCK immunoreactivity was slightly inhibited by a competitive NMDA antagonist, AP5, however, a non-competitive NMDA antagonist, MK-801 did not alter the increased immunolabeling of CCK (Fig. 3B). On the other hand, the increase of NMDA-induced CCK immunoreactivity was counteracted by both MK-801 and AP5 (Fig. 4B). These data indicate that CCK

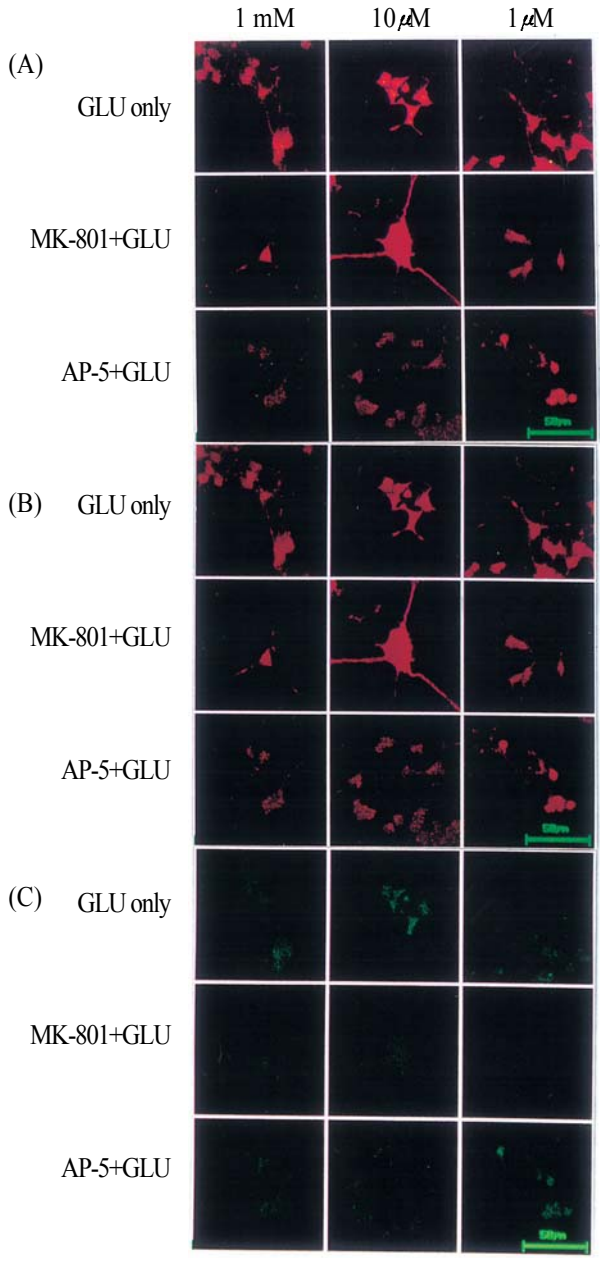

Fig. 3. Influences of MK-801 and AP5 on the CCK and NPY immunolocalization upon glutamate (GLU)-induced neuroexcitation in the PC12 cells. Shown are a representative series of confocal sections through the GLU-stimulated cells at the concentrations indicated. Panel A represents the merged images of CCK and NPY immunoreactivity, panel B is the representative image of CCK immunolabeling, and panel C indicates the cells expressing NPY only. Images are representative of at least three independent experiments.

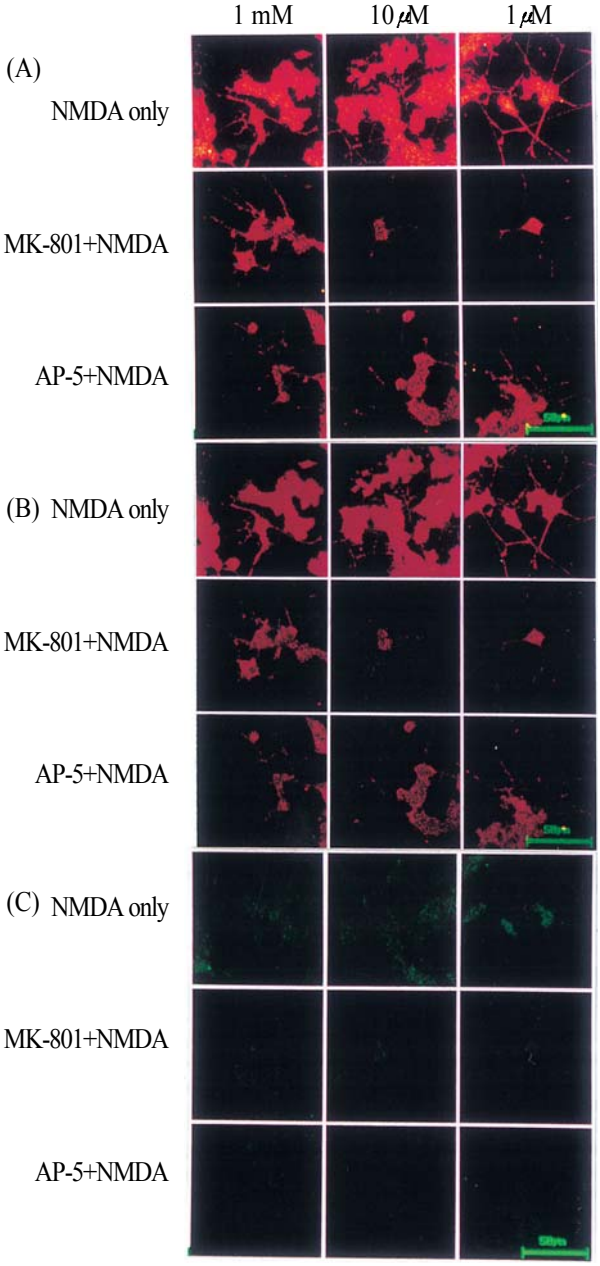

Fig. 4. Influences of MK-801 and AP5 on the CCK and NPY immunolocalization upon NMDA-induced neuroexcitation in the PC12 cells. Shown are a representative series of confocal sections through the NMDA-stimulated cells at the concentrations indicated. Panel A represents the merged images of CCK and NPY immunoreactivity, panel B is the representative image of CCK immunolabeling, and panel C indicates the cells expressing NPY only. Images are representative of at least three independent experiments.

expression can be regulated by the ionotropic NMDA receptor, but not by non-NMDA receptor, although we can not exclude the mediation *via* metabotropic glutamate receptors. This finding of NMDA modulation in CCK expression is well in line with the results of previous *in vivo* and *in vitro* studies [16-18]. Furthermore, the finding is consistent with a report stating that KA does not cause significant changes in CCK expression in contrast to NMDA *in vivo* [16]. Although we cannot offer exact mechanism underlying this NMDA receptor-mediated CCK expression, our data clearly indicate the physiological interactions between CCK and NMDA.

The fact that CCK immunoreactivity was markedly enhanced by NMDA stimulation, but not by KA, is of a great interest. Gronier and Debonnel [19] reported that CCK in dorsal hippocampus is involved in the NMDA response to σ ligands. The evidence that CCK interacts with σ ligands was first demonstrated in colonic motility of gastrointestinal tract [20]. Whereas σ receptor facilitates the glutamate neurotransmission *via* activation of NMDA receptors, the activation of KA is not altered by the σ ligands [21,22]. These findings implicate the existence of a functional association between CCK and σ receptor-mediated effects, and this may explain the expression of CCK in response to NMDA treatment, which was observed in the present study.

In recent years, many interesting results on the NPY release upon glutamatergic stimulation have accumulated, raising a potential of NPY as a new therapeutic target since NPY exerts an inhibitory influence on neuronal excitations [13,23]. In the present study, GLU and NMDA treatment enhanced NPY immunoreactivity, while KA treatment did not, and the enhancement of GLU-induced immunolabeling was effectively altered by MK-801, whereas AP5 only slightly altered the increase. On the other hand, the enhanced immunolabeling of NPY by NMDA was reversed by both MK-801 and AP-5 (Fig. 4C). Previous studies in our laboratory have shown NPY as a neuropeptide expressed in a functional relationship linking the activity of glutamatergic, dopaminergic, and GABAergic system in C6 glioma cells [24]: we proposed that the expression of NPY reflects a dynamics of the neuronal information generated by interactions between classical neurotransmitters, not by a single transmitter alone. The distribution of GLU-mediated NPY expression may differ, depending on brain regions *in vivo*. Interestingly, it has been reported that the microiontophoretic application of NPY produced a response similar to NMDA-induced neuronal firing, and that the response was mediated via σ receptors, not NPY receptors, in CA3 regions of dorsal hippocampus [25]. The σ-ligand potentiation induced by NMDA appeared to be selective, since quisqualate or KA did not have any effect. The physiological role of σ receptors is quite controversial: there is no evidence to indicate which subtype of σ receptors is located on the NMDA receptor complex or is activated by allosteric modulation of NMDA receptor. Further studies are needed to verify the mechanisms underlying the CCK and NPY expression upon glutamatergic neuroexcitation: mediation through σ ligands, regulation at transcriptional or translational level, and functional interrelationship with other classical neurotransmitters.

Taken together, we conclude that the expressions of CCK and NPY upon glutamatergic neuroexcitation are modulated by NMDA receptor and possibly by σ ligands, since the expressions of CCK and NPY are not induced by KA. Furthermore, our data clearly indicated that NPY and CCK coexist. Moreover, the expression of NPY and CCK is closely regulated by NMDA receptor activation, and these neuroactive peptides might play important roles in functional modifications of neurons, which underlie a protective mechanism against glutamate-induced neurotoxicity.

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