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Effect of Cholecystokinin on Serotonin Release from Cultured Neurons of Fetal Rat Medulla Oblongata

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ABSTRACT

Serotonergic neurons in medulla oblongata play an important role in the endogenous descending pain inhibitory system. To illucidate the factors involved in the regulation of medullary serotonergic neurons, we studied the effects of cholecystokinin (CCK) and agents acting on various second messenger systems on 5-hydroxytryptamine (5-HT) release from cultured neurons of rat fetal (gestational age 14th day) medulla oblongata. Cultured cells maintained for 10 days *in vitro* were stimulated for 48 hours with CCK or other neuropeptides at 10 micromolar concentration. CCK (10 μM) and substance P (10 μM) significantly increased 5-HT release. However, somatostatin, proctolin, thyrotropin releasing hormone, and interleukin-6 did not have any effects on 5-HT release. Nimodipine (1 μM), a calcium channel blocker, almost completely, and calmidazolium (1 μM), a calmodulin antagonist, significantly inhibited the CCK-induced 5-HT release. The total 5-HT content (intracellular 5-HT plus released 5-HT) was significantly increased by CCK. However, the intracellular 5-HT content was not significantly changed by CCK. Forskolin (5 μM), an adenylate cyclase activiator, but not 2 μM phorbol myristate acetate (PMA), a protein kinase C activator, significantly enhanced 5-HT release. The total 5-HT content (intracellular 5-HT plus released 5-HT) was significantly increased by forskolin. However, the intracellular 5-HT content was not significantly changed by forskolin. PMA had no effect on intracellular 5-HT levels. These results suggest that CCK regulates serotonergic neurons in the medulla oblongata by enhancing 5-HT secretion through calcium influx and calmodulin, and that cyclic AMP system but not protein kinase C system is involved in 5-HT release.

Key Words: Serotonin secretion, Medulla oblongata, Cholecystokinin, Culture

INTRODUCTION

Nucleus raphe magnus and other nuclei of medulla oblongata contain many 5-hydroxytryptamine (5-HT) neurons that project to dorsolateral funiculus in spinal cord. Stimulation of these 5-HT neurons is important for endogenous pain inhibition (Fields and Basbaum, 1978; Olivera *et al.*, 1979). Substance P-, thyrotropin releasing hormone (TRH)- (Dean *et al.*, 1994), somatostatin- (Taber-Pierce *et al.*, 1985), cholecystokinin (CCK)-like immunoreactivities (Kubota *et al.*, 1986), were reported to be located in neuronal cells of medulla oblongata.

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To illucidate the factors in the regulation of medullary serotonin neurons, we studied the effects of CCK and agents acting on various second messenger systems on 5-HT release from cultured neurons from rat fetal medulla oblongata. We report that CCK, through calcium influx and calmodulin, enhanced 5-HT secretion, and an evidence suggesting that cyclic AMP system but not protein kinase C system is involved in 5-HT release.

MATERIALS AND METHODS

Drugs used

Dulbecco's modified Eagle's medium (DMEM), pargyline HCl, 5-hydroxytryptamine (5-HT), somatostatin, proctolin, thyrotropin releasing hormone, and forskolin were purchased from Sigma (St. Louis, MO). Nimodipine, calmidazolium, and phorbol myristate acetate were from RBI (Natick, MA). Cholecystokinin octapeptide, and substance P were from Penninsula Labratories (Belmont, CA). Hank's balanced salt solution (HBSS) was from Gibco BRL. Interleukin-6 and fluoxetine HCl were kindly supplied by Dr. K. H. Pyun (Genetic Engineering Research Institute, KIST) and Eli Lilly & Co. (Indianapolis, IN), respectively.

Neuronal cultures from fetal rat medulla oblongata

Methods of neuronal cultures from fetal rat medulla oblongata were modified from Yamamoto *et al.* (1981). Two-mm paramedian saggital strips of medulla oblongata were obtained from Sprague-Dawley rat embryos (gestational age 14th day). After mechanical dissociation with pasteur pipette, cells suspended in Dulbecco's modified Eagle's medium (DMEM) were centrifuged for 3 min at 500-1,000 rpm. Cells resuspended with DMEM containing Defined N2 media (Bottenstein, 1983) were plated at an approximate density of 0.5×10^6 cells/well on 24-multiwell plate coated with poly-L-lysine (10 μg/ml). Cells were maintained for 10 days at 37°C in a humid 5% CO_2 incubator. Media were changed every 3 days. In the 5-HT release experiment, cultured cells at 10th day *in vitro* were rinced with Hank's balanced salt solution (HBSS), and exposed to the test reagents for 48 hours in HBSS containing pargyline (30 μM), a monoamine oxidase inhibitor, and fluoxetine (0.2 μM), a selective serotonin reuptake inhibitor, at 37°C in a humid 5% CO_2 incubator.

5-HT assay

5-HT levels were determined by high-performance liquid chromatography with electrochemical detection (Saller and Salama, 1984; Park *et al,* 1993). After exposure to test reagents, media were collected and filtered through a 0.45 μm Millipore HV-4 filter unit, 25 μl of the filtrate was injected onto C_{18} μ-Bondapak column (Waters). As a mobile phase, 0.5 M monobasic sodium phosphate (adjusted to pH 3.7) containing sodium octanesulfonic acid (1 mM), disodium EDTA, and 10 % acetonitrile was used. The flow rate was 1 ml/min and the oxidation potential was 0.55 V. Data were presented as means±S.E. and expressed as percent control. For comparison of two or more means Student's *t*-test or Dunnett's test was used, respectively.

RESULTS AND DISCUSSION

Cultured neuronal cells from rat fetal medulla oblongata, maintained for 10 days *in vitro* were stimulated for 48 hours with CCK or other neuropeptides at 10 micromolar concentration. CCK (10 μM) and substance P (10 μM) significantly increased 5-HT release (Fig. 1). This result suggests that these neuropeptides play an important role in the regulation of 5-HT release. However, somatostatin, proctolin, TRH, and interleukin-6 did not have any effects on 5-HT release. To study the involvement of calcium ion in the CCK-induced 5-HT release, the effects of nimodipine, a calcium channel blocker, and calmidazolium, a calmodulin antagonist, on the CCK-induced 5-HT release were investigated. Nimodipine (1 μM) almost completely, and calmidazolium (1 μM) significantly inhibited the CCK-induced 5-HT release (Fig. 2). These results indicate that calcium influx through calcium channel, and intracellular calmodulin play an important role

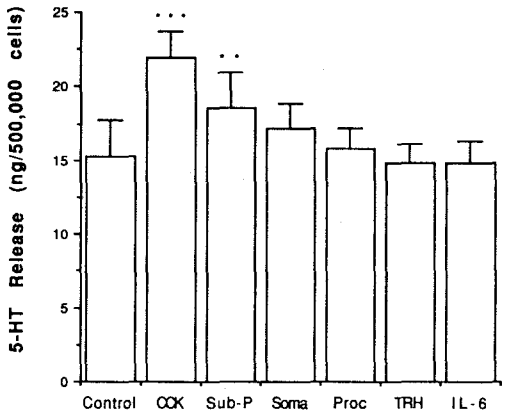

Fig. 1. Effects of cholecystokinin (CCK), substance P (Sub-P), somatostatin (Soma), proctolin (Proc), TRH and IL-6 on serotonin (5-HT) release from cultrued neuronal cells from rat fetal medulla oblongata. The cells (0.5 million cells /well) were treated with 1 ml of 10 μM of neuropeptides. The 5-HT level was measured by HPLC. The vertical bars indicate the standard error of the mean (**, $p<0.05$; ***, $p < 0.01$ compared to the control group; n=3 independent experiments).

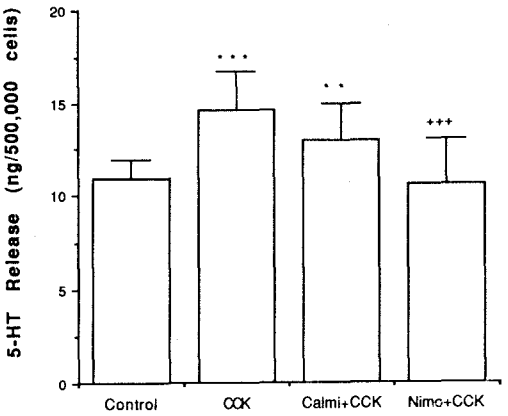

Fig. 2. Effects of pretreatment with nimodipine or calmidazolium on 5-HT secretion induced by CCK. The cultrued neuronal cells (0.5 million cells/well) were pretreated with either 1 μM nimodipine (Nimo) or calmidazolium (Calmi) for 30 min and then the cells were incubated at 37℃ with CCK (10 μM) for 48 hr. The vertical bars indicate the standard error of the mean (**, $p<0.05$; ***, $p<0.01$ compared to the control group; +++, $p<0.05$ compared to the CCK-treated group; n=3 independent experiments).

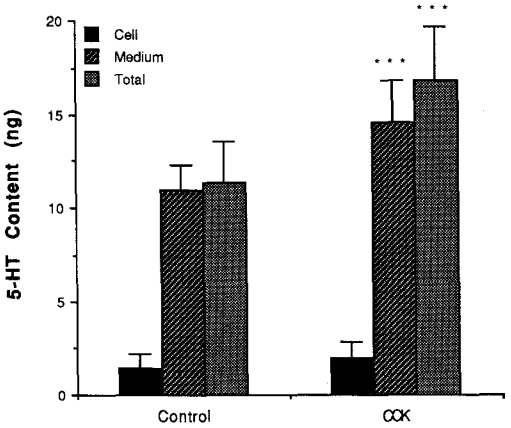

Fig. 3. Effect of CCK on 5-HT secretion and the intracellular content of 5-HT. The neuronal clls (0.5 million cells/well) were treated with 1 ml of control medium or 10 μM of CCK and the 5-HT level was measured by HPLC. The total 5-HT level was calculated as the sum of intracellular 5-HT and 5-HT secreted into the medium during the 48 hr of incubation. The vertical bars indicate the standard error of the mean (***, $p<0.01$ compared to the control group; n=3 independent experiments).

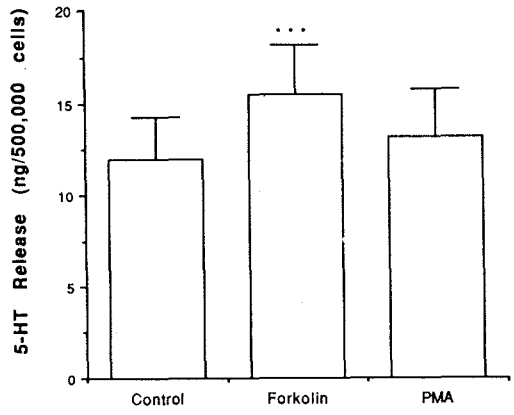

Fig. 4. Effects of forskolin and PMA on serotonin (5-HT) release from cultured neuronal cells. The neuronal cells (0.5 million cells/well) were treated with either 5 μM of forskolin or 2 μM of PMA. The 5-HT level was measured by HPLC. The vertical bars indicate the standard error of the mean (***, $p<0.01$ compared to the control group; n=3 independent experiments).

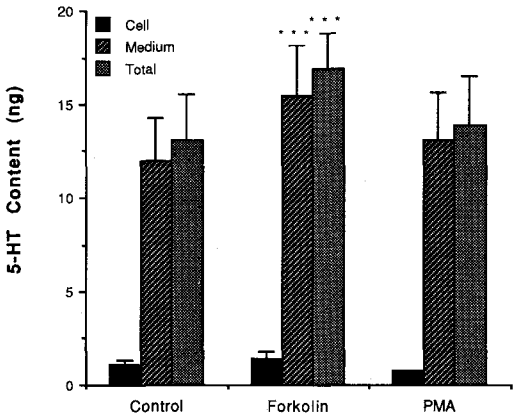

Fig. 5. Effect of forskolin and PMA on 5-HT secretion and the intracellular content of 5-HT. The neuronal cells (0.5 million cells/well) were treated with 1 ml of control medium, 5 μM of forskolin or 2 μM of PMA and the 5-HT level was measured by HPLC. The total 5-HT level was calculated as the sum of intracellular 5-HT and 5-HT secreted into the medium during the 48 hr of incubation. The vertical bars indicate the standard error of the mean (***, $p<0.01$ compared to the control group; n=3 independent exeriments).

in the CCK-induced 5-HT release. As shown in Fig. 3, the total 5-HT content (intracellular 5-HT plus released 5-HT) was significantly increased by CCK, implying that CCK increases 5-HT biosynthesis in the cells. However, the intracellular 5-HT content was not significantly changed by CCK.

To study the involvement of other second messenger systems in 5-HT release, we examined the effects of forskolin, an adenylate cyclase activiator, and phorbol myristate acetate (PMA), a protein kinase C activator on 5-HT release. Forskolin (5 μM), but not PMA (5 μM), significantly enhanced 5-HT release (Fig. 4). These results suggest that cyclic AMP system but not PKC system is involved in 5-HT release. As shown in Fig. 5, the total 5-HT content (intracellular 5-HT plus released 5-HT) was significantly increased by forskolin. However, the intracellular 5-HT content was not significantly changed by forskolin, implying that forskolin increases 5-HT biosynthesis in the cells.

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=국문초록=

연수 신경세포 배양에서 세로토닌 분비에 대한 Cholecystokinin의 작용

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연수의 세로토닌 신경계는 내재성 하행성 동통 억제계 (endogenous descending pain inhibitory system) 에 있어서 중추적인 역할을 하고 있다. 연수의 세로토닌 신경세포에 대한 cholecystokinin (CCK) 및 second messenger systems에 작용하는 약물들의 작용을 알아보기 위하여, 쥐의 태자 (태생 14일) 로부터 연수를 분리하여 10일동안 배양한 후 5-hydroxytryptamine (5-HT) 의 분비에 대한 cholecystokinin (CCK) 및 second messenger systems에 작용하는 약물의 영향을 연구하였다. 배양 10일된 세포에 여러 neuropeptide들을 10 μM 농도로 48 시간동안 자극한 결과, CCK와 substance P에 의하여 5-HT의 분비가 증가됨을 관찰하였다. Somatostatin, proctolin, thyrotropin releasing hormone, 및 interleukin-6 은 5-HT의 분비에 있어서 아무런 영향이 없었다. 어떠한 second messenger가 CCK에 의한 5-HT 분비에 연관되어 있나를 알아보기 위하여 calcium channel 봉쇄제인 nimodipine, 그리고 calmodulin 길항제인 calmidazolium의 영향을 살펴본 결과 nimodipine (1 μM)은 거의 완전하게, 그리고 calmidazolium (1 μM)은 부분적으로 유의하게 CCK에 의한 5-HT의 분비를 억제하였다. 또한 adenyl cyclase의 활성도를 높이는 forskolin (5 μM)은 5-HT의 분비를 증가시켰지만 protein kinase C (PKC)를 활성화시키는 phorbol myristate acetate (PMA)는 2 μM 농도에서 5-HT의 분비에 아무런 영향을 미치지 아니하였다. 이상의 연구결과, calcium channel을 통한 calcium influx와 세포내 calmodulin이 CCK에 의한 5-HT분비 증가에 있어서 중요한 역할을 함을 제시한다. 또한, 5-HT의 분비에 있어서 cyclic AMP system이 중요한 역할을 하나, PKC system은 5-HT의 분비에 연관이 없음을 제시하고 있다.