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Short Communication

Effect of H-7, A Protein Kinase Inhibitor, on Cyclic AMP-Dependent Inhibition of Monoamine Transport in PC12 Cells

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Introduction

Cyclic AMP (cAMP) regulates activity of catecholaminergic neurons in various aspects. It regulate tyrosine hydroxylase, a rate-limiting enzyme in catecholamine biosynthesis (1): the enzyme is activated by phosphorylation with cAMP-dependent protein kinase (protein kinase A) (2), and its expression is enhanced by protein kinase A pathway (3). cAMP elevates intracellular level of tetrahydrobiopterin (4, 5), an essential cofactor for tyrosine hydroxlase (1). Although cAMP itseif dose not induce exocytotic catecholamine release from PC12 cells (6), it modifies the amine release induced by other stimulants from PC12 cells and adrenal chromaffin cells: both stimulatory (7) and inhibitory effects (8) of cAMP were reported indicating the complexity of its function in amine release. Concerning to the amine transport, Kadowaki et al. (9) and Cool et al. (10) reported an increase in amine uptake by cAMP through plasma membranes. Recently, we found that cAMP down-regulated vesicular monoamine transport in PC12 cells and thereby inhibited catecholamine reuptake and elevated its level in the medium (6). Extracellular levels of monoamine neurotransmitters are dependent on rates of their release to and reuptake from the extracellular fluid. Since the vesicular storage capacity for amines is far larger than the cytosolic pool size (6), vesicular amine transport allows continuous reuptake from the extracellular fluid. Furthermore, unlike peptide neurotransmitters, biogenic amines are synthesized in the cytosolic ompartment (11-14) and therefore have to be transported into secretory vesicles for exocytotic release. Vesicular amine transport is thus vital to both release and reuptake. Consequently, among of these various actions of cAMP, regulation of vesicular monoamine transport might give serious effect on extracellular neurotransmitter levels and, therefore, neuronal activities.

In the present study, we examine effect of protein kinase inhibitor, H-7, on cAMP-dependent modulation of amine uptake by intact and digitionin-permeabilized PC12 cells to know whether protein phosphorylation is involved in this cAMP action.

Materials and Methods

PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7% fetal bovine serum, 7% horse serum, 100 μg/ml streptomycin, and 100 units/ml penicillin (15). Cells were seeded on 12-well plates and were used for experiments after 2 to 3 days of culture.

For norepinephrine (NE) uptake by intact PC12 cells, they were incubated with 5.92 μM NE in culture medium for 60 min in a CO_2 incubator. The cells were then washed with 1 ml each of the culture medium and serum-free DMEM. NE incorporated was extracted with 0.1 M perchloric acid/1 mM EDTA/0.1% sodium bisulfite, and analyzed by HPLC/electrochemical detector (ECD) (6, 13).

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Vesicular monoamine transport was measured with using digitonin-permeabilized PC12 cells in terms of serotonin (5HT) uptake. PC12 cells were treated with 1000-150 μM digitonin for 15 min at 25℃ and washed twice with 50 mM Hepes-Tris, pH 7.4/6 mM magnesium chloride/0.32 M sucrose (HTMS buffer). Permeabilized cells were then incubated with 50 μM 5HT in 50 mM HTMS buffer in the presence and absence of 2 mM APT for 30 min at 25℃. After the cells were washed twice with HTMS buffer, 5HT incorporated was extracted with 0.1 M acetic acid and quantified by HPLC/ECD (6). Vesicular amine uptake was measured as an ATP-dependent 5HT uptake.

Results and Discussion

Agents which increase increase intracellular cAMP down regulated vesicular monoamine transport in PC12 cells and thereby inhibited amine uptake by intact cells from the medium (6). Since an intracellular messenger, cAMP, mainly functions via the protein kinase A pathway, we examined effects of protein kinase inhibitors on cAMP-induced inhibition of monoamine uptake by intact PC12 cells. Among the inhibitors tested K252a and staurosporin interfered the cAMP action and increased creased the amine uptake, suggesting involvement of protein phosphorylation process in cAMP-modulation of monoamine transport (17). Contrarily, a protein kinase inhibitor, H-7, decreased amine uptake by intact cells (Fig. 1).

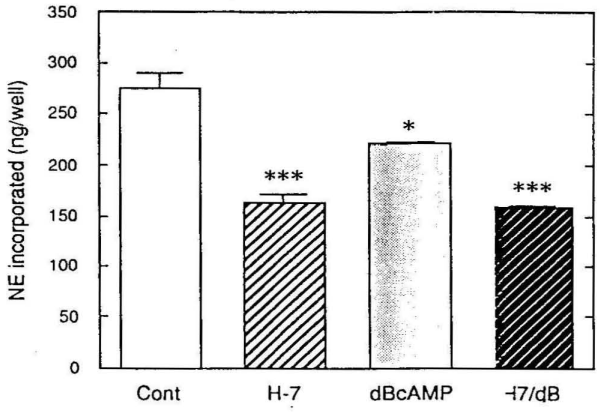

Fig. 1. Effect of protein kinase inhibitor, H-7 on the norepinephrine (NE) uptake and on cAMP-dependent inhibition of NE uptake by intact PC12 cells. Pheochromocytoma PC12 cells cultured in a 12 well plate were incubated with 5.92 μM NE in the absence (Cont), and presence of 100 μM H-7, 1 mM dBcAMP or both of them (H7/dB) for 60 min in a CO_2 incubator. NE incorporated into the cells was measured as described in the text. Values are the means ± S.E. (n=3). ***P<0.01, *P<0.5 by Student's t test for difference from the control value.

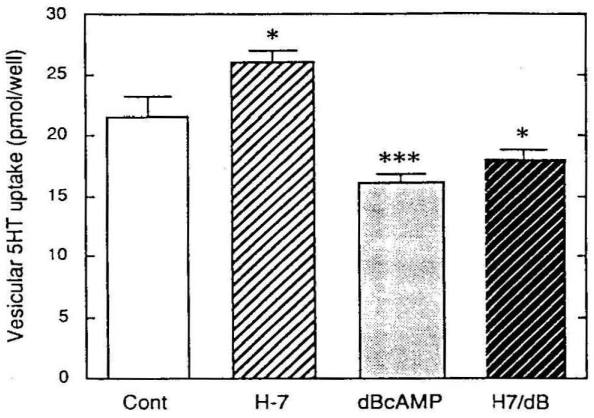

Fig. 2. Enhancement of vesicular 5HT uptake by digitonin-permeabilized PC12 cells by pretreatment of the cells with H-7. PC12 cells in a 12 well plate were cultured in the absence (Cont), and presence of 100 μM H-7, 1 mM dBcAMP or both of the agents (H7/dB) for 30 min in a CO_2 incubator. Cells were permeabilized by incubating with 100 μM digitonin in HTMS buffer for 15 min at 25℃ and then vesicular 5HT uptake was measured as described in the text. Values are the means ±S.E. (n=3). ***P <0.01. *P<0.5 by student's t-test for difference from the control vaiue.

Inhibition of the amine uptake by H-7 was dose-dependent and IC_{50} was observed at around 30 nM (not shown). Inhibitory effect of H-7 and dBcAMP on amine uptake was neither synergistic nor additive (Fig. 1). H-8, another protein kinase inhibitor, which has the same chemical group (isoquinoline) as that H-7 has, also decreased the amine uptake by intact PC12 cells. However, a protein kinase C activator, SC-9 which contains a chmical group (naphthalene) similar to isoquinoline showed even stronger inhibitory effect than H-7 or H-8 (not shown). The results suggest that inhibition of amine uptake by H-7 may not be resulted from their inhibitory action on protein kinase but from their direct interaction with amine transporters.

Amines in the extracellular fluid is incorporated into secretory vesicles via two types of amine transport systems, one involving the plasma membrane and the other the vesicular membrane. Therefore, we then examined effect of H-7 on vesicular monoamine transport by using cells of which plasma membranes were permeabilized by digitionin treatment. With the digitonin-permeabilized PC12 cells, an addition of H-7 (100 μM) to the amine (5HT) uptake mixture did not reduce 5HT uptake, indicating no inhibitory effect of this protein kinase inhibrior on vesicular monoamine transport (not shown). On the hand, when intact cells were incu-

bated with H-7 prior to digitonin treatment, 5HT uptake by these cells measured after digitonin-permeabilization was increased (Fig. 2) in spite of the inhibitory effect of H-7 on amine uptake by intact cells (Fig. 1). In the case of dBcAMP, pretreatment of intact cells by this compound lowered the vesicular 5HT uptake (5HT uptake measured after permeabilization of the cells) (Fig. 2). Pretreatment of intact cells with both H-7 and dBcAMP weakened this inhibitory effect of dBcAMP on vesicular transport. These results indicated that H-7 decreased monoamine uptake by intact PC12 cells by inhibiting the amine transport through plasma membranes but not that through vesicular membranes. Furthermore, pretreatment of intact PC12 cells with H-7 increased the vesicular 5HT uptake measured after digitonir-permeabilization, supporting our idea that protein phosphorylation process may be involved in cAMP-dependent down-regulation of vesicular monoamine transport.

In monoaminerrgic neurons, vesicular amine transport is crucial for both neurotransmitter release and its reuptake. Since cAMP regulates both vesicular transport and biosynthesis of amines, cAMP might be the most important endogenous messenger in controling the activities of monoaminergic neurons. Studies on mechanisms of cAMP action on vesicular amine transoort are thus essential for understanding physiology and pharmacology of monoanminergic neurons.

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