Effects of protein kinase inhibitors and protein phosphatase inhibitors on cyclic AMP-dependent down-regulation of vesicular monoamine transport in pheochromocytoma PC12 Cells

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Abstract Cyclic AMP down-regulates vesicular monoamine transport in PC12 cells and thereby decreased catecholamine reuptake from the extracellular fluid. We examined the effects of protein kinase inhibitors and protein phosphatase inhibitors on this cAMP action. Treatment of cells with a protein kinase inhibitor, K252a, increased vesicular amine transport and cellular amine uptake, thereby antagonizing the regulatory action of cAMP. In contrast, a protein phosphatase inhibitor, okadaic acid, had the opposite effect on the amine transport, i.e. it enhanced the cAMP action. These results suggest the involvement of a protein phosphorylation process in the cAMP-dependent modulation of vesicular monoamine transport.

Key words: Amine transport; Secretory vesicle; Cyclic AMP; Protein phosphorylation; Pheochromocytoma; Serotonin

1. Introduction

Monoamines such as dopamine and serotonin (5-HT) in neuronal cells are synthesized in the cytosolic compartment and, therefore, have to be transported in secretory vesicles for exocytotic release. On the other hand, cellular ability to reuptake extracellular amines is dependent on activities of amine transport systems in both the plasma membrane and the secretory vesicle membrane [1–3]. Since the cyctosolic pool size for the amines is rather small [4], amine transport from the cytosol to vesicles is required to allow continuous reuptake of extracellular amines. Many psychostimulants and sympathomimetic agents such as amphetamine increase extracellular amine levels in terminal dopaminergic areas of the brain [5] by disturbing the amine storage in the secretory vesicles [6,7]. Thus, vesicular monoamine transport is crucial for exocytotic release and reuptake of amines, both of which processes determine the extracellular amine levels and hence neuronal activities. In a previous study, we demonstrated that treatment of PC12 cells with dibutyryl cyclic AMP (dBcAMP) or other agents that elevate cellular cAMP increased the extracellular catecholamine level by inhibiting the uptake process of the cells [4]. Furthermore, this inhibition was shown to be due to the down-regulation of vesicular monoamine transport by cAMP [4].

In the present study, in order to elucidate the mechanism of this cAMP action on the vesicular amine transport, we examined the effect of protein kinase inhibitors and protein phosphatase inhibitors on catecholamine uptake by intact PC12 cells and on vesicular 5-HT uptake by digitonin-permeabilized PC12 cells. The action of cAMP was inhibited by protein kinase inhibitors and enhanced by protein phosphatase inhibitors, suggesting the involvement of a protein phosphorylation process in this cAMP-dependent down regulation of vesicular monoamine transport.

2. Experimental

2.1. Pheochromocytoma cell culture

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 [8]. Cells were seeded in 3.5-cm plastic dishes or 12-well plates and used for experiments 2 or 3 days later.

2.2. Norepinephrine (NE) uptake by intact cells

Catecholamines were measured as described previously [4,9]. In brief, PC12 cells in 3.5-cm dishes were incubated in a CO_2 incubator for 60 min with 1 ml of culture medium containing 1 μg/ml of NE (5.92 μM). The cells were then washed with 1 ml each of the culture medium and serum-free DMEM. NE incorporated into the cells was extracted with 0.5 ml of 0.1 M PCA/1 mM EDTA/0.1% sodium bisulfite. Precipitated proteins were removed by brief centrifugation, and a 10-μl sample of the supernatant was analyzed on a high-performance liquid chromatography (HPLC) apparatus equipped with an electrochemical detector (Coulochem Model 5100A). The precipitate was dissolved in 1 M NaOH and used for protein determination by the Bradford method [10].

2.3. Serotonin-uptake by digitonin-permeabilized cells

PC12 cells were made permeable with digitonin and then subjected to an assay of 5-HT uptake as described previously [4,11]. Cells were treated with 100–150 μM digitonin in the culture medium for 15 min at 25°C and then washed twice with 1 ml of 50 mM HEPES-Tris, pH 7.4/6 mM magnesium chloride/0.32 M sucrose (HTMS buffer). Incubation for 5-HT uptake was performed in 1 ml of HTMS buffer (1 ml in 3.5 cm-dish) in the presence and absence of 2 mM ATP at 37°C for 30 min. Uptake was initiated by the addition of 5-HT (final 50 μM), and stopped by removing the uptake mixture. This was followed by washing the cells twice with 1 ml of HTMS buffer. Incorporated 5-HT was extracted with 250 μl of 0.1 M PCA/1 mM EDTA/0.1% sodium bisulfite and determined by HPLC using a fluorescence detector according to Hasegawa and Ichiyama [12] or by an HPLC apparatus equipped with an electrochemical detector (Eicom ECD-100) [11].

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Abbreviations: dBcAMP, dibutyryl cyclic AMP; NE, norepinephrine; 5-HT, serotonin; HPLC, high-performance liquid chromatography; PCA, perchloric acid; HTMS buffer, 20 mM HEPES-Tris, pH 7.4/6 mM magnesium chloride/0.32 M sucrose.

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3. Results

3.1. Norepinephrine uptake by intact cells

In order to test a possible involvement of protein phosphorylation in the regulation of catecholamine transport by cAMP, we examined the effects of inhibitors of protein kinase and of protein phosphatase. K252a, a protein kinase inhibitor, increased the NE uptake activity of intact PC12 cells and antagonized the inhibitory effect of dBcAMP or forskolin when used in combination with either of these compounds (Fig. 1A). Staurosporin, another kinase inhibitor, also increased the catecholamine uptake to about 140% at a dose of 2.0 μM. The stimulatory effect of K252a on NE uptake was concentration dependent in either the presence or absence of dBcAMP, but the dose-response curve in the presence of dBcAMP shifted down and rightward (Fig. 1B); i.e., dBcAMP suppressed the uptake

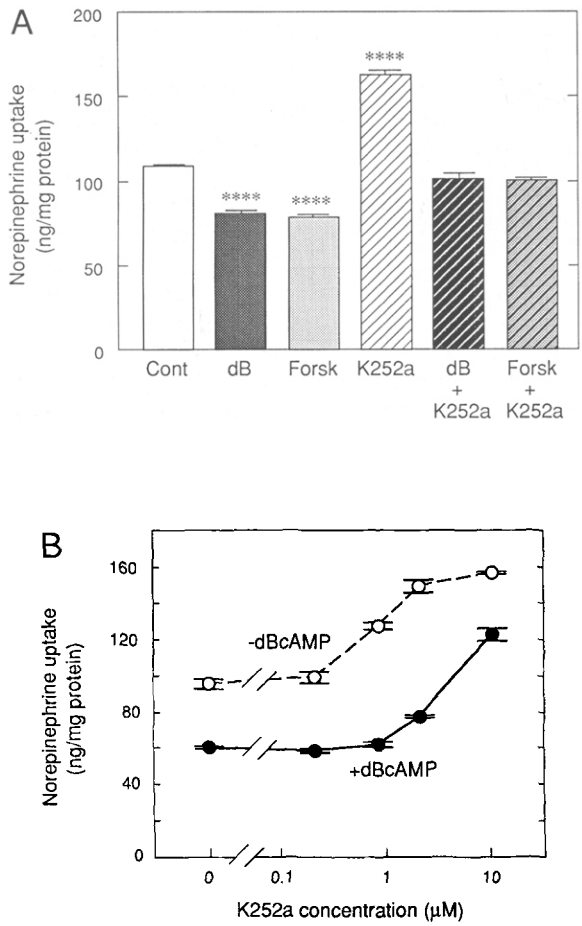

Fig. 1. Effect of K252a on cAMP inhibition of norepinephrine (NE) uptake by intact PC12 cells. (A) Effect of K252a on dBcAMP- and forskolin-dependent inhibition of the NE uptake. PC12 cells cultured in 3.5 cm dishes were incubated with 1 μg/ml (=5.92 μM) NE for 60 min in the presence of 1 mM dBcAMP (dB), 50 μM forskolin (Forsk), 2.14 μM K252a, the combination of K252a and dB or Forsk, or in the absence of any of them (Cont), in a CO_2 incubator. K252a was added simultaneously to the medium when used in combination with either dBcAMP or forskolin. Values are the means ± S.E.M. (n = 5). ****Different from the control value at $P < 0.001$ (Student's t-test). (B) Effect of K252a concentration on NE uptake in the presence (○) and absence (●) of 1 mM dBcAMP. NE uptake by PC12 cells in 3.5-cm dishes was measured in the presence of various concentrations of K252a as described above. Values are the means ± S.E.M. (n = 4).

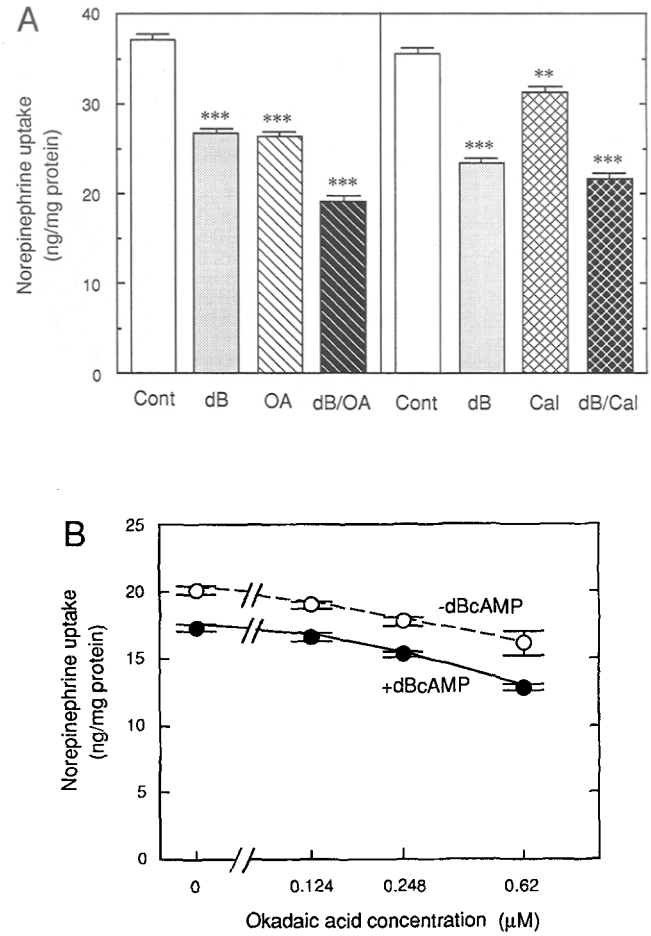

Fig. 2. Effect of phosphatase inhibitors on cAMP inhibition of norepinephrine (NE) uptake by intact PC12 cells. (A) Effects of calyculin A and okadaic acid on NE uptake. PC12 cells in 3.5 cm dishes were incubated for 60 min with 1 μg/ml NE in the absence (Cont), and presence of 1 mM dBcAMP (dB), 0.62 μ M okadaic acid (OA), 10 nM calyculin A (Cal), dBcAMP plus okadaic acid, or dBcAMP plus calyculin A in a CO_2 incubator. The effect of okadaic acid (0.62 μM) was also examined in the presence of 2.14 μM K252a. Values are the means ± S.E.M. (n = 4). Different from the respective control values at: ***$P < 0.01$ and **$P < 0.02$ (Student's t-test). (B) Effect of okadaic acid concentration. NE uptake activity of intact PC12 cells was measured as above in the presence of various concentrations of okadaic acid with (○) or without (●) 1 mM dBcAMP. Values are the means ± S.E.M. (n = 4).

activity of the cells, and thus a higher dose of K252a was required to overcome the effect of dBcAMP and to increase the uptake process. These results are compatible with the hypothesis that the inhibitory effect of cAMP on vesicular monoamine transport is mediated by protein phosphorylation and suggest the possibility that some protein(s) being involved in the regulation of the amine transport is partly phosphorylated by K252a-sensitve kinase(s) even without the dBcAMP treatment.

In contrast, inhibitors of protein phosphatase showed an opposite effect to that of protein kinase inhibitors on NE uptake. Okadaic acid (0.62 μM) and calyculin A (40 nM) inhibited NE uptake by PC12 cells by about 25% and 10%, respectively (Fig. 2A). In the presence of dBcAMP, okadaic acid showed greater inhibition at all doses examined (up to 0.62 μM, Fig. 2B). Calyculin A behaved similarly to okadaic acid in the pres-

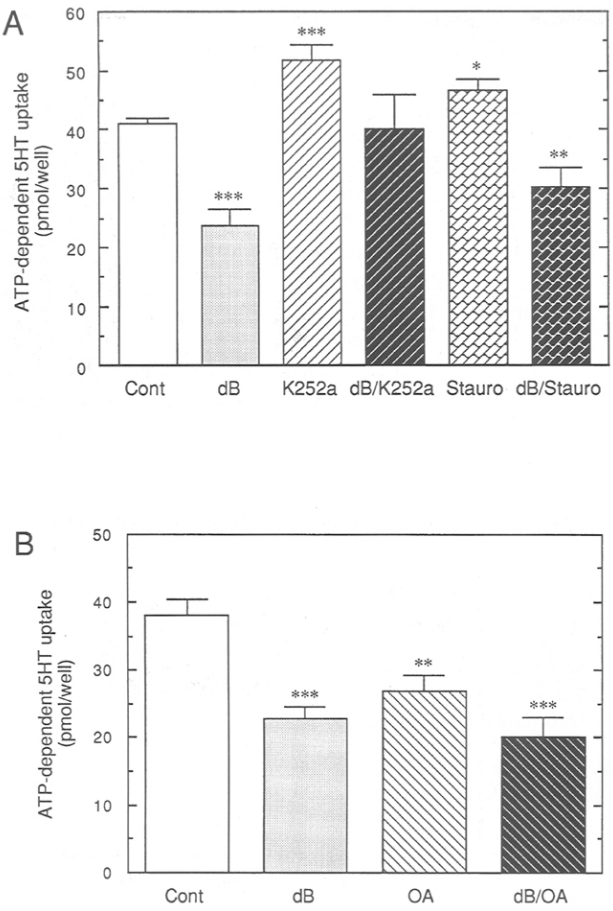

Fig. 3. Effect of protein kinase inhibitors and a protein phosphatase inhibitor on vesicular monoamine transport by digitonin-permeabilized PC12 cells. (A) Effect of protein kinase inhibitors. PC12 cells in 12-well plates were cultured for 30 min in the presence of 1 mM dBcAMP (dB), 2.14 μM K252a, dBcAMP plus K252a, 2 μM staurosporin (Stauro), dBcAMP plus staurosporin, or none of them (Cont) for 30 min in a CO_2 incubator. The cells were then permeabilized by digitonin treatment and then incubated with 50 μM 5-HT in the presence and absence of 2 mM ATP for 30 min at 25°C. Vesicular monoamine transport was measured as ATP-dependent 5-HT uptake, which was calculated by subtracting the 5-HT uptake obtained in the absence of ATP from that obtained in the presence of ATP. Values are the means ± S.E.M. ($n = 4$). (B) Effect of okadaic acid. PC12 cells cultured in 12-well plates were cultured for 30 min in the presence of 1 mM dBcAMP, 0.62 μM okadaic acid (OA), or dBcAMP plus okadaic acid, or in the absence of any of them (Control) for 30 min in a CO_2 incubator. Vesicular 5-HT uptake was measured as described above after the digitonin-permeabilization of the cells. Values are the means ± S.E.M. ($n = 4$). In (A) and (B), significant differences from each control value are marked: $^{***}P < 0.01$, $^{**}P < 0.02$, and $^{*}P < 0.05$ (Student's t-test).

ence of dBcAMP. Higher doses of both okadaic acid and calyculin A were not used because PC12 cells lost adhesion to the culture dish at high concentrations of the agents. These results together with those obtained with protein kinase inhibitors suggest that the rate of catecholamine uptake by PC12 cells is regulated by a balance of protein phosphorylation and dephosphorylation activities and that cAMP inhibition of catecholamine transport was due to stimulation of protein phosphorylation, probably by protein kinase A.

3.2. *Vesicular monoamine transport: 5-HT uptake by permeabilized cells*

In a previous study, we demonstrated that the inhibitory effect of cAMP on catecholamine uptake by intact cells resulted from the down-regulation of the process of amine transport through the secretory vesicular membrane [4]. Since we developed an assay method for vesicular monoamine transport by using digitonin-permeabilized cells, the plasma membrane of which allows free movement of the amines [4,11], we further examined the effect of protein kinase inhibitors and protein phosphatase inhibitors on vesicular monoamine transport with this permeabilized cell system. The vesicular monoamine transport measured as ATP-dependent 5-HT uptake by permeabilized PC12 cells was lowered by the pretreatment of intact cells with dBcAMP prior to the digitonin-permeabilization (Fig. 3). However, previous studies showed that the addition of dBcAMP [4] or forskolin [11] to the reaction mixture for 5-HT uptake by already permeabilized cells had no effect on the vesicular transport activity. Thus, the inhibitory effect on the uptake process caused by dBcAMP treatment remained after the permeabilization. Similarly, the stimulatory effect of K252a treatment of intact cells remained after the digitonin-permeabilization: when PC12 cells were cultured for 30 min in the presence of K252a and then permeabilized, significant elevation of vesicular 5-HT uptake was observed (Fig. 3A). Addition of K252a to the uptake mixture did not affect the vesicular transport activity. The stimulation of 5-HT uptake by K252a pretreatment was observable at its concentrations as low as 20 nM and showing its maximal level at 1–2 μM. The maximal value varied from experiment to experiment, being 120% to 150% of the value of control cells. The effect of dBcAMP pretreatment on vesicular 5-HT uptake was antagonized by the simultaneous addition of K252a to the PC12 culture (Fig. 3A). Staurosporin and H-7, both known as protein kinase inhibitors, also showed effects similar to that of K252a.

On the other hand, okadaic acid, an inhibitor of protein phosphatase, decreased the vesicular monoamine transport, as expected from the results with intact PC12 cells. Pretreatment of PC12 cells with okadaic acid (0.62 μM) decreased the vesicular monoamine transport (ATP-dependent 5-HT uptake) measured after the digitonin-permeabilization to about 60% of the control. Simultaneous addition of this reagent with dBcAMP into the cell culture enhanced the inhibitory effect of dBcAMP on vesicular 5-HT uptake (Fig. 3B). Thus, all of the results with protein kinase inhibitors and protein phosphatase inhibitors strongly suggest that vesicular monoamine transport is down-regulated by a cAMP-sensitive protein phosphorylation, namely, via a protein kinase A pathway.

4. Discussion

Cyclic AMP is known to activate tyrosine hydroxylase, a rate-limiting enzyme in catecholamine biosynthesis [13], by phosphorylating the enzyme protein via protein kinase A action [14–16], thereby increasing the DA production in PC12 cells [4,15]. However, as we demonstrated in a previous study [4], cAMP also down-regulated the monoamine transport through secretory vesicle membranes. Thus, cAMP influenced the intra and extracellular catecholamine levels not only by stimulating its synthesis but also by regulating vesicular monoamine transport. In the present study, we examined the effect of protein

kinase inhibitors and protein phosphatase inhibitors on this cAMP action on vesicular monoamine transport and catecholamine uptake activity of PC12 cells, since the majority of the regulatory actions of cAMP are mediated via protein kinase A-dependent protein phosphorylation [17]. Vesicular monoamine transport was measured as ATP-dependent 5-HT uptake by digitonin-permeabilized PC12 cells [4]. A protein kinase inhibitor, K252a, antagonized and a protein phosphatase inhibitor, okadaic acid, enhanced the dBcAMP effect on the vesicular amine transport measured in the permeabilized cell system (Fig. 3), supporting the idea that protein phosphorylation is involved in the mechanism of the cAMP action. As the amine storage capacity of the vesicular compartment is far larger than the cytosolic pool size [4], the effects of either of dBcAMP, protein kinase inhibitors, or protein phosphatase inhibitors on vesicular monoamine transport are necessarily reflected in the cellular uptake of catecholamines from the medium. Indeed, the inhibitory effect of dBcAMP on NE uptake by intact cells was antagonized by K252a, and strengthened by okadaic acid and calyculin A. Furthermore, in the absence of dBcAMP, K252a and okadaic acid enhanced and inhibited, respectively, the NE uptake by intact PC12 cells in a concentration-dependent manner (Figs. 1 and 2). These results suggest that vesicular amine transport and hence catecholamine uptake activity of the cells is regulated by a balance of protein-phosphorylating and -dephosphorylating activities, or the level of phosphorylation of a certain protein(s) responsible for the control of vesicular amine transport. In addition, a significant fraction of such protein(s) is suggested to be present in the phosphorylated state even without dBcAMP treatment of the cells, since K252a alone was able to increase vesicular 5-HT transport and NE uptake activities of the cells. cAMP may down-regulates vesicular monoamine transport by elevating the phosphorylation level of such a protein(s) via the protein kinase A pathway. It is yet to be elucidated what protein(s) is the target for phosphorylation and whether it is phosphorylated directly by protein kinase A or via some protein phosphorylation cascade. Recently, cDNAs for the chromaffin granule amine transporter and the synaptic vesicle amine transporter were cloned and sequenced [18–21]. Both types of these vesicular monoamine transporters were shown to contain consensus sequences for phosphorylation by protein kinase A in their cytoplasmic domains [18,20]. This is of particular interest to us since the vesicular monoamine transporter would be the one to be examined as the target of cAMP-dependent regulation because of its primary role in vesicular monoamine transport.

Regulation of the extracellular level of neurotransmitters is crucial for neuronal activity. Many of the sympathomimetic agents, antidepressants, and psychostimulants are known to elevate extracellular monoamine levels in the brain by blocking the amine transport through either plasma membrane [22] or vesicular membrane [5], thus disturbing the amine storage function. In PC12 cultures, dBcAMP was more effective in increasing extracellular DA than amine transport inhibitors such as reserpine and nomifensine, since cAMP stimulated, at the same time, the de novo DA synthesis besides inhibiting vesicular amine transport. The observed effect of dBcAMP on extra and intracellular catecholamine levels in PC12 cells suggests that cAMP functions in a similar fashion also in catecholaminergic and serotonergic neurons of central nervous system and that it, thereby, participates in intrinsic mechanisms for antidepressant or psychostimulant activity. Indeed, infusion of 8-bromo-cAMP or forskolin by the brain microdialysis technique was reported to increase the DA level in a dialysate from rat striatum [23]. We also observed by brain microdialysis the increased DA output by dBcAMP infusion of the rat striatum. Furthermore, dBcAMP, unlike high K+, maintained the extracellular DA at a high level as long as the perfusion with the agent was continued (N. Nakanishi et al., unpublished results).

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