κ-Opioid inhibits catecholamine biosynthesis in PC12 rat pheochromocytoma cell

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Abstract It was reported that nicotine-induced dopamine release in the rat pheochromocytoma cell line, PC12 cells, was inhibited by κ-opioid. However, it is not known whether inhibition of catecholamine biosynthesis is involved in the inhibitory mechanisms of κ-opioids in PC12 cells. U-69593 (a κ-opioid agonist: ≥100 nM) significantly inhibited the nicotine-induced increase of tyrosine hydroxylase (TH, a rate-limiting enzyme in biosynthesis of catecholamine) enzyme activity and TH mRNA levels. These inhibitory effects were completely reversed by naloxone and nor-binaltorphimine dihydrochloride (nor-BNI), a specific κ-antagonist, whereas pertussis toxin (PTX) only partially reversed this inhibitory effect. Also, U-69593 (≥100 nM) significantly inhibited the nicotine-induced increase of cAMP production. This inhibitory effect was completely reversed by naloxone and nor-BNI, whilst only partially reversed by PTX. Moreover, U-69593 (≥100 nM) significantly inhibited the nicotine-induced increase of both the TH protein level and intracellular catecholamine levels. These results indicate that the anti-cholinergic actions of κ-opioid can be explained partially by its inhibition of both TH enzyme activity and TH synthesis, through suppression of the cAMP/protein kinase A pathway. It would also appear that the PTX-sensitive G-protein mediates the inhibitory effect of this pathway, at least in part.

Key words: κ-Opioid; Tyrosine hydroxylase; cAMP; Pertussis toxin; PC12 cell

1. Introduction

Adrenal chromaffin cells produce opioids as well as catecholamine [1–4]. The biological effects of opioids are believed to be mediated via specific receptors (κ, δ and μ), which belong to the family of GTP binding protein (G-protein)-coupled receptors [5]. It was demonstrated that stimulation of these opioid receptors leads to inhibition of adenylate-cyclase (AD) through pertussis toxin (PTX)-sensitive G-protein ($G_{i/o}$), resulting in the reduction of cAMP levels [6,7].

Although the precise role of opioids in chromaffin cells is unclear, growing evidence suggests that opioids exert their action through the inhibition of catecholamine release in a paracrine and/or autocrine manner. Normal adrenal chromaffin cells produce only δ-opioid peptides such as enkephalin, which inhibit basal and nicotine-induced catecholamine secretion [2,4,8,9]. This property of normal chromaffin cells may be changed as a result of their transformation to pheochromocytomas. Indeed, human pheochromocytomas produce κ-opioid peptides such as dynorphin in addition to enkephalin and have a κ-opioid binding site [10–12]. Furthermore, Margioris et al. [3] demonstrated that PC12 cells, a rat pheochromocytoma cell line, which is an appropriate model to investigate pheochromocytomas, contain and secrete dynorphin in addition to enkephalin [1,3]. Moreover, it was demonstrated in several studies that κ-opioid agonists, but not δ- or μ-opioid agonists, significantly inhibited nicotine-induced dopamine release in PC12 cells [13,14]. These findings indicate that κ-opioids are the most potent inhibitors of catecholamine release in PC12 cells. However, it is unclear whether inhibition of catecholamine biosynthesis is involved in the anti-cholinergic effect of κ-opioids in PC12 cells.

In adrenal medullary cells, tyrosine hydroxylase (TH) is a rate-limiting enzyme in the biosynthesis of catecholamine. TH activity can be regulated by both short- and long-term mechanisms. Short-term regulation of enzyme activity occurs at the post-transcriptional level. Central to this regulation is the phosphorylation of TH, which results in activation of the enzyme [15]. Indeed, TH is phosphorylated and activated by a variety of protein kinases including cAMP protein kinase A (PKA) [16–18]. A long-term regulation has been shown to be exerted at the TH protein synthesis level following TH gene transcription [19]. Similar to their effect on TH enzyme activity, several protein kinases, including PKA, also induce an increase in levels of TH mRNA [19].

Nicotine is a ligand of the nicotinic acetylcholine receptor in adrenal medullary cells. Both TH enzyme activity and TH mRNA are increased by nicotine treatment in the rat pheochromocytoma cell line, PC12 [15,20]. Moreover, the cAMP-mediated pathway appears to play a key role in both the nicotine-induced TH enzyme activity and TH mRNA levels [20].

To gain new insight into the mechanisms underlying the anti-cholinergic action of opioids, we investigated the effects of opioids on nicotine-stimulated catecholamine biosynthesis in PC12 cells.

2. Materials and methods

2.1. Reagents

Unless otherwise noted, all reagents were purchased from Wako Seiyaku (Tokyo, Japan). U-69593, DPDPE and DAGO (κ-, δ- and μ-opioid agonists, respectively), nor-binaltorphimine dihydrochloride (nor-BNI: a specific κ-antagonist) were purchased from Sigma Chemical (St. Louis, MO, USA). The concentrations of κ-agonist, U-69593, used in our experiments (1 nM–1 μM) were chosen according to Venihaki et al. [13,21]. Toxicity of U-69593 to PC12 cells was negli-

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gible under our experimental conditions [21]. The concentration of nor-BNI was set at 100 nM according to Venihaki et al. [21].

2.2. Cell culture

The PC12 cell line (RCB009) was obtained from the RIKEN Cell Bank (Ibaraki, Japan). Cells were grown in 75 cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Gaithersburg, MD, USA) containing 10% inactivated horse serum (Gibco BRL) and 10% fetal bovine serum (Gibco BRL) in a humidified atmosphere of 5% CO_2/95% O_2 at 37°C. The culture medium was changed three times per week. Cells were removed from the flasks for subculture and for plating into assay dishes using a Ca^{2+}/Mg^{2+}-free solution of 172 mM NaCl, 5.4 mM KCl, 1 mM NaH_2PO_4 and 5.6 mM glucose, at pH 7.4. After about 2 min in this solution, the cells were detached by tapping the side of the flask. The cells (1×10^6) were plated into 35 mm polystyrene dishes and cultured with 2 ml of DMEM for 2 days under similar conditions as described above, and then used for experiments in a serum-starved condition.

2.3. TH enzyme activity

TH enzyme activity was measured using a method previously reported by Kumai et al. [22]. Experiments were initiated by replacing the medium with HEPES-buffered Krebs buffer containing various concentrations (10 nM–1 μM) of U-69593 or U-69593 (1 μM) in the presence of PTX (100 ng/ml) or naloxone (1 M) or nor-BNI (100 nM), and the cells were incubated at 37°C for 10 min. Then, cells were homogenized in 0.25 M sucrose (50 volumes) using a glass tissue grinder. The standard incubation medium consisted of the following components in a total volume of 250 μl: 100 μl tissue homogenate, 40 μl of 1 M sodium acetate buffer (pH 6.0), 40 μl of 1 mM L-tyrosine or D-tyrosine, 20 μl of 1 M 6-methyl-5,6,7,8-tetra-hydropterine in 1 M 2-mercaptoethanol, 20 μl of 20 mM catalase and 30 μl water. The medium was incubated at 37°C for 30 min, and the reaction stopped with 1 M perchloric acid containing dihydroxy benzylamine as an internal standard and then 0.2 M EDTA in an ice bath. Then, 1 M potassium carbonate and 0.2 M Tris–HCl (pH 8.5) containing 1% EDTA were added. The 3-(3,4-di-dihydroxyphenyl)-alanine (DOPA) was extracted using the aluminum oxide method. 40 μl of extracted medium was mixed with 0.1 N NaOH and TSK-GEL ODS-120T (TOSOH, Japan) and analyzed by high performance liquid chromatography (HPLC). The mobile phase consisted of the following components: 50 mM sodium acetate, 20 mM citric acid, 12.5 mM sodium octyl sulfate, 1 mM di-*n*-butylamine and 0.134 mM EDTA. All separations were performed isocratically at a flow rate of 0.6 ml/min at 28°C. The detector potential was maintained at +0.65 V. The TH enzyme activity was calculated as the amount of DOPA formed from tyrosine per mg of protein per minute.

2.4. Northern blot analysis

For Northern blot analysis, total RNA was extracted from the samples using an ISOGEN kit (Nippon Gene, Tokyo, Japan). The RNA concentration was determined spectrophotometrically (at 260 nm). RNA (10 μg sample) was fractionated by electrophoresis on 1% agarose/5% formaldehyde gels (80 V, 2 h). After staining with ethidium bromide and a visual inspection of the UV fluorescence to confirm the presence of equal amounts of 18S and 28S ribosomal RNA in each lane, the RNA was transferred to a nitrocellulose membrane and hybridized to ^{32}P-labeled probes. The following probe was used: a 1.9 kbp *Eco*RI fragment of pTHT1 encoding TH. Plasmid pTHT1 contained the full-length cDNA for human TH type 1 cDNA. This plasmid was developed by T. Nagatsu and was provided by the RIKEN Gene Bank (Ibaraki, Japan). The probe was labeled using a random primer extension labeling kit (New England Nuclear, Boston, MA, USA). Rat G3PDH cDNA was used as an internal standard (Clontech, Palo Alto, CA, USA). Hybridization signals were scanned in an image analyzer (BAS2000, Fuji, Tokyo).

2.5. Western blot analysis

Western blot analyses were performed as previously described [23]. In brief, the cells were solubilized with 0.1% sodium dodecyl sulfate (SDS) containing 1% Triton X-100, 1% sodium deoxycholate and 20 mM Tris–HCl, pH 7.4. The supernatant containing 10 mg protein was separated on 10% SDS–polyacrylamide gels, and then transferred to nitrocellulose using a Bio-Rad Transblot apparatus. After transfer, the nitrocellulose sheets were incubated for 1 h with BLOTTO buffer (5% skimmed milk, 0.05% Triton X-100, 100 mM NaCl, 200 mM Tris–HCl, pH 7.4). The nitrocellulose membranes were washed three times for 10 min with TBST solution (0.05% Triton X-100, 20 mM Tris–HCl, pH 7.4, 150 mM NaCl), and then for 1 h with 1 mg/ml of the monoclonal antibody to TH (Boehringer, Germany). The nitrocellulose membranes were then washed three times for 10 min with TBST solution, and then incubated for 1 h with horseradish peroxidase-labeled Protein A (Amersham, UK). Finally, the blots were washed three times, incubated with ECL reagent (Amersham, UK) for 1 min and then exposed to Polaroid films (ISO 3000).

2.6. Measurement of cAMP production

The cAMP production by the cells was determined as previously described [24]. Briefly, cells were washed twice with EM (Eagle's minimal essential medium) and preincubated in EM containing 0.2 mM 3-isobutyl-1-methylxanthine (IBMX) for 5 min. Experiments were initiated by replacing the medium with HEPES-buffered Krebs buffer containing test substances and 0.2 mM IBMX, and the cells were then incubated at 37°C for 10 min. The reaction was terminated by adding 100 μl of 1 N HCl, followed by incubation on ice for 30 min. The cAMP in the acid extract was then measured with a cAMP kit (Yamasa, Chousi, Japan).

2.7. Determination of intracellular catecholamine levels

Cells were treated with U-69593 (10 nM–1 μM) for 24 h. Then, the catecholamine levels in cells were determined as previously described [24], using a catecholamine autoanalyzer (TOSOH, H8030, Japan) with built in HPLC and a spectrofluorometer.

2.8. Statistical analysis

All data are expressed as mean ± S.D. The significance of differences in the data was determined by an analysis of variance (ANOVA). P values less than 0.05 were considered significant.

3. Results

3.1. Effect of U-69593 (κ-opioid agonist) on nicotine-induced TH enzyme activity

As shown in Fig. 1, 10 nM of U-69593 (κ-opioid agonist) had no effect on TH enzyme activity. At 100 nM and over (100 nM and 1 μM), U-69593 significantly inhibited the nicotine-induced increase of the TH enzyme activity by 24 and 57%, respectively. This inhibitory effect was completely abolished by naloxone and nor-BNI, a specific κ-antagonist, suggesting that a κ-opioid receptor was involved in the inhibitory effect of U-69593. Pretreatment of PC12 with 100 ng/ml PTX for 15 h [25] partially prevented the inhibitory effect of U-69593. DPDPE and DAGO (δ- and μ-opioid agonists, respectively) did not alter the nicotine-induced TH enzyme activity (data not shown).

3.2. Effect of U-69593 (κ-opioid agonist) on nicotine-induced TH mRNA expression

As shown in Fig. 2, 10 nM of U-69593 had no effect on the

Table 1
Effects of U-69593 on intracellular catecholamine content

Nicotine (μM)	U-69593	Dopamine (ng/mg protein)
10	none (control)	1651 ± 50
10	10 nM	1511 ± 62
10	100 nM	1371 ± 78*
10	1 μM	1099 ± 81*

PC12 cells were treated with U-69593 (10 nM–1 μM) for 24 h. Then, intracellular catecholamine levels (dopamine) were measured as described in Section 2. Since the major catecholamine synthesized in PC12 cells is dopamine and the production of noradrenaline and adrenaline was negligible, findings on dopamine levels are presented. Control experiments were done with nicotine (10 μM) alone. *Significantly different ($P < 0.05$) from controls.

TH mRNA level. At 100 nM and over (100 nM and 1 μM), U-69593 significantly inhibited the nicotine-induced increase of TH mRNA levels by 37 and 63%, respectively. This inhibitory effect was completely abolished by naloxone and nor-BNI, whereas PTX partially prevented this inhibitory effect of U-69593. DPDPE and DAGO did not alter the nicotine-induced TH mRNA levels (data not shown).

3.3. Effect of U-69593 (κ-opioid agonist) on nicotine-induced cAMP production

Since the increase of cAMP plays a key role in the nicotine-induced TH activity as well as the TH mRNA level, we next examined the effect of U-69593 on nicotine-induced cAMP production. As shown in Fig. 3a, 1 and 10 nM of U-69593 had no effect on the cAMP level. At 100 nM and over (100 nM and 1 μM), U-69593 significantly inhibited the nicotine-induced increase of the cAMP production by 34 and 58%, respectively. Similar to their effect on the TH enzyme activity and the TH mRNA level, naloxone and nor-BNI completely abolished the inhibitory effect of U-69593 on cAMP, while PTX only partially prevented the inhibition (Fig. 3b). In contrast to U-69593, DPDPE and DAGO, δ- and μ-opioid agonists, respectively, did not affect the nicotine-induced cAMP production.

3.4. Effects of U-69593 (κ-opioid agonist) on the nicotine-induced TH protein level and intracellular catecholamine levels

To confirm the decrease of catecholamine synthesis following TH mRNA reduction, the effects of U-69593 on the TH protein level and intracellular catecholamine levels were examined. PC12 cells were treated with U-69593 (10 nM–1 μM) for 24 h. Then, TH protein and intracellular catecholamine levels were examined by Western blot analysis and HPLC, respectively. As shown in Fig. 4, 10 nM of U-69593 had no effect on the TH protein level. At 100 nM and over (100 nM and 1 μM), U-69593 significantly inhibited the nicotine-induced increase of TH protein by 24 and 44%, respectively. Also, as shown in Table 1, 10 nM of U-69593 had no effect on intracellular catecholamine levels. At 100 nM and over (100 nM and 1 μM), U-69593 significantly inhibited the nicotine-induced increase of intracellular catecholamine levels by 18 and 34%, respectively.

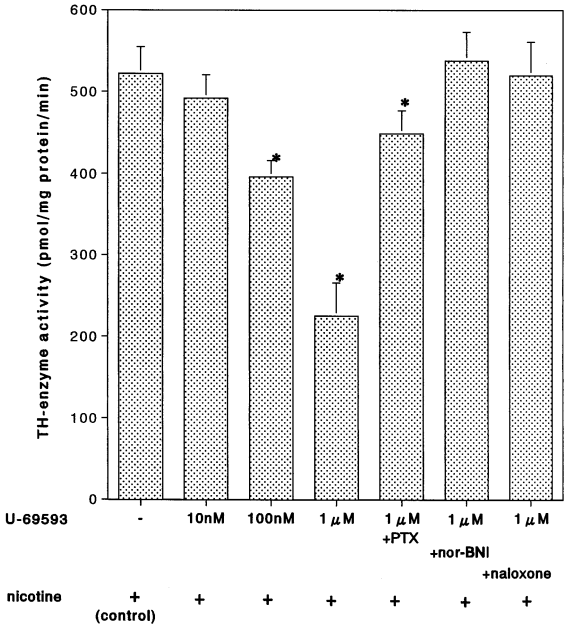

Fig. 1. Effect of U-69593 (κ-opioid agonist) on nicotine-induced TH enzyme activity. PC12 cells were incubated for 10 min with various concentrations (10 nM–1 μM) of U-69593, U-69593 (1 μM) in the presence of PTX (100 ng/ml) or naloxone (1 μM) or nor-BNI (100 nM), as indicated. Then, TH enzyme activity was measured as described in Section 2. The values represent the means ± S.D. ($n = 4$). Control experiments were done with nicotine (10 μM) alone. *Significantly different ($P < 0.05$) from controls.

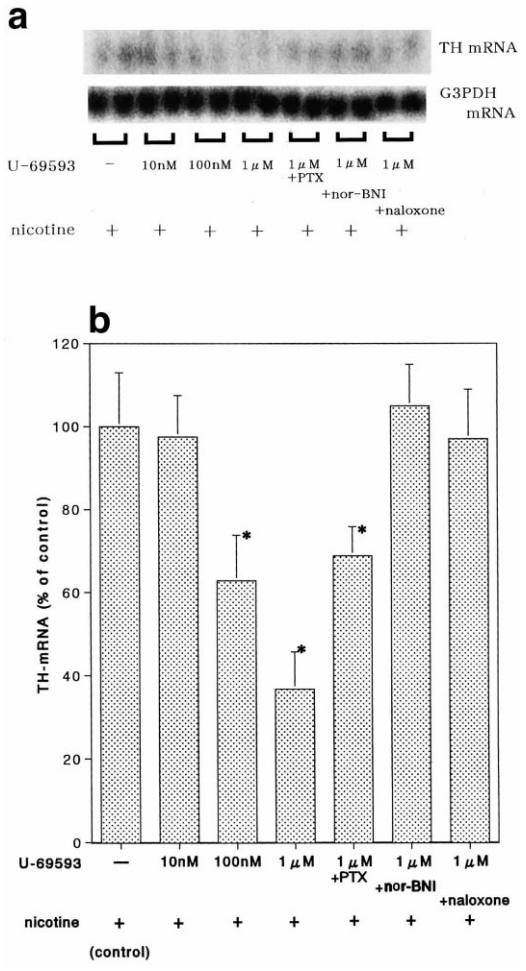

Fig. 2. Effect of U-69593 (κ-opioid agonist) on nicotine-induced TH mRNA expression. (a) PC12 cells were incubated for 8 h with various concentrations (10 nM–1 μM) of U-69593, U-69593 (1 μM) in the presence of PTX (100 ng/ml) or naloxone (1 μM) or nor-BNI (100 nM), as indicated. Total cellular RNA (10 μg/lane) from porcine adrenal medullary cells was characterized by Northern blot analysis as described in Section 2. The lower panel shows the control mRNA (G3PDH) containing equivalent amounts of total mRNA. Values from duplicate determinations are shown. Control experiments were done with nicotine (10 μM) alone. (b) The values represent the means ± S.D. ($n = 4$) of the radioactivity (photostimulated luminescence minus background) of each TH mRNA level. *Significantly different ($P < 0.05$) from controls.

4. Discussion

The results of the present study showed that the nicotine-induced increases of both TH enzyme activity and the TH

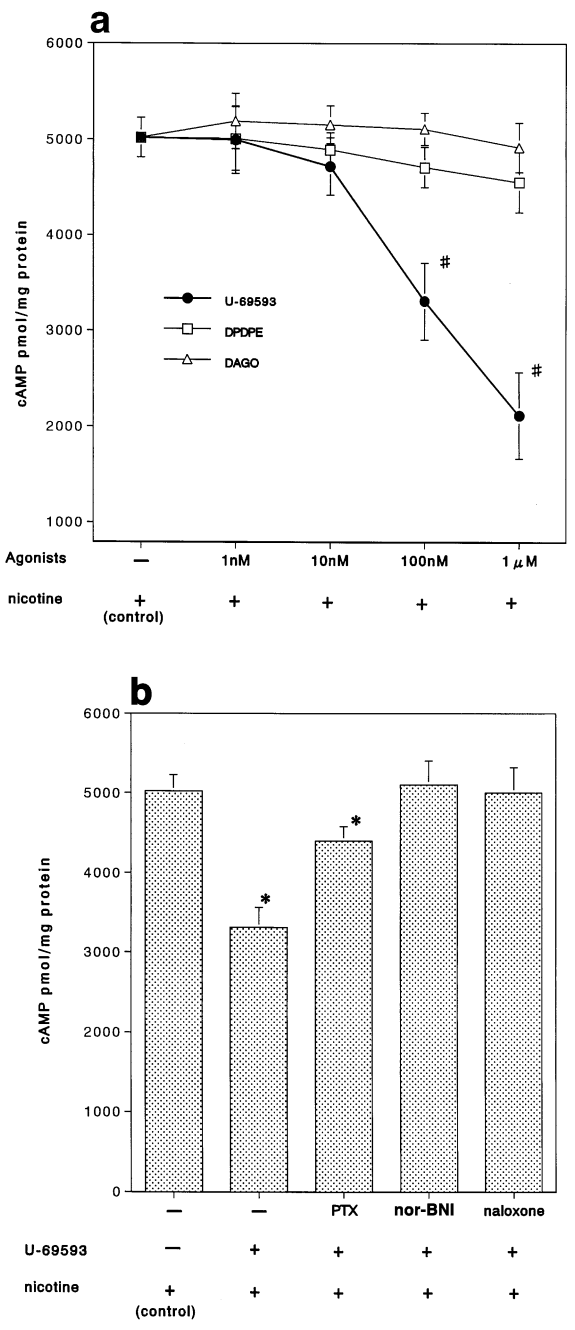

Fig. 3. Effect of U-69593 (κ-opioid agonist) on nicotine-induced cAMP production. (a) PC12 cells were incubated with various concentrations (1 nM–1 μM) of U-69593, DPDPE and DAGO (κ-, δ- and μ-opioid antagonists, respectively) for 10 min. (b) PC12 cells were incubated for 10 min with U-69593 (1 μM) in the presence of PTX (100 ng/ml) or naloxone (1 μM) or nor-BNI (100 nM), as indicated. Then intracellular cAMP was subjected to EIA as described in Section 2. Control experiments were done with nicotine (10 μM) alone. *Significantly different ($P < 0.05$) from control.

mRNA level were attenuated by U-69593 (κ-opioid agonist) in parallel (Figs. 1 and 2). This suggests that an inhibitory effect on catecholamine biosynthesis may be involved in the anti-cholinergic action of κ-opioids. In addition, we demonstrated that U-69593 markedly suppressed the increase of TH protein levels induced by nicotine (Fig. 4). Therefore, it is likely that U-69593 induced a reduction in TH gene expression, resulting in a decrease of the TH protein level in PC12 cells. Furthermore, we showed that U-69593 markedly suppressed intracellular catecholamine levels (Table 1), supporting the suggestion that κ-opioid stimulation inhibits catecholamine biosynthesis.

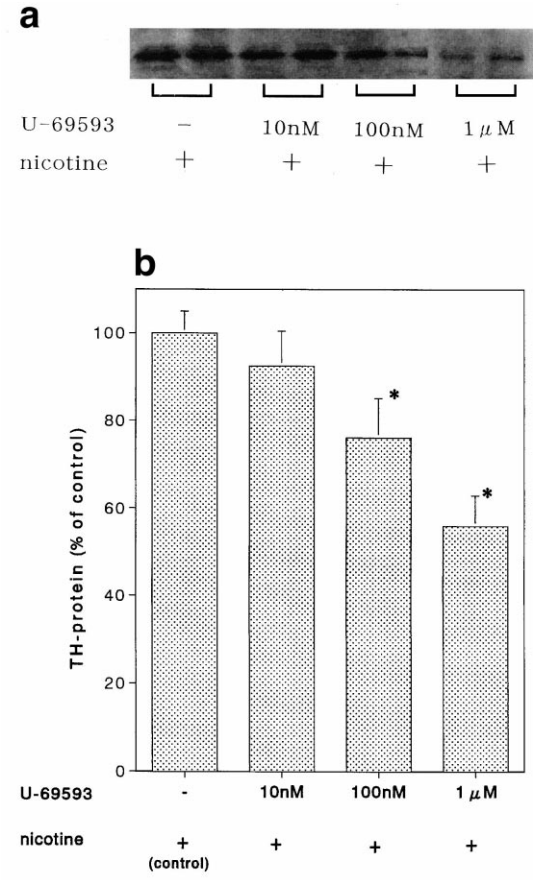

Fig. 4. Effects of U-69593 (κ-opioid agonist) on TH protein level. (a) PC12 cells were incubated for 24 h with U-69593 (10 nM–1 μM). Then, the TH protein level was measured by Western blot as described in Section 2. (b) The values represent the means ± S.D. ($n = 4$). Control experiments were done with nicotine (10 μM) alone. *Significantly different ($P < 0.05$) from controls.

It was also shown in the present study that U-69593 inhibited the nicotine-induced increase of cAMP production (Fig. 3a). Moreover, it was noteworthy that this inhibition occurred at the same threshold concentration of U-69593 (100 nM) as the inhibition of the nicotine-induced increase of TH mRNA levels and TH enzyme activity. This would suggest that the inhibitory effect of U-69593 on the nicotine-induced increase of TH enzyme activity and TH mRNA levels are mediated, at least in part, by the cAMP/PKA pathway.

U-69593 markedly suppressed the nicotine-induced increase of cAMP production, and this inhibitory effect was partially abolished by pretreatment with PTX (Fig. 3b). These findings indicate that $G_{i/o}$ may be involved in the inhibitory mechanism of the κ-opioid agonist, U-69593. As such, it is possible that U-69593 inhibits AD via $G_{i/o}$, which leads to an inhibition of the cAMP/PKA pathway. This in turn results in the inhibition of TH enzyme activity and TH synthesis.

While naloxone and nor-BNI completely abolished the inhibitory effect of U-69593 on nicotine-induced cAMP production, PTX only partially abolished this inhibitory effect of

U-69593 (Fig. 3b). The reasons for this difference are unclear. However, one possible explanation for this difference is that inhibitory mechanisms other than that of $G_{i/o}$, such as the PTX-insensitive G-protein, as suggested by Ozawa et al. may contribute to the inhibitory effects of U-69593 [26]. Further studies (i.e. test the level of ADP ribosylation) will be needed to clarify this point.

The effective concentrations of U-69593 that we described in the present study (far greater than its K_d value in PC12 cells (2.18 nM) [27]) are rather high. This indicates that the effects of U-69593 we observed here are not mediated by an opioid receptor, which is consistent with the inhibitory effect of U-50488 (another κ-agonist) [14,28]. However, Venihaki et al. [13] demonstrated that U-69593 inhibited nicotine-induced catecholamine secretion in PC12 cells at high concentrations, which is comparable to that observed in our study. They also showed that the inhibitory effect of U-69593 was reversed by nalaxone and claimed that the U-69593 effects were mediated by opioid receptors, which is in agreement with our study. The precise reasons for these discrepancies are unclear and further studies will be needed to determine whether opioid receptors are involved in the inhibitory mechanisms of U-69593. Also, the effect of using different drugs (U-69593 vs. U-50488) should be considered.

It was previously reported [13] that U-69593 did not alter intracellular catecholamine levels in PC12 cells and that the anti-cholinergic effects of κ-opioids were due to the inhibition of catecholamine release rather than biosynthesis. While this obviously disagrees with the results presented here, the reasons for this difference are unclear. Venihaki et al. [13] did not examine the effects of U-69593 on the TH enzyme activity and TH synthesis in PC12 cells. Also, differences in exposure time to U-69593 (24 h vs. 12 h) could be significant.

In contrast to the inhibitory effects of U-69593, DPDPE and DAGO (δ- and μ-opioid agonists, respectively) had no significant effects on the nicotine-induced TH enzyme activity and TH synthesis, confirming that δ- and μ-opioids play a minimal role in the anti-cholinergic effect in chromaffin cells [13,14].

Kampa et al. [12] recently demonstrated that the κ-sites were the dominant opioid binding sites in human pheochromocytomas. Combining our findings presented here with previous studies, it would appear that κ-opioid may inhibit catecholamine biosynthesis as well as catecholamine release in human pheochromocytomas. Alternatively, differences in tissue content of κ-opioid peptide may contribute to the clinical manifestations of pheochromocytomas, by the inhibition of catecholamine biosynthesis [10,11].

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