

## Stimulation of Noradrenaline Release by T-588, a Cognitive Enhancer, in PC12 Cells

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**ABSTRACT**—Previously, we reported that (*R*)-(–)-1-(benzo[*b*]thiophen-5-yl)-2-[2-(*N,N*-diethylamino)ethoxy] ethanol hydrochloride (T-588), a novel putative cognitive enhancer, stimulated noradrenaline (NA) release from rat cerebral cortical slices. In this study, we investigated the effects of T-588 compared to other secretagogues on NA release from PC12 cells. Addition of as little as 10  $\mu$ M T-588 stimulated [<sup>3</sup>H]NA release in a dose-dependent and an extracellular Ca<sup>2+</sup>-independent manner from PC12 cells. Ten micromolar ionomycin-, 300  $\mu$ M adenosine-5'-*O*-( $\gamma$ -thiotriphosphate)- and 10  $\mu$ M forskolin-induced extracellular Ca<sup>2+</sup>-dependent [<sup>3</sup>H]-NA release was further enhanced by 30  $\mu$ M T-588. Cytosolic synaptophysin and 25-kDa synaptosome-associated protein immunoreactivity was increased by addition of T-588 in a dose-dependent manner. Interestingly, increases in synaptic vesicle-related proteins triggered by T-588 had a 4-min lag time and were completely dependent on extracellular CaCl<sub>2</sub>. These findings suggest that T-588 stimulates NA release from PC12 cells in a Ca<sup>2+</sup>-independent manner. T-588 also induced the translocation of synaptic vesicles in a Ca<sup>2+</sup>-dependent manner.

**Keywords:** T-588, Noradrenaline release, Synaptic vesicle, Synaptophysin, PC12 cell

Activity-dependent changes in synaptic efficacy are believed to underlie the processes of learning and memory (1). Synaptic transmission is mediated by neurotransmitters released from presynaptic nerve terminals. The expression of plasticity in neuronal networks involves the modulation of synaptic function, and the potentiation or suppression of neurotransmitter release may be an important mechanism of this modulation (2, 3). Analysis of rat hippocampal CA3 synapses indicates that enhanced release of neurotransmitter is a crucial event in the induction of long term potentiation, a synaptic mechanism believed to be involved in learning and memory (4). Moreover, a relationship between the central noradrenergic and cholinergic systems has been reported to affect learning and memory processes (5–7). In Alzheimer's disease, abnormalities of the noradrenergic system in the brain have been reported (8–11). Therefore, noradrenaline (NA) is believed to play an important role in the enhancement of cognitive function.

Previously, we reported that learning and memory im-

pairment in rats is ameliorated by injection of T-588, (*R*)-(–)-1-(benzo[*b*]thiophen-5-yl)-2-[2-(*N,N*-diethylamino)ethoxy]ethanol hydrochloride (12–15). T-588 administered daily during the chronic phase improved the working memory deficit, as measured by a three-panel runway task (13), and the spatial memory deficit (15) following transient forebrain ischemia in rats. Additionally, in an *in vivo* microdialysis study, oral administration of 3 or 30 mg/kg T-588 to rats significantly increased acetylcholine and NA overflow in cortical and hippocampal dialysates (16). We also reported that T-588 stimulates NA release from rat cerebral cortex in an *in vitro* study and that T-588-stimulated NA release was extracellular Ca<sup>2+</sup>- and Ca<sup>2+</sup>/calmodulin-independent (17). However, the effects of T-588 on NA release from other neuronal cell types and on mobilization of synaptic vesicle-related proteins have not been studied.

PC12 cells are capable of storing and releasing catecholamines (18) and are often used to analyze the mechanisms of neurotransmitter release. Synaptic vesicle-related proteins such as syntaxin, 25-kDa synaptosome-associated protein (SNAP-25), synaptobrevin/vesicle-associated membrane protein (VAMP), and synaptophysin are expressed in PC12 cells (19). Stimulation with high K<sup>+</sup> and ATP

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causes an influx of  $\text{Ca}^{2+}$  through  $\text{Ca}^{2+}$  channels, and the subsequent release of neurotransmitters such as NA from PC12 cells (20–24). In this study, we examined the effect of T-588 on NA release in PC12 cells and compared it to the effects of various secretagogues. We found that T-588 stimulates NA release from PC12 cells in an extracellular  $\text{Ca}^{2+}$ -independent manner, and T-588 also induces translocation of synaptic vesicles in an extracellular  $\text{Ca}^{2+}$ -dependent manner.

## MATERIALS AND METHODS

### Materials

T-588 was synthesized by Toyama Chemical Co., Ltd. (Toyama). Ionomycin and forskolin were purchased from Sigma (St. Louis, MO, USA). Adenosine-5'-O-( $\gamma$ -thiotriphosphate) (ATP $\gamma$ S) was purchased from Boehringer Mannheim (Mannheim, Germany). Mouse anti-synaptophysin monoclonal antibody was purchased from Chemicon Int. (Temecula, CA, USA). Rat anti-SNAP-25 monoclonal antibody, anti-syntaxin monoclonal antibody, and other materials were obtained from Wako Pure Chemical (Osaka). Anti-mouse IgG and 1-[7,8- $^3\text{H}$ ]NA (1.37 TBq/mmol) were purchased from Amersham (Buckinghamshire, UK).

### Cell culture

PC12 cells (D-type) (25) were cultured on collagen-coated dishes in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) heat-inactivated fetal bovine serum and 5% horse serum. They were kept at 37°C in a humidified 5%  $\text{CO}_2$ /95% air environment. The medium was changed every 48 h until the cells were grown to a subconfluent state.

### Measurement of [ $^3\text{H}$ ]NA release

[ $^3\text{H}$ ]NA release from prelabeled PC12 cells was determined as described previously (24). Subconfluent cells were labeled with 25 nM [ $^3\text{H}$ ]NA for 2 h in modified Tyrode HEPES buffer (137 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 20 mM HEPES; pH 7.0) containing 0.1 mM ascorbic acid. The pH of the buffer was lowered to avoid degradation of NA. The PC12 cells were washed and detached from the dish under a gentle stream of  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free PBS (pH 7.4). Cells were washed by centrifugation at  $200 \times g$  for 2 min at 4°C and suspended in ice-cold  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free Tyrode's buffer (pH 7.4). Cell suspensions (50–70  $\mu\text{g}$  protein/tube) were incubated at 37°C in Tyrode's buffer (total volume, 0.2 ml) containing 0.2% BSA with or without test compounds for 8 min. Reactions were terminated by addition of 0.5 ml of ice-cold HEPES buffer (137 mM NaCl, 20 mM HEPES, 5 mM EGTA and 5 mM EDTA; pH 7.4), followed by a 30-s centrifugation at  $1000 \times g$  at 4°C. The radioactivity in the supernatants was estimated with a liquid scintillation spectrometer. Data are normalized as percentages of non-stimulated release.

### Immunoblotting analysis

PC12 cell suspensions (1.0–1.5 mg protein/tube) were incubated at 37°C in Tyrode's buffer (total volume, 1 ml) with or without T-588 for the given times and then centrifuged for 2 min at  $200 \times g$  and 4°C. In some experiments,  $\text{CaCl}_2$  was omitted and 0.2 mM EGTA was added to the incubation mixture. The cell pellets were suspended in ice-cold homogenate buffer (0.25 M sucrose, 1 mM  $\text{MgCl}_2$ , 0.5 mM EDTA, 10 mM HEPES, 0.5 mM phenylmethanesulfonyl fluoride, 0.5 mM benzamidine, 1 mM dithiothreitol; pH 7.4). Cells were homogenized at 4°C with twenty strokes of a glass/teflon homogenizer, and the total homogenate was centrifuged at  $40,000 \times g$  for 30 min at 4°C to prepare cytosolic and membrane fractions. These fractions were used for immunodetection.

Samples dissolved in Laemmli sample buffer (5 or 6  $\mu\text{g}$ /lane) were electrophoresed in SDS/10% or 12% polyacrylamide gels, and transferred in a semidry transfer system (1 mA/cm $^2$ , 80 cm $^2$ , 60 min) at room temperature onto a nitrocellulose membrane. The nitrocellulose membrane was incubated with PBS containing 0.1% Tween 20 and 1% milk powder to block nonspecific binding; and then it was incubated with the first antibody (anti-synaptophysin antibody, diluted 1:2500, anti-SNAP-25 antibody, diluted 1:500, or anti-syntaxin antibody, diluted 1:500) and the second antibody (horseradish peroxidase linked anti-mouse IgG, diluted 1:2000–4000). Subsequently, membrane-bound horseradish peroxidase-labeled antibodies were detected using an enhanced chemiluminescence detection system (ECL kit, Amersham). Images of reactive bands were analyzed by laser densitometry. The intensity of each band was dependent on the protein concentration.

### Statistics

Values are reported as the means  $\pm$  S.E.M. In the case of multiple comparisons, the significance of differences was determined by ANOVA followed by Dunnett's test. For pairwise comparisons, Student's 2-tailed *t*-test was used. A probability value of  $P < 0.05$  was considered significant.

## RESULTS

### Effect of T-588 on [ $^3\text{H}$ ]NA release

We investigated the effects of T-588 on [ $^3\text{H}$ ]NA release from prelabeled PC12 cells. Table 1 shows that T-588 stimulates [ $^3\text{H}$ ]NA release in a dose-dependent manner from PC12 cells. The stimulatory effects of T-588 at concentrations of 30–300  $\mu\text{M}$  were significant. Perlman et al. (26) previously reported that ionomycin, a  $\text{Ca}^{2+}$  ionophore, stimulates NA release in an extracellular  $\text{Ca}^{2+}$ -dependent manner from PC12 cells. In our study, addition of 10  $\mu\text{M}$  ionomycin induced [ $^3\text{H}$ ]NA release from PC12 cells; the absolute [ $^3\text{H}$ ]NA release (% of total incorporated NA) was  $18.5 \pm 0.9\%$  ( $n = 5$ )

**Table 1.** Effects of T-588 on [<sup>3</sup>H]NA release from PC12 cells

Additions	[ <sup>3</sup> H]NA release (%)	
	None	10 $\mu$ M ionomycin
None	100.0 $\pm$ 4.9	225.8 $\pm$ 12.1
T-588 (10 $\mu$ M)	125.6 $\pm$ 4.6	246.0 $\pm$ 1.5
(30 $\mu$ M)	131.1 $\pm$ 4.9 <sup>a</sup>	263.2 $\pm$ 11.2
(100 $\mu$ M)	130.3 $\pm$ 4.7 <sup>a</sup>	274.0 $\pm$ 10.3 <sup>a</sup>
(300 $\mu$ M)	136.9 $\pm$ 4.2 <sup>a</sup>	270.7 $\pm$ 4.7 <sup>a</sup>

Prelabeled PC12 cells were incubated for 8 min at 37°C with vehicle or T-588 in the presence or absence of 10  $\mu$ M ionomycin. Each experiment was done with duplicate or triplicate determinations and data are normalized as percentages of non-stimulated release. The absolute [<sup>3</sup>H]NA releases (% of total incorporated NA) were 8.2  $\pm$  0.4 and 18.5  $\pm$  0.9 (means  $\pm$  S.E.M. of 3–5 independent experiments) in non-stimulated and ionomycin-stimulated PC12 cells, respectively. <sup>a</sup>P<0.05, significantly different from vehicle alone (non-stimulated) or ionomycin alone.

after 10  $\mu$ M ionomycin and 8.2  $\pm$  0.4% (n = 5) after the vehicle (control). The stimulatory effect of ionomycin was significantly enhanced by T-588 at concentrations of 100 and 300  $\mu$ M (Table 1). Table 2 shows the effect of extracellular CaCl<sub>2</sub> on T-588-stimulated NA release. Addition of 30  $\mu$ M T-588 stimulated [<sup>3</sup>H]NA release to the same extent in the presence or absence of extracellular CaCl<sub>2</sub>. Addition of ATP $\gamma$ S induced [<sup>3</sup>H]NA release in an extracellular CaCl<sub>2</sub>-dependent manner, and maximal [<sup>3</sup>H]NA release (13.6  $\pm$  0.8%, n = 3) was obtained by 300  $\mu$ M ATP $\gamma$ S, as described previously (21, 23, 24, 27). Additional [<sup>3</sup>H]NA release by T-588 was observed in the presence of 300  $\mu$ M ATP $\gamma$ S. Addition of forskolin (10  $\mu$ M) also slightly increased [<sup>3</sup>H]NA release in a CaCl<sub>2</sub>-dependent manner. The effect of T-588 was additive to the effect of forskolin in the presence of extracellular CaCl<sub>2</sub>. On the other hand, in the absence of extracellular CaCl<sub>2</sub>, addition of ATP $\gamma$ S or forskolin did not induce [<sup>3</sup>H]NA release. Additional [<sup>3</sup>H]NA release by T-588 was observed in the presence of ATP $\gamma$ S or forskolin. Furthermore, 50 mM K<sup>+</sup> and 100 nM phorbol 12-myristate 13-acetate induced CaCl<sub>2</sub>-dependent [<sup>3</sup>H]NA release from PC12 cells (24), which was also enhanced by 30  $\mu$ M T-588 (data not shown). These results suggest that T-588 stimulates NA release in an extracellular Ca<sup>2+</sup>-independent manner and additionally stimulates Ca<sup>2+</sup>-dependent NA release.

#### Effect of T-588 on the translocation of synaptic vesicles

Next, we investigated the effects of T-588 on the translocation of synaptic vesicle-related proteins, such as synaptophysin, SNAP-25 and syntaxin, by western blotting. Clift-O'Grady et al. (28) reported that centrifugation (at 27,000  $\times$  g) of post-nuclear homogenates of PC12 cells separates synaptic vesicle-size vesicles, which remain in the supernatant, from larger membranes, which sediment. We prepared the

**Table 2.** T-588-stimulated [<sup>3</sup>H]NA release in a Ca<sup>2+</sup>-independent manner

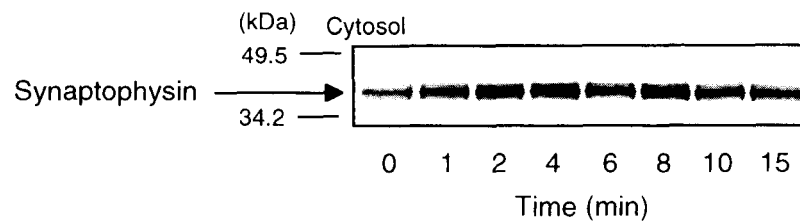
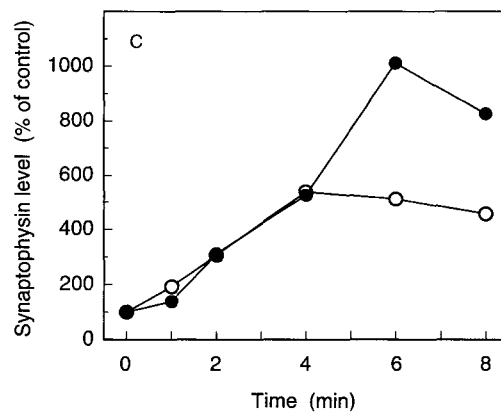
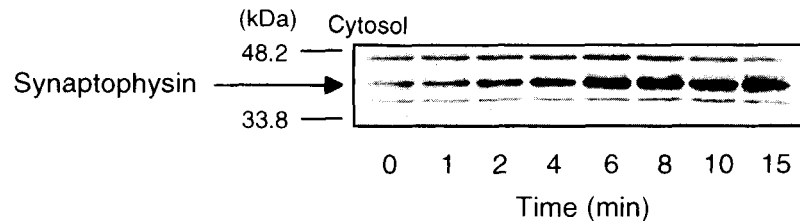
Additions	[ <sup>3</sup> H]NA release (%)	
	None	30 $\mu$ M T-588
1 mM CaCl <sub>2</sub>		
None	100.0 $\pm$ 5.2	130.1 $\pm$ 0.7 <sup>b</sup>
ATP $\gamma$ S (300 $\mu$ M)	140.5 $\pm$ 6.8 <sup>a</sup>	166.7 $\pm$ 10.1 <sup>b</sup>
Forskolin (10 $\mu$ M)	114.4 $\pm$ 3.9 <sup>a</sup>	140.2 $\pm$ 4.0 <sup>b</sup>
CaCl <sub>2</sub> -free (0.2 mM EGTA)		
None	100.0 $\pm$ 9.4	143.5 $\pm$ 5.6 <sup>b</sup>
ATP $\gamma$ S (300 $\mu$ M)	106.4 $\pm$ 1.6	139.9 $\pm$ 3.3 <sup>b</sup>
Forskolin (10 $\mu$ M)	107.2 $\pm$ 2.1	141.0 $\pm$ 2.4 <sup>b</sup>

Prelabeled PC12 cells were incubated for 8 min at 37°C with vehicle, ATP $\gamma$ S or forskolin in the presence or absence of 30  $\mu$ M T-588. Some assay mixtures were further supplemented with 1 mM extracellular CaCl<sub>2</sub>. One experiment was done with triplicate determinations and data are normalized as percentages of non-stimulated release in the presence or absence of 1 mM CaCl<sub>2</sub>. The absolute [<sup>3</sup>H]NA releases (% of total incorporated NA) were 9.7  $\pm$  0.5 and 6.4  $\pm$  0.6 (means  $\pm$  S.E.M. of 3 independent experiments) in the presence or absence of extracellular CaCl<sub>2</sub>, respectively. <sup>a</sup>P<0.05 and <sup>b</sup>P<0.05, significantly different from vehicle alone and without T-588, respectively.

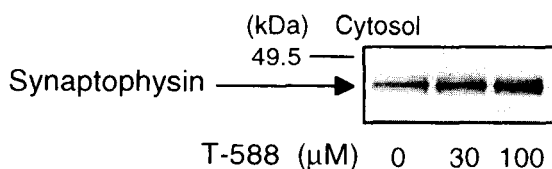
cytosolic and membrane fractions from PC12 cells by their method with minor modifications. The cytosolic fraction contained a substantial amount of free synaptic vesicles and the membrane fraction contained plasma membranes, synaptic vesicles that bound with plasma membrane, and a few free synaptic vesicles. Although a gradual increase in synaptophysin immunoreactivity was observed in the cytosolic fraction from non-stimulated PC12 cells (Fig. 1A), 100  $\mu$ M T-588 stimulated greater increases in synaptophysin (Fig. 1B). The increase in synaptophysin immunoreactivity by T-588 was much greater than in non-stimulated cells at 6 and 8 min post-treatment (Fig. 1C). Next, we investigated the effect of T-588 at 8 min on translocation of synaptophysin (Fig. 2, Table 3). The stimulatory effects of T-588 at 8 min were dose-dependent and remarkable at concentrations from 30–100  $\mu$ M. The increased immunoreactivity of synaptophysin by 30 and 100  $\mu$ M T-588 was 136.9  $\pm$  15.2% and 162.0  $\pm$  19.8%, respectively. In the absence of extracellular CaCl<sub>2</sub>, however, increases in synaptophysin in the cytosolic fraction by 100  $\mu$ M T-588 were not observed (Fig. 3A and Table 3).

We also investigated the effect of T-588 on the translocation of SNAP-25. Although SNAP-25 has been believed to be a presynaptic plasma membrane protein, it was reported recently that significant pools of SNAP-25 are localized to synaptic vesicles (29). Shimazaki et al. (30) reported that a 28-kDa protein in PC12 cells, which was cleaved by botulinum toxin A, was recognized by an anti-SNAP-25 antibody, and it was indicated that the 28-kDa protein was SNAP-25.

## A. None

B. T-588 (100  $\mu$ M)

**Fig. 1.** Increase of synaptophysin immunoreactivity in the cytosolic fraction from T-588-stimulated PC12 cells. PC12 cells were incubated with vehicle (A) or 100  $\mu$ M T-588 (B) at 37°C for the indicated periods. Equal amounts of cytosolic proteins (6  $\mu$ g protein/lane) from PC12 cells were analyzed by Western blotting with anti-synaptophysin antibody. Data represent one of two separate experiments. Images of reactive bands were analyzed by laser densitometry (C). Data are from a typical experiment, are normalized as percentages of non-incubated immunoreactivity, and represent means of 2 to 5 independent experiments.  $\circ$ : None,  $\bullet$ : 100  $\mu$ M T-588.



**Fig. 2.** Dose-dependent increase of synaptophysin immunoreactivity by T-588. PC12 cells were incubated with vehicle or the indicated concentration of T-588 at 37°C for 8 min, and synaptophysin immunoreactivity in the cytosolic fraction was analyzed by Western blotting. Data are from a typical experiment and represent one of 5 to 8 independent experiments.

In our experimental preparations, an approximately 28-kDa protein was immunoreactive with anti-SNAP-25 antibody, and this band was expressed markedly in the membrane fraction (Fig. 3B: lower panel) and slightly in the cytosolic fraction (Fig. 3B: upper panel). A gradual increase in SNAP-25 was observed over time in the cytosolic fraction of non-stimulated PC12 cells in the presence of  $\text{CaCl}_2$ : 139% at 8 min compared with that at 0 min. Incubation with 100  $\mu$ M T-588 increased SNAP-25 in the cytosolic fraction but not the membrane fraction (Fig. 3B). At 8 min, 100  $\mu$ M T-588 induced increases in SNAP-25 in the cytosolic fraction ( $153.9 \pm 13.7\%$ ,  $n=8$ ), compared with the control value. At 8 min, the effect of T-588 was not observed in the absence of extracellular  $\text{CaCl}_2$  (Fig. 3B: upper panel and Table 3). In our

**Table 3.** Synaptophysin and SNAP-25 immunoreactivity in the cytosolic fraction from T-588-treated PC12 cells

Treatment	Amounts of immunoreactive protein (% of control)	
	1 mM CaCl <sub>2</sub>	CaCl <sub>2</sub> -free (0.2 mM EGTA)
Synaptophysin		
None	100	100
T-588 (100 $\mu$ M)	162.0 $\pm$ 19.8	76.1 $\pm$ 16.2
SNAP-25		
None	100	100
T-588 (100 $\mu$ M)	153.9 $\pm$ 13.7	89.7 $\pm$ 11.3

PC12 cells were incubated with vehicle or 100  $\mu$ M T-588 at 37°C for 8 min in the absence or presence of 1 mM CaCl<sub>2</sub>. The 0.2 mM EGTA was further added to the CaCl<sub>2</sub>-free buffer. Synaptophysin or SNAP-25 levels were analyzed by Western blotting. Equal amounts of cytosolic proteins (5 or 6  $\mu$ g protein/lane) from PC12 cells were analyzed with anti-synaptophysin antibody or anti-SNAP-25 antibody. Quantitative determination was performed as described under Materials and Methods. Data are normalized as percentages of non-stimulated immunoreactivity in the presence or absence of 1 mM CaCl<sub>2</sub>, and presented as means  $\pm$  S.E.M. of 3 to 8 independent experiments.

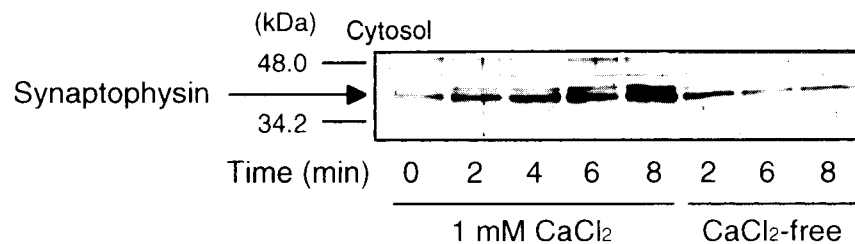
preparation, immunoreactivity of syntaxin was marked in the membrane fraction, but not the cytosolic fraction, and this was not affected by T-588 (data not shown). Ten micromolar ionomycin also markedly increased synaptophysin and SNAP-25 immunoreactivity in the cytosolic fraction from 2–4 min. At 8 min in the presence of extracellular CaCl<sub>2</sub>, synaptophysin and SNAP-25 immunoreactivity increased by 334.1% and 414.9%, respectively. These findings suggest that T-588 increases the translocation of vesicles, which contain synaptophysin and SNAP-25, to the cytosolic fraction, in an extracellular Ca<sup>2+</sup>-dependent manner and after a lag time.

## DISCUSSION

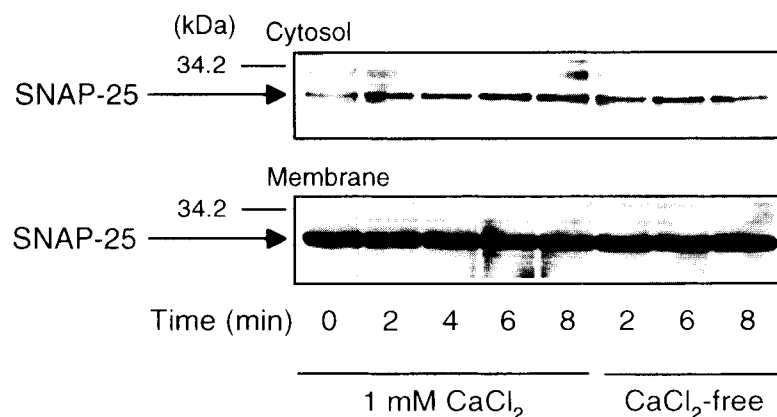
### *Effect of T-588 on [<sup>3</sup>H]NA release*

Previously, we reported that oral administration of 10 or 30 mg/kg T-588 to rats significantly increased NA overflow in an in vivo microdialysis study (16) and that T-588 stimulated NA release from rat cerebral cortical slices in an in vitro study (17, 31). In the present work, we investigated the effects of T-588 on [<sup>3</sup>H]NA release from PC12 cells, which

### A. Synaptophysin



### B. SNAP-25



**Fig. 3.** The effects of T-588 on synaptophysin or SNAP-25 immunoreactivity and extracellular CaCl<sub>2</sub> dependency. PC12 cells were incubated with vehicle or 100  $\mu$ M T-588 at 37°C for the indicated periods in the absence or the presence of 1 mM CaCl<sub>2</sub>. The CaCl<sub>2</sub>-free buffer contained 0.2 mM EGTA. Equal amounts of cytosolic or membrane proteins (5–6  $\mu$ g protein/lane) were analyzed by Western blotting with anti-synaptophysin antibody (A) or anti-SNAP-25 antibody (B). Data are from a typical experiment and represent one of three separate experiments.

are often used as neuronal models. T-588 stimulated [ $^3\text{H}$ ]NA release in a dose-dependent and in an extracellular  $\text{Ca}^{2+}$ -independent manner from PC12 cells. Although T-588 at mM concentrations stimulated NA release from rat cerebral cortical slices (17), T-588-stimulated [ $^3\text{H}$ ]NA release from PC12 cells was observed at  $\mu\text{M}$  concentrations (Table 1). Serum and brain levels of T-588 after single oral administration to rats at 10 mg/kg, which is an effective dose in vivo, were calculated to be about 0.3  $\mu\text{M}$  and 3  $\mu\text{M}$ , respectively (12, 13). It is probable that T-588 is effectively taken up into PC12 cells, not taken up into the brain slices.

The effects of T-588 on [ $^3\text{H}$ ]NA release by various secretagogues from PC12 cells were also examined. The  $\text{Ca}^{2+}$  ionophore ionomycin provides uniform  $\text{Ca}^{2+}$  entry across the entire plasma membrane (32) and causes neurotransmitter release (26, 33). High  $\text{K}^+$  and ATP concentrations also cause an influx of  $\text{Ca}^{2+}$  through  $\text{Ca}^{2+}$  channels (20, 23), possibly clustered in active zones, and neurotransmitter release (20, 21). The effect of T-588 was additive to  $\text{Ca}^{2+}$ -induced [ $^3\text{H}$ ]NA release by ionomycin and ATP $\gamma\text{S}$  from PC12 cells (Tables 1 and 2). NA release from PC12 cells was also stimulated by agents stimulating cyclic AMP accumulation such as forskolin, and the effect of T-588 was additive to the effect of forskolin. T-588 also enhanced [ $^3\text{H}$ ]NA release by high  $\text{K}^+$  or phorbol 12-myristate 13-acetate from PC12 cells (data not shown). These results suggest that T-588 stimulates NA release 1) in brain slices and in other neuronal cells, 2) in lower concentrations in PC12 cells than in brain slices, 3) in a  $\text{Ca}^{2+}$ -independent manner, and 4) additionally to a  $\text{Ca}^{2+}$ -dependent NA release system by ATP $\gamma\text{S}$  or forskolin in PC12 cells.

#### *Effect of T-588 on the synaptic vesicle-related proteins*

Neurotransmitters are released from nerve terminals by  $\text{Ca}^{2+}$ -dependent exocytotic processes: interaction between synaptic vesicle proteins and presynaptic plasma membrane proteins. However, it was recently reported that nitric oxide (NO) donor-stimulated synaptic vesicle release from hippocampal synaptosomes is independent of a rise in intracellular free  $\text{Ca}^{2+}$  concentrations (34). Meffert et al. (35) also reported that biochemical modification by NO alters synaptic protein interactions that regulate neurotransmitter release and synaptic plasticity. These reports raise the possibility that T-588 stimulated NA release by modification of synaptic proteins, like NO. We investigated the effects of T-588 on the translocation of synaptic vesicle-related proteins in PC12 cells. Synaptophysin, a major synaptic vesicle protein, is a hexameric homo-oligomer, which in electron micrographs exhibits structural features common to channel-forming proteins (36). Linstedt and Kelly (37) reported that when PC12 cells were warmed to 37°C for 30 min, some synaptophysin redistributed into the supernatant fraction, which corresponds to the cytosolic fraction in our experiments. As in

their report, synaptophysin in the cytosolic fraction increased after incubation at 37°C without stimulants (Fig. 1A). Addition of T-588 further increased synaptophysin in a time and dose-dependent manner (Fig. 1B). Interestingly, the increase in synaptophysin by T-588 was observed after a 4-min lag, and this was completely dependent on extracellular  $\text{CaCl}_2$  (Fig. 1C and Table 3). The effect of T-588 requires several minutes (lag time), as previously shown in brain slices (17). Clift-O'Grady et al. (28) reported that synaptophysin is localized to small synaptic-like microvesicles, not to the large dense cored vesicles that take up and release [ $^3\text{H}$ ]NA in PC12 cells. In contrast, James and Richard (38) found that synaptophysin is present on both types of vesicles in PC12 cells. Although we could not determine which types of vesicles are sensitive to T-588, it seems that T-588 stimulates the translocation of NA-containing vesicles in PC12 cells.

It was reported recently that significant pools of SNAP-25 are localized to synaptic vesicles, comprising 3% of the total vesicle protein (29). In our preparation, only a slight amount of SNAP-25 was present in the cytosolic fraction, but it was abundantly detected in the membrane fraction. SNAP-25 in the cytosolic fraction from T-588-stimulated PC12 cells increased in an extracellular  $\text{Ca}^{2+}$ -dependent manner (Fig. 3). Ionomycin, which releases NA in a  $\text{Ca}^{2+}$ - and synaptic vesicles-dependent manner (39), also increased synaptophysin and SNAP-25 in the cytosolic fraction. These findings suggest that T-588 enhances the translocation of vesicles to the cytosol from the membrane fraction containing early endosomes and plasma membrane binding vesicles in an extracellular  $\text{Ca}^{2+}$ -dependent manner.

#### *[ $^3\text{H}$ ]NA release and vesicle translocation by T-588*

Our results present a major mystery: the translocation of vesicle-related proteins by T-588 was  $\text{Ca}^{2+}$ -dependent, while NA release by T-588 was  $\text{Ca}^{2+}$ -independent. T-588 may effect vesicle targeting of the plasma membrane. Targeting is proposed to occur through the formation of a 7S complex composed of two vesicle proteins, VAMP and synaptotagmin, along with two target membrane proteins, SNAP-25 and syntaxin (40, 41). NO donors increase formation of the VAMP/SNAP-25/syntaxin, which is the core complex of the 7S and 20S complex, and stimulate  $\text{Ca}^{2+}$ -independent release (34, 35). Thus, it is probable that T-588 enhances and/or stabilizes the formation of complexes on plasma membranes without  $\text{Ca}^{2+}$  and stimulates NA release. In this scenario, T-588 is able to stimulate NA release without translocation of vesicles and in the absence of  $\text{CaCl}_2$ .

In conclusion, we found that 1) T-588 at  $\mu\text{M}$  concentrations stimulates NA release from PC12 cells in a  $\text{Ca}^{2+}$ -independent manner, and 2) T-588 also increases the translocation of vesicles in a  $\text{Ca}^{2+}$ -dependent manner. The rule and/or contribution of translocation of vesicles by T-588 on NA release should be determined. Also, because the precise

target of T-588 has not been established, further studies are needed.

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