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## Effects of substance P on nicotine-induced intracellular $\text{Ca}^{2+}$ dynamics in bovine adrenal chromaffin cells

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### Abstract

Substance P (SP) is colocalized with ACh in splanchnic nerves that innervate into adrenal medulla and the peptide has been shown to inhibit nicotinic agonists-induced catecholamine secretion. To elucidate the effects of SP on cytosolic  $\text{Ca}^{2+}$  dynamics, the present study was conducted using fura-2-loaded isolated bovine adrenal chromaffin cells. Stimulation of the cells with nicotine (10–100  $\mu\text{M}$ ) produced a rapid rise of cytosolic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ), the peak level of which increased in a dose-dependent manner, followed by a gradual decay. In the presence of 10  $\mu\text{M}$  SP, the dose-response relationship of the peak levels shifted downward. Quantitative analyses implied that SP inhibits the nicotine-induced  $\text{Ca}^{2+}$  influx in a noncompetitive manner. Nicotinic acetylcholine receptor is composed of two major functional domains: an agonist-binding site and an ionophore or channel domain. Agonist binding activates ionophore / channel domain and causes mainly  $\text{Na}^+$  influx. This  $\text{Na}^+$  influx depolarizes the cell and activates voltage-dependent  $\text{Ca}^{2+}$  channels. Based on this fact, the present results indicate that SP does not block nicotine binding sites but interferes with other sites of nicotinic receptor / channel molecule, most probably a channel domain. It was suggested that SP colocalized with ACh in splanchnic nerves functions as a physiological modulator of catecholamine secretion by non-competitively suppressing ACh-induced cytosolic  $\text{Ca}^{2+}$  dynamics in bovine adrenal chromaffin cells.

Key words: adrenal chromaffin cells, bovine,  $\text{Ca}^{2+}$ , fura-2, substance P

### Introduction

Catecholamine secretion from adrenal chromaffin cells is regulated mainly by acetylcholine released from splanchnic nerve terminals. Recently, various non-cholinergic factors such as neuropeptides<sup>12)</sup> and nitric oxide<sup>6,9)</sup> are identified in adrenal medulla and these substances are

thought as cotransmitters or modulators of ACh-regulated catecholamine secretion. Substance P (SP), an undeca peptide, is one of these non-cholinergic neurotransmitters which are colocalized with ACh in splanchnic nerves<sup>13,16,30,40)</sup>, implying that this peptide shares some roles with ACh in regulation of catecholamine secretion. So far, two major effects of SP on ACh-induced

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catecholamine secretion have been reported ; 1) inhibition of secretion<sup>15,17,22,23,29,32,37,38,39</sup> and 2) protection of desensitization<sup>4,5,17,18,26</sup>. Nicotinic acetylcholine receptor is composed of two major functional domains : an agonist-binding site and an ionophore or channel domain. Agonist binding activates ionophore / channel domain and causes mainly Na<sup>+</sup> influx. This Na<sup>+</sup> influx depolarizes the cell and activates voltage-dependent Ca<sup>2+</sup> channels. The possible site of SP action is believed to be this nicotinic acetylcholine receptor since SP is without effect when secretion is induced by depolarization with a high potassium solution or by some muscarinic agonists<sup>29,39</sup>.

In chromaffin cells, stimulation with ACh or nicotine induces a large increase in cytosolic Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>). It has been reported that in adrenal chromaffin cells the membrane capacitance, a linear index of frequency of exocytosis, increases proportionally to a power of approximate 1.5 of total amount of Ca<sup>2+</sup> which entered the cell<sup>10</sup>, suggesting that there is a non-linear relationship between the agonist-induced [Ca<sup>2+</sup>]<sub>i</sub> levels and the degree of granular exocytosis. So far, little has been reported on the effect of SP on ACh-triggered intracellular Ca<sup>2+</sup> dynamic. In the current study, we carried out quantitative experiments by recording changes in [Ca<sup>2+</sup>]<sub>i</sub> in response to nicotine in the presence or the absence of SP. The results obtained were then analyzed to define the mode of inhibitory action of SP on nicotine-induced [Ca<sup>2+</sup>]<sub>i</sub> dynamics with the aid of enzyme kinetics by using Lineweaver-Burk plots. Similar analyses were also made for the inhibitory effect of hexamethonium (C<sub>6</sub>), a typical competitive nicotinic antagonist, to compare the effects between these two inhibitors.

## Materials and Methods

### Solutions

Following solutions were used ; *Standard*

*HEPES-Buffered Ringer Solution (HBS)* is composed of 130 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM NaHCO<sub>3</sub>, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 11.1mM glucose, and 15mM HEPES (2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid) (Dojindo Laboratories, Kumamoto, Japan). pH was adjusted to 7.4 with NaOH. *Physiological saline* containing 0.9% NaCl, 200 U/ml Penicillin (Gibco BRL, Grand Island, NY, USA), 200 µg/ml Streptomycin (Gibco BRL, Grand Island, NY, USA), and 0.25 µg/ml Fungizone (Gibco BRL, Grand Island, NY, USA). *Solution A* is composed of Ca<sup>2+</sup>-free HBS supplemented with 200 U/ml Penicillin, 200 µg/ml Streptomycin, and 0.25 µg/ml Fungizone. *Solution B* is composed of Ca<sup>2+</sup>-free HBS supplemented with 200 U / ml Penicillin, 200 µg/ml Streptomycin, 0.25 µg/ml Fungizone, and 0.1% bovine serum albumin (BSA ; Fraction V, Sigma Chemical, St Louis, MO, USA). *Solution C* is composed of Ca<sup>2+</sup>-free HBS containing 0.1% BSA. *Digestion medium* is Ca<sup>2+</sup>-free HBS supplemented with 200 U/ml collagenase (type II, Worthington Biochemicals, Freehold, NJ, USA) and 4 U/ml DNase (Grade II, Boehringer Mannheim, Mannheim, Germany). *BSA solution* is composed of standard HBS containing 4% BSA. *Culture medium* : DMEM (Gibco BRL, Grand Island, NY, USA) supplemented with 10% FCS (Irvine Scientific, Santa Ana, CA, USA) and 100 U/ml Penicillin and 100 µg/ml Streptomycin. *Perfusion solution* : standard HBS supplemented with 0.1% BSA. *Preparation and primary culture of isolated bovine chromaffin cells*

Bovine chromaffin cells were isolated by collagenase digestion. Bovine adrenal glands were obtained from a local slaughterhouse (Hokkaido Ebetsu Meat Inspection Center). Bilateral adrenal glands were excised, and surrounding fat was removed. After rinsing with *Physiological saline* the glands were retrogradely infused with *Solution B* through adrenal vein to

flash out remaining blood and were kept in *Solution A*. The Digestion medium was then retrogradely circulated at a rate of 3 ml/min at 37°C for 30 min followed by the second circulation with a fresh *Digestion medium* for an additional 1 hr. Adrenal medulla were isolated and resuspended in *Solution C*, and filtered through 350  $\mu$ m nylon mesh (the first suspension). Tissue fragments remained on the mesh were re-digested at 37°C with vigorous shaking for 30 min. The digested tissue was again filtered through 350  $\mu$ m mesh (the second suspension). Both suspensions were mixed, centrifuged (25 x g) for 2 min, and the cell pellet was resuspended in *Solution C*. The cell suspension was overlaid on 4% BSA solution, centrifuged (25 x g) for 2 min, and the pellet was resuspended in *Culture medium* and incubated at 37°C under humidified air with 5% CO<sub>2</sub>. Cells cultured for 2–10 days were used for experiments. All the experiments were carried out by using *Perifusion solution*.

#### *[Ca<sup>2+</sup>]<sub>i</sub> measurement*

After rinsing adrenal chromaffin cells three times with *Perifusion solution*, cells were loaded with 3  $\mu$ M Fura-2/AM (Dojindo Laboratories, Kumamoto, Japan) and left for 1 hr at room temperatures. Microfluorometric measurements of  $[Ca^{2+}]_i$  were carried out as reported elsewhere<sup>14</sup> with a P-1 system (Nikon, Tokyo, Japan). Briefly, Fura-2-loaded cells were attached on the surface of non-fluorescent cover glass which was glued to an original stainless chamber. The chamber was placed on a stage of an inverted microscope (TMD-2, Nikon, Tokyo, Japan) and the cells were perifused at a rate of 1 ml/min. Ratiometry was done by selecting a single cell. Calcium concentrations were calculated as reported by Grynkiewicz *et al.*<sup>11</sup>.

#### *Statistical analyses*

Results were shown as means  $\pm$  SE. Student's *t*-test was adopted in most statistical analyses. For analyses of Lineweaver-Burk plots, regression lines were obtained by the least

square method and significance was evaluated with correlation coefficients. Ninety-five percent confidence limit was calculated for each regression line and used to accomplish stoichiometric analyses.

## Results

### *Effects of nicotine on [Ca<sup>2+</sup>]<sub>i</sub>*

Chromaffin cells were stimulated with 10–100  $\mu$ M nicotine for 2 min. Fig. 1 shows that nicotine rapidly increased  $[Ca^{2+}]_i$ , which was followed by a gradual decay. Time at which  $[Ca^{2+}]_i$  reached a peak level varied from experiment to experiment but it was always within 30 sec after the stimulation. As shown in Fig. 1B, mean peak  $[Ca^{2+}]_i$  level increased as nicotine concentration increased. Repetitive stimulation of the cells with nicotine, especially with higher concentrations, caused a gradual decay of the peak levels (data not shown). As this desensitization phenomenon may complicate further analyses, the stimulation was made only once on one preparation and analyses were carried out with each peak  $[Ca^{2+}]_i$  value. Stoichiometric analyses by adopting Lineweaver-Burk plot showed that there was significant linear relationship between reciprocals of the second power of nicotine concentrations and reciprocals of peak  $[Ca^{2+}]_i$  levels ( $r=0.815$ ) (Fig. 2).

### *Effects of SP on stoichiometry of nicotine-induced [Ca<sup>2+</sup>]<sub>i</sub> responses*

Addition of 10  $\mu$ M SP 1 min before and during the stimulation with nicotine significantly lowered the peak  $[Ca^{2+}]_i$  levels at all nicotine concentrations tested ( $P<0.01$ ) (Fig. 1A). At 10  $\mu$ M nicotine alone, an average of the peak  $[Ca^{2+}]_i$  level attained  $740 \pm 92$  nM ( $n=10$ ) and this level was reduced to  $314 \pm 75$  nM ( $n=10$ ) by the addition of SP ( $P<0.01$ ). At a maximal nicotine concentration (100  $\mu$ M) tested, the mean peak level significantly reduced from  $1,271 \pm 197$  nM ( $n=11$ ) to  $528 \pm 124$  nM ( $n=9$ ) by the peptide ( $P<0.01$ ). The dose-response curve

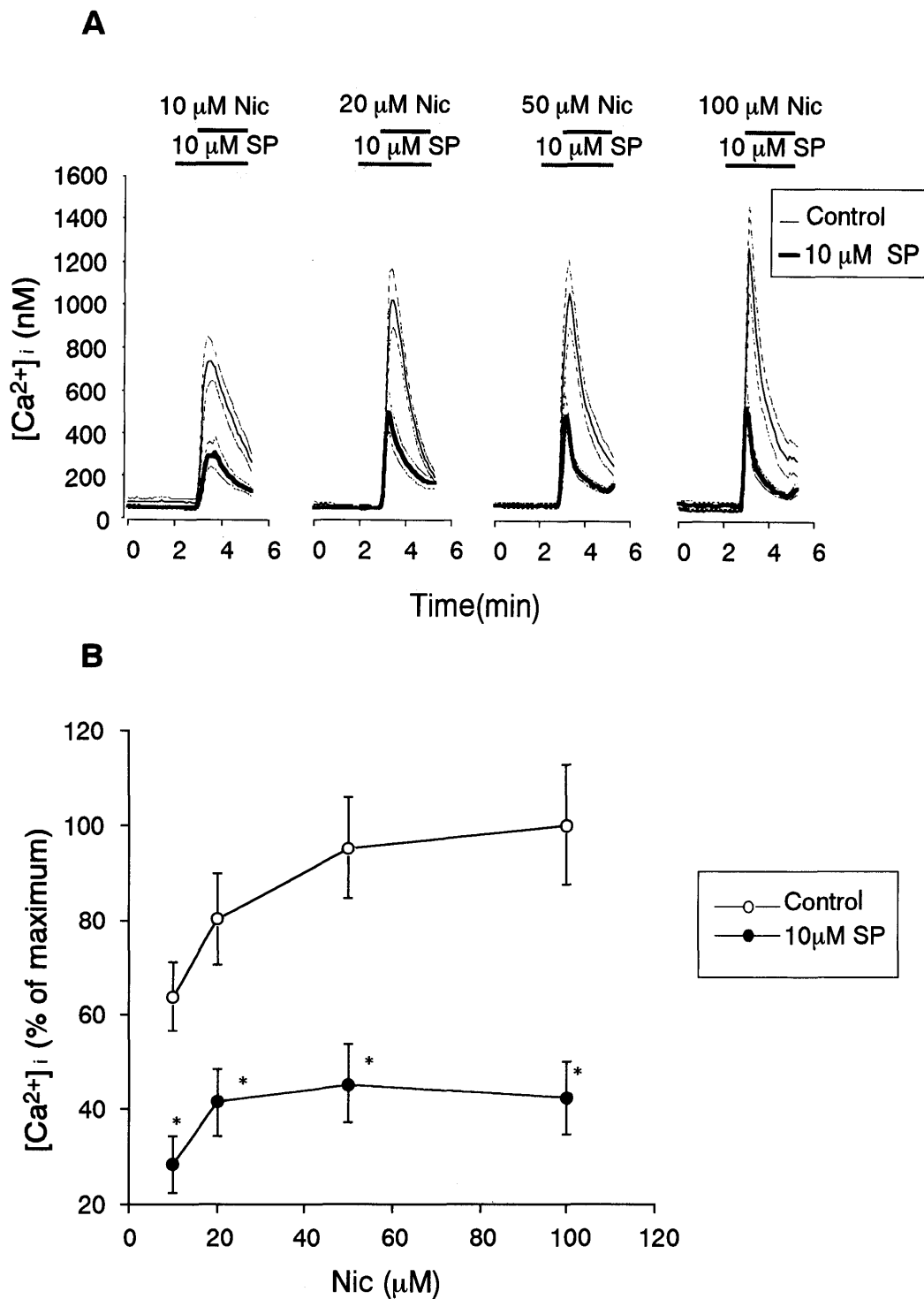


Fig. 1 A; Effects of SP on the time courses of  $[Ca^{2+}]_i$  changes induced by increasing concentrations of nicotine. Bovine adrenal chromaffin cells were stimulated with 10, 20, 50, and 100  $\mu M$  nicotine for 2 min and  $[Ca^{2+}]_i$  was monitored. Values are the means  $\pm$  SE obtained from 9–11 experiments. Thin solid lines indicate  $[Ca^{2+}]_i$  responses in the control group and thick ones denote those treated with 10  $\mu M$  SP. Thin dotted lines above and beneath each line show SE. B; Effects of SP on the dose-response relationships. An average value of peak  $[Ca^{2+}]_i$  induced with 100  $\mu M$  nicotine in the absence of SP was set as 100% (maximum) and each peak  $[Ca^{2+}]_i$  in each experiment was averaged and plotted as percent of maximum. Open circles denote the means in the control group and filled circles show those in the SP-treated group. Vertical lines show SE. \*:  $P < 0.01$  ( $[Ca^{2+}]_i$  in the control vs  $[Ca^{2+}]_i$  in the presence of SP at each nicotine concentration.)

was found to be shifted downward in the presence of  $10 \mu\text{M}$  SP, in which a maximal response was obtained at a nicotine concentration of as low as  $20 \mu\text{M}$  (Fig. 1B). No further increase was observed with higher nicotine concentrations. SP itself was without effect on unstimulated resting  $[Ca^{2+}]_i$  levels. Stoichiometric analyses for these data by adopting Lineweaver-Burk plot showed that there was also significant linear relationship between reciprocals of the second power of nicotine concentrations and reciprocals of peak  $[Ca^{2+}]_i$  levels ( $r=0.971$ ) (Fig. 2).

Theoretical maximal responses ( $V_{\max}$ ) obtained by extrapolating the regression lines gave  $1,059 \text{ nM}$  for control and  $339 \text{ nM}$  for SP-treated group, respectively (Fig. 2). As 95% confidence limit area did not overlap each other, it was concluded that  $V_{\max}$  of  $[Ca^{2+}]_i$  was significantly reduced in the presence of SP. No inhibition by SP was found in  $30 \text{ mM K}^+$ - or  $500 \mu\text{M}$  methacholine-, a muscarinic agonist in chromaffin cell, induced  $[Ca^{2+}]_i$  response (data

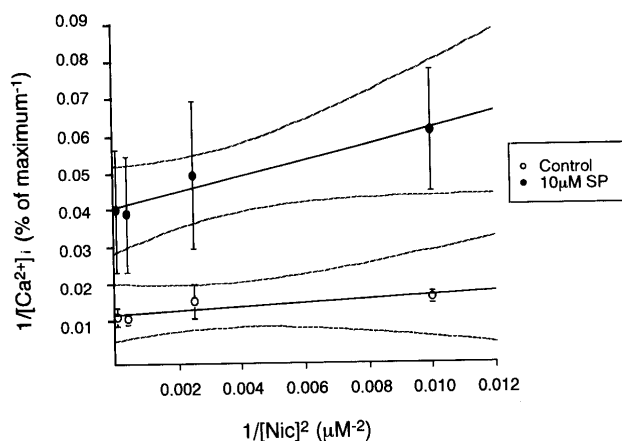


Fig. 2 Lineweaver-Burk plots and regression lines obtained from the dose-response relationship. Results were expressed as percentage of the mean maximal response of  $[Ca^{2+}]_i$  obtained with  $100 \mu\text{M}$  nicotine. Solid lines denote regression lines and dotted lines indicate 95% confidence limits of respective lines. Open circles show the means in the control group and filled circles show those in the SP-treated group. Vertical lines denote SE ( $n=9-11$ ).

not shown).

#### *Effects of $C_6$ on stoichiometry of nicotine-induced $[Ca^{2+}]_i$ responses*

We carried out similar experiments by using  $C_6$ , a classical competitive antagonist of nicotinic acetylcholine receptor (Fig. 3). In this series of experiments,  $10 \mu\text{M}$  nicotine induced an averaged peak  $[Ca^{2+}]_i$  level of  $496 \text{ nM} \pm 71 \text{ nM}$  ( $n=10$ ), which was somewhat lower than the value obtained in counterpart experiments for SP effects probably due to unexpected variations in experimental conditions. This peak level significantly decreased to  $287 \pm 55 \text{ nM}$  ( $n=14$ ) by the addition of  $1 \mu\text{M}$   $C_6$  ( $P<0.05$ ). This inhibition by  $C_6$  became unclear as nicotine concentration increased (Fig. 3A). From the dose-response curve, it was found that there was no significant inhibition in  $[Ca^{2+}]_i$  response to nicotine at concentrations higher than  $10 \mu\text{M}$  (Fig. 3B).  $C_6$  itself was without effect on unstimulated resting  $[Ca^{2+}]_i$  levels.

Stoichiometric analyses by Lineweaver-Burk plot indicated that theoretical  $V_{\max}$  obtained by extrapolating the regression lines ( $r=0.924$  for control and  $r=0.994$  for  $C_6$ -treated group, respectively) gave  $598 \text{ nM}$  for control and  $555 \text{ nM}$  for  $C_6$ -treated group, respectively (Fig. 4). As 95% confidence limit area overlapped each other, it was concluded that  $V_{\max}$  of  $[Ca^{2+}]_i$  response was not changed by  $C_6$  pretreatment.

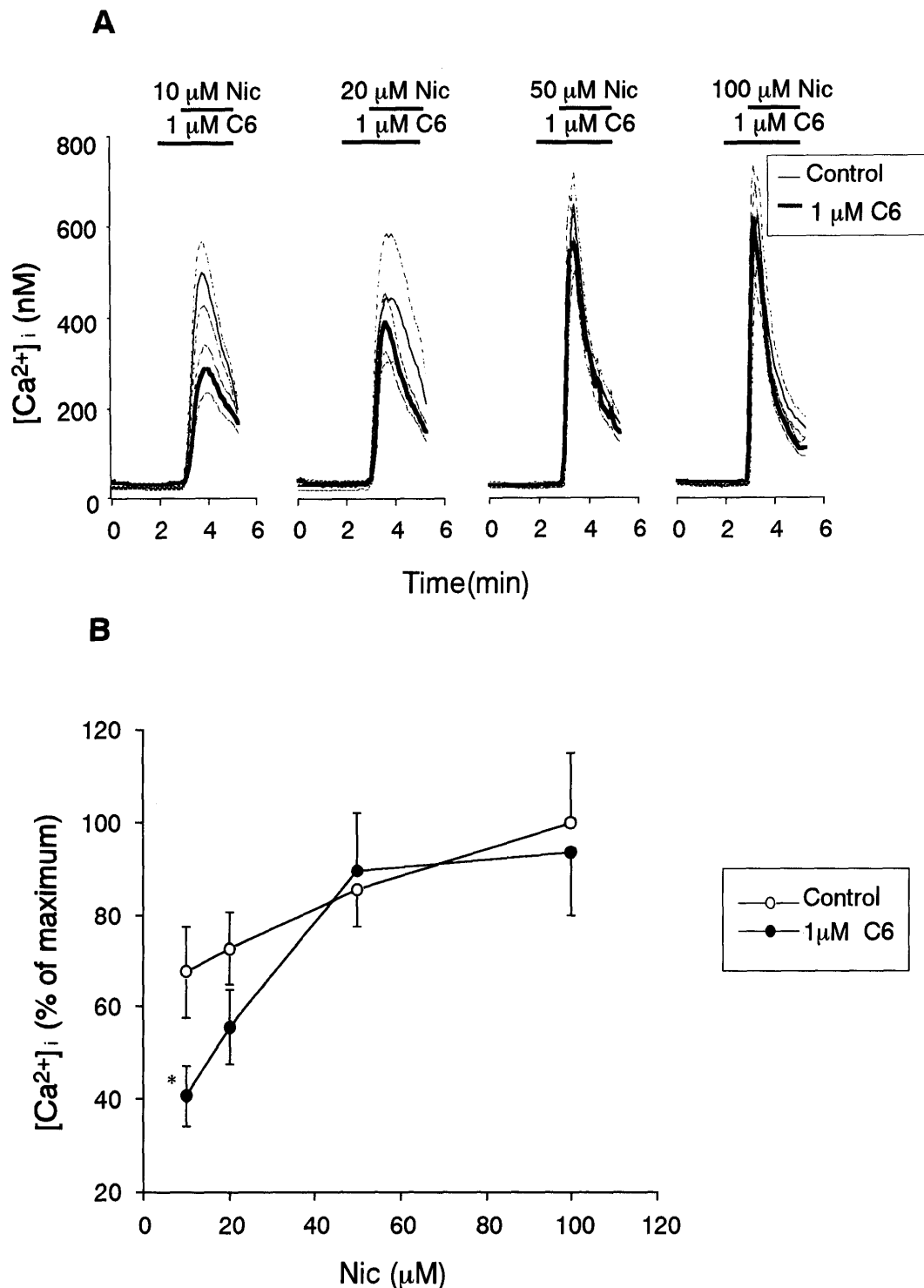


Fig. 3 A; Effects of C<sub>6</sub> on the time courses of mean [Ca<sup>2+</sup>]<sub>i</sub> changes induced by increasing concentrations of nicotine. Bovine adrenal chromaffin cells were stimulated with 10, 20, 50, and 100 μM nicotine for 2 min and [Ca<sup>2+</sup>]<sub>i</sub> was monitored. Values are the means ± SE obtained from 10–14 experiments. Thin solid lines indicate [Ca<sup>2+</sup>]<sub>i</sub> responses in the control group and thick ones denote those treated with 1 μM C<sub>6</sub>. Thin dotted lines above and beneath each line show SE. B; Effects of C<sub>6</sub> on the dose-response relationships. An average value of peak [Ca<sup>2+</sup>]<sub>i</sub> induced with 100 μM nicotine in the absence of C<sub>6</sub> was set as 100% (maximum) and each peak [Ca<sup>2+</sup>]<sub>i</sub> in each experiment was averaged and plotted as percent of maximum. Open circles indicate the means in the control group and filled circles denote those in the C<sub>6</sub>-treated group. Vertical lines show SE. \* : P < 0.05 ([Ca<sup>2+</sup>]<sub>i</sub> in the control vs [Ca<sup>2+</sup>]<sub>i</sub> in the presence of C<sub>6</sub> at each nicotine concentration.)

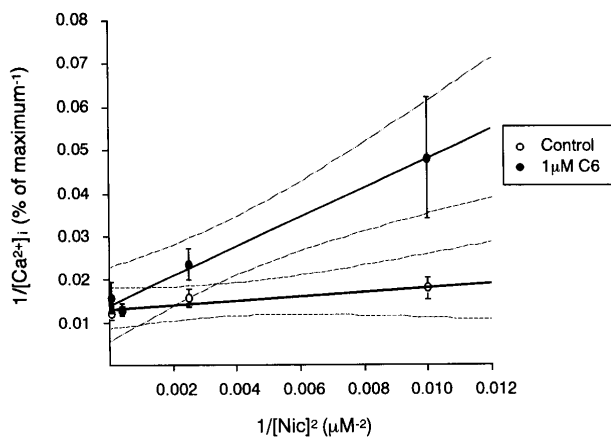


Fig. 4. Lineweaver-Burk plots and regression lines obtained from the dose-response relationship. Results were expressed as percentage of the mean maximal response of  $[Ca^{2+}]_i$  obtained with  $100 \mu M$  nicotine. Solid lines denote regression lines and dotted lines indicate 95% confidence limits of respective lines. Open circles show the means in the control group and filled circles show those in the  $C_6$ -treated group. Vertical lines denote SE ( $n=10-14$ ).

### Discussion

SP is known as a neurotransmitter of sensory nerve terminals. Belcher and Ryall<sup>1)</sup> and Krnjevic and Letic<sup>19)</sup> reported that SP inhibited acetylcholine-induced activation of cat spinal Renshaw cells, suggesting that SP may ubiquitously be involved in modulating cholinergic regulation. Immunocytochemical studies showed that SP exists in adrenal medulla<sup>21)</sup>, and not only sensory nerves but also splanchnic nerves which innervate into adrenal medulla contain this peptide<sup>13,16,30,40)</sup>. An inhibitory effect by SP on nicotinic agonists-induced catecholamine secretion in chromaffin cells was first reported by Livett *et al.*<sup>23)</sup>. In addition to the inhibitory effect, a protective effect of the peptide on acetylcholine-induced desensitization was reported subsequently<sup>4)</sup>, indicating that SP possesses both inhibitory and facilitatory effects on catecholamine secretion. These dual effects of SP were examined and confirmed in experi-

ments in which inward currents<sup>7)</sup>,  $^{22}Na^+$  uptake<sup>28,33,34)</sup>,  $^{45}Ca^{2+}$  uptake<sup>3)</sup>, and receptor binding<sup>2,27,35,36)</sup> were investigated as indices<sup>24,25)</sup>.

Real-time monitoring of  $[Ca^{2+}]_i$  is a useful tool to assess cellular mechanisms by which SP exerts its inhibitory effect as  $Ca^{2+}$  is an intracellular second messenger that links receptor activation and exocytosis. If SP has some modulatory effects on nicotinic receptor, there would be a strong possibility that  $[Ca^{2+}]_i$  may be influenced since receptor activation by nicotine can firstly activate voltage-dependent  $Ca^{2+}$  channels and induces  $Ca^{2+}$  influx. In the present experiments, temporal dynamics of  $[Ca^{2+}]_i$  were directly monitored to explore possible mechanisms of SP since, except for work done by Boksa<sup>3)</sup>, no report has been available so far on the effect of SP on receptor-activated  $Ca^{2+}$  dynamics. Thus we can estimate agonist-receptor stoichiometry by measuring  $[Ca^{2+}]_i$  dynamics more directly. When the amount of secretion is chosen as an index of cell function, stoichiometric analyses on the effects of SP may become erroneous since, in bovine adrenal chromaffin cells, it was reported that the membrane capacitance by which the degree of exocytosis can be monitored electrically increases proportionally to a power of approximate 1.5 of total amount of  $Ca^{2+}$  which entered the cell<sup>10)</sup>.

A linear relationship was obtained between reciprocals of the second power of nicotine concentrations and reciprocals of peak  $[Ca^{2+}]_i$  levels. This result is compatible with a view that there are two binding sites for ACh on one nicotinic acetylcholine receptor molecule and binding of two molecules of agonist induces a quantum  $[Ca^{2+}]_i$  change by activating voltage-dependent calcium channels. Thus receptor activation appears to linearly correlate with  $[Ca^{2+}]_i$  increases and this process does not require any mediators which amplify or attenuate signaling between receptor activation and  $[Ca^{2+}]_i$

increases. Therefore,  $[Ca^{2+}]_i$  dynamics rather than catecholamine secretion can be an appropriate index to directly and quantitatively assess stoichiometry of agonist-receptor interaction.

Quantitative analyses with Lineweaver-Burk plots revealed that the  $V_{max}$  of the SP-treated cells was lowered to one third of that of the control group. In contrast, the  $V_{max}$  of the  $C_6$ -treated group and the control group was coincided. These results suggest that SP noncompetitively inhibits the nicotinic receptor-activated  $[Ca^{2+}]_i$  increases, while  $C_6$ , as widely accepted, competitively suppresses the intracellular  $Ca^{2+}$  signaling.

A next question is the site of interaction of SP in nicotinic receptor molecule. Although the present study does not give any direct insights into possible sites of action of SP, previous studies have proposed a view that SP may directly interact with receptor-ion channel complex. Supports for this view are: 1) SP was ineffective on responses induced by high  $K^+$  or muscarinic agonists but was effective only on those elicited by nicotinic agonists<sup>29,39</sup>, 2) SP can bind with nicotinic receptors<sup>27</sup>, and 3) SP reduces nicotinic agonist-induced  $^{22}Na^+$  influx<sup>28,33,34</sup>. A nicotinic receptor is composed of 5 subunits, each of which possesses hydrophilic extracellular domain, intracellular loops, and hydrophobic membrane-spanning region. The membrane-spanning region, M2, is believed to be an ion channel. Any subtypes of nicotinic receptor have two  $\alpha$  subunits and adjacent  $\beta$  (nerve type receptor),  $\gamma$ , and  $\delta$  (muscle and electric organ type) subunits. Two agonist binding sites are extracellularly located in-between each of  $\alpha$  subunit and two of other subunits. Furthermore, agonist binding sites and ion channel are thought to be very proximal. It is known that there are not only agonist- or competitive antagonist-binding sites in a receptor molecule but also noncompetitive antagonist-binding site within an ionic channel domain of the receptor<sup>31</sup>.

Léna and Changeux stated that, although the biochemical properties of neuronal nicotinic acetylcholine receptor remain largely unexplored and the interpretation of the electrophysiological data in terms of the allosteric scheme remains speculative, the noncompetitive blockers interact with two kinds of sites; a unique high-affinity site that is sensitive to histrionicotoxin and whose affinity and access is facilitated by agonist binding, and 10–30 low affinity sites (per molecule of nicotinic acetylcholine receptor in Torpedo membrane preparations)<sup>20</sup>. Previous reports also showed that SP inhibited binding of a noncompetitive antagonist, phencyclidine, to nicotinic receptor in the presence of carbachol<sup>36</sup>. Photoaffinity labeling showed that SP bound with M2 of  $\delta$  subunit<sup>2</sup>. An inhibitory effect of SP on acetylcholine-induced inward current was found to be strongly dependent on  $\beta$  subunit of a mutational nicotinic receptor of neuron type which is expressed with genetic recombination<sup>35</sup>. Further studies remain to clarify interacting sites of SP with nicotinic receptor.

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