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Report

Agonist-Induced Substance P Receptor Down-Regulation in Rat Central Nervous System

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Rat brain slices were incubated with substance P (SP), and the SP receptors on the membranes from those slices were characterized by a ^{3}H-SP binding technique. The number of substance P receptors measured in the extensively washed membrane preparations pretreated with 3×10^{-5} *M* SP was reduced by 30% compared with that in nontreated membranes. This reduction was dependent on the incubation time and temperature. The metabolic inhibitors sodium azide and 2,4-dinitrophenol protected SP receptors from the reduction. The characteristics of ^{3}H-SP incorporation into rat brain slices were similar to those of SP receptor down-regulation, that is, the ^{3}H-SP incorporation was time, temperature, and energy dependent. Thus these results indicate that the processes of ligand incorporation and receptor down-regulation are closely associated phenomena. These observations may be important in elucidating the phenomenon of SP-induced desensitization.

KEY WORDS: Substance P receptors; down-regulation; rat brain slices, ^{3}H-substance P incorporation.

INTRODUCTION

The 11-amino acid peptide, substance P (SP), a member of the tachykinin family, is a putative neurotransmitter widely distributed in the mammalian nervous system. The regional distribution of SP receptors in rat central nervous system had been previously shown by autoradiography (1,2). SP may play some physiological role via its specific receptors in the central nervous system (CNS). An increase in SP concentration in the receptor biophase may result in adaptive changes culminating in a loss of cellular responsiveness to the agonist, a phenomenon known as desensitization. Desensitization is a widespread phenomenon in a variety of neurotransmitter receptor systems. Several *in vitro* models have been used to study desensitization of SP-induced effects. These include an increase in coronary blood flow (3), the hypotensive effect (4), the secretion of saliva from rat submaxillary gland (5), and the contraction of guinea pig ileum preparation (6). Further, the desensitization of SP-induced K^+ release in rat parotid has been well characterized (7). However, desensitization of the SP response has not been studied in great detail in the CNS, and its mechanisms are almost completely unknown. Sjödin (8) suggests the internalization of SP receptor into guinea pig pancreatic acinar cells. Sugiya *et al.* (9) indicate SP-induced homologous desensitization of SP receptor in rat parotid acinar cells, which involves the down-regulation or sequestration of SP binding sites.

We have studied the characteristics of ^{3}H-SP incorporation and SP-induced reduction of ^{3}H-SP binding activity in rat CNS, which may be important in elucidating the phenomena of SP-induced desensitization.

MATERIALS AND METHODS

SP Receptor Down-Regulation Experiments

Whole brain, except cerebellum, from adult male Wistar rats was minced using a razor blade. The resulting slices were incubated at 37°C with and without nonradioactive SP (3×10^{-5} *M*) with shaking in a Krebs–Ringer (KR) solution of the following composition (m*M*)—NaCl, 118; KCl, 4.8; $CaCl_2$, 2.5; $MgCl_2$, 1.1; NaH_2PO_4, 1.15; $NaHCO_3$, 25; and glucose, 11.1—pregassed with 5% CO_2–95% O_2 gas. At the end of the incubation period the slices were washed with ice-cold KR solution. The washed slices were homogenized in 5 ml of 0.32 *M* sucrose with a Teflon–glass homogenizer and centrifuged at 1000*g* for 10 min. The supernatant (S_1) was centrifuged at 17,000*g* for 15 min and the supernatant discarded. The pellet (P_2) was suspended in 50 m*M* Tris–HCl buffer containing 100 m*M* NaCl and 0.1 m*M* guanosine 5′-diphosphate (GDP), dispersed by a polytron (setting 6), and allowed to stand at room temperature for 1 hr. The suspension was centrifuged at 48,000*g* for 10 min and the pellet was washed three times with ice-cold 50 m*M* Tris–HCl buffer (pH 7.4). The resulting pellet (corresponding to the membrane fraction) was used for the receptor binding experiments.

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SP Receptor Binding Experiment

The membrane fraction was suspended in 50 m*M* Tris–HCl buffer (pH 7.4) containing 0.2% bovine serum albumin (BSA), 10 m*M* $MgCl_2$, and several peptidase inhibitors, leupeptin (4 μg/ml), chymostatin (2 μg/ml), bacitracin (40 μg/ml) (Sigma Chemical Co., St Louis, Mo.), and captopril (2 μg/ml) (Sankyo Co. Ltd., Japan). The suspension was incubated with ^{3}H-SP (0.7 n*M*, 60 Ci/mmol, Amersham, U.K.) in a final volume of 0.5 ml. Incubation was carried out at room temperature for 60 min. The incubation mixture was filtered under vacuum through Whatman GF/B glass-fiber filters (Whatman Ltd. England) pretreated with 0.1% polyethyleneimine. The filters were washed four times with ice-cold 50 m*M* Tris–HCl buffer. Individual filters were placed in scintillation vials to which 8 ml of a xylene-based scintillation fluid (Univer-Gel, Nakarai Chemicals, Ltd.) was added and the radioactivity was measured in the liquid scintillation spectrometer (LSC-602, Aloka) at about a 30% counting efficiency. Nonspecific binding was obtained in the presence of 10^{-6} *M* nonradioactive SP and was subtracted from the total binding in the absence of nonradioactive SP to estimate the specific binding. To study the characteristics of SP-induced down-regulation of the ^{3}H-SP binding, membranes from slices preincubated with or without 3×10^{-5} *M* nonradioactive SP were incubated with increasing concentrations (0.05–5 n*M*) of ^{3}H-SP in the absence or presence of 10^{-6} *M* nonradioactive SP for 1 hr at room temperature. Specific binding was analyzed using Scatchard plots (10). The level of nonspecic binding of ^{3}H-SP was 20 to 40% of the total binding. Proteins were determined by a modification of the method of Lowry *et al.* (11).

^{3}H-SP Incorporation

The slices from rat whole brain, except cerebellum, were suspended in KR solution containing 0.2% BSA and several peptidase inhibitors described above (pH 7.4). Incubation of the suspension with ^{3}H-SP (4×10^{-6} *M*, 2.5 mCi/mmol) was carried out at 37°C. At the end of the incubation period ice-cold saline was added and the mixture was centrifuged at 650*g* for 10 sec to stop the reaction. The slices were washed three times with ice-cold saline containing 0.2 *M* acetic acid and weighed. A major part of bound ^{3}H-SP was released by washing with 0.2 *M* acetic acid in saline. After the washed slices were dissolved with 1 *M* NaOH, 1 *M* HCl was added to neutralize the solution and the radioactivity was measured in 10 ml of dioxane-based scintillation fluid.

Extraction of the SP-like Immunoreactivity (SPLI) from Rat Brain Slices

The slices from rat whole brain, except cerebellum, were suspended in KR solution containing 0.2% BSA and several peptidase inhibitors described above, incubated with or without SP (10^{-5} *M*) for 1 hr at 37°C, and centrifuged at 650*g* for 10 sec. The slices were washed three times with ice-cold saline containing 0.2 *M* acetic acid. The washed slices were weighed, boiled in 2 *M* acetic acid for 10 min, dispersed by a polytron (setting 6), and centrifuged at 20,000*g* for 20 min. The supernatant was lyophilized, dissolved with 0.02 *M* sodium phosphate buffer containing 0.15 *M* NaCl and 0.1% BSA, and used for radioimmunoassay to determine the amount of SPLI.

Radioimmunoassay

The anti-SP serum was raised in guinea pigs according to the method reported by Powell *et al.* (12) and diluted to 1:50,000 with 0.02 *M* sodium phosphate buffer (pH 7.5) containing 0.15 *M* NaCl and 0.1% BSA. Sample, antiserum, and ^{125}I-labeled Tyr^8-SP (NEN; 2200 Ci/mmol) were incubated for 20 hr at 0°C. Free label was then separated from antibody-bound label by adsorption using 0.2% charcoal. After being centrifuged at 1500*g* for 10 min, the radioactivity in the supernatant was measured. The minimum detectable amount of SP by this assay was 5 pg/incubation. The affinities of two new mammalian tachykinins, neurokinin A and neurokinin B, to this anti-SP serum were found to be 10- and 100-fold less than that of SP, respectively.

Statistical analysis was performed using Student's *t* test.

RESULTS AND DISCUSSION

When the rat brain slices were incubated with 3×10^{-5} *M* SP at 37°C, the specific ^{3}H-SP binding to the extensively washed membranes from those slices decreased gradually with increasing times of incubation with the agonist, and a significant reduction, by 30%, was observed after 60 min (Fig. 1). Further incubation caused little more reduction.

At 0°C no significant reduction was observed at any time of the incubation (Fig. 1). Thus, the SP-induced loss of ^{3}H-SP binding was time and temperature dependent. Similar findings have been reported in muscarinic receptor regulation (13), enkephalin receptor regulation (14), and β-adrenergic receptor regulation (15).

Figure 2 shows the effects of pretreatment with various concentrations of SP on ^{3}H-SP specific binding. SP-induced SP receptor down-regulation was not observed at less than

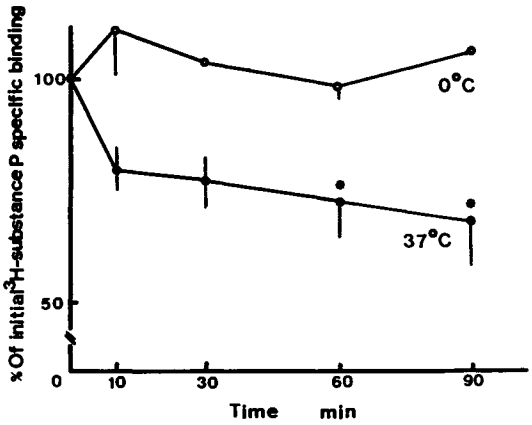

Fig. 1. Time course and effect of temperature on the SP-induced decrease in ^{3}H-SP binding. Membranes from slices treated with or without 3×10^{-5} *M* SP for different time periods at 37°C (●) or 0°C (○) were incubated with ^{3}H-SP (0.7 n*M*) for 1 hr at room temperature. Specific binding of ^{3}H-SP was determined by subtracting nonspecific binding in the presence of 10^{-6} *M* nonradioactive SP from total binding in the absence of nonradioactive SP, expressed as a percentage of that in nonpretreated membranes (control). (*) Significantly different from control, $P < 0.02$.

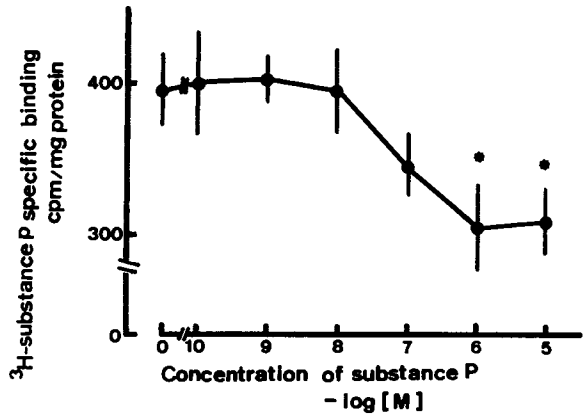

Fig. 2. Effect of SP concentration on SP receptor down-regulation. Rat brain slices were incubated with 10^{-10} to 10^{-5} *M* SP. The specific binding of ^{3}H-SP (0.7 n*M*) to the membranes from those slices was determined as described in the legend to Fig. 1. (*) Significantly different from control, $P < 0.05$.

10^{-8} *M* SP. Pretreatment with 10^{-7} *M* SP decreased ^{3}H-SP specific binding considerably but not significantly. Significant down-regulation was observed at more than 10^{-6} *M* SP.

When the extensively washed membranes from rat brain slices treated with SP were incubated with increasing concentrations (0.05–5 n*M*) of ^{3}H-SP, ^{3}H-SP specific binding increased according to the increasing concentration of ^{3}H-SP and was saturable (Fig. 3). Analysis by Scatchard plot gave K_d and B_{max} values of 0.74 n*M* and 18.8 fmol/mg protein in control membranes and 0.76 n*M* and 13.1 fmol/mg protein in treated membranes, indicating a reduction in the B_{max} value, that is, a decrease in the number of SP binding sites by about 30% in treated membranes.

The agonist-induced reduction in ^{3}H-SP binding may simply be due to the presence of residual SP bound to the receptors and not because of a true disappearance of receptor sites To exclude this possibility the membranes from SP-treated slices were extracted with boiling 2 *N* acetic acid, and the SPLI content was determined by radioimmunoassay. The result showed that, at most, only a concentration of 10^{-10} *M* SP remained bound in the pretreated membranes, which was the same as that thought to be derived from endogeneous SP in control membranes and which caused no significant decrease in the ^{3}H-SP binding in our assay. Furthermore, the affinity of the ^{3}H-SP binding did not change between control and pretreated membranes. Thus, the loss of binding activity could not be attributed to the competition of ^{3}H-SP with ligand that remained after preparation of the membrane fragments.

Incubation of ^{3}H-SP with rat brain slices resulted in a saturable incorporation of radioactivity. The time course of the ^{3}H-SP incorporation (Fig. 4) was similar to that of the SP-induced decrease in ^{3}H-SP binding, that is, the incorporation increased with increasing times of incubation.

The incorporation was temperature dependent. As shown in Fig. 4, at 0°C the incorporation of ^{3}H-SP into the slices was greatly reduced. This temperature dependency was also similar to that of the SP-induced decrease in ^{3}H-SP binding.

We checked the possibility that SP-degraded products were incorporated instead of SP. Rat brain slices were incu-

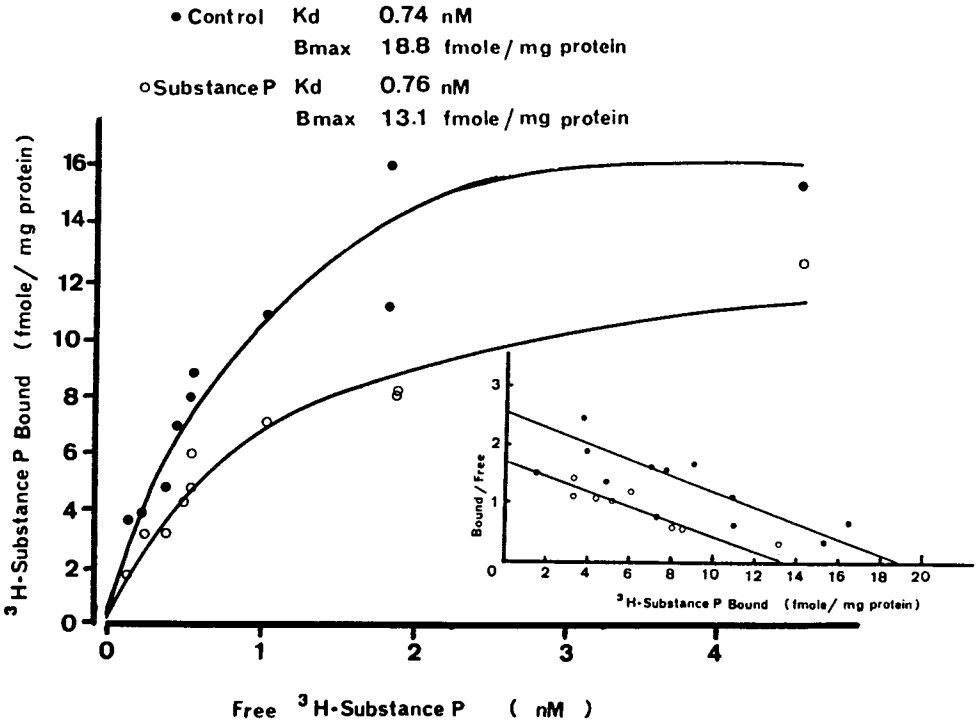

Fig. 3. Saturation curves of the ^{3}H-SP specific binding to the membranes pretreated with (○) or without (●) 3×10^{-5} *M* SP for 1 hr at 37°C. Specific binding of ^{3}H-SP (0.05–5 n*M*) was determined as described in the legend to Fig. 1. Inset: Scatchard plot of the data presented in the saturation curves.

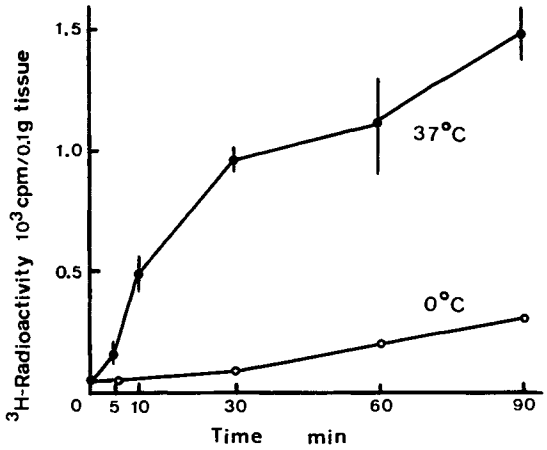

Fig. 4. Time course of the ^{3}H-SP incorporation into rat brain slices. Rat brain slices were incubated with 4×10^{-6} *M* ^{3}H-SP for different time periods at 37°C (●) or 0°C (○). The radioactivity in the slices washed with ice-cold saline containing 0.2 *M* acetic acid was measured as described under Materials and Methods.

bated with 10^{-5} *M* SP for 1 hr at 37°C and washed with ice-cold saline containing 0.2 *M* acetic acid. The extract from the slices obtained by boiling with 2 *M* acetic acid was subjected to radioimmunoassay to determine the amount of SPLI. As shown in Fig. 5, in the control slices 6.7 ng/g wet weight of SPLI was observed and in SP-treated slices 17.6 ng/g wet weight of SPLI was observed. Thus it is suggested that SP itself can be incorporated into the rat brain slices. Furthermore, the incorporation of ^{3}H-SP is suggested to occur postsynaptically, since using the rat brain synaptosomal P_2 fraction, no incorporation was observed (data not shown). To characterize the SP-induced receptor down-regulation, the requirements of the down-regulation and ligand incorporation processes for metabolic energy were studied using the metabolic inhibitors sodium azide (NaN_3) and 2,4-dinitrophenol (DNP). Both DNP at 1 m*M* and NaN_3 at 10 m*M* almost completely protected the slices against the down-regulation caused by 3×10^{-5} *M* SP (Fig. 6).

Also, the incorporation of ^{3}H-SP into the slices was decreased in the presence of these inhibitors (Fig. 7).

To examine the possibility that these metabolic inhibitors may affect the change due to a simple change in receptor occupancy, the ability of the inhibitors to decrease the amount of ^{3}H-SP bound to membranes was determined. Both inhibitors caused no decrease in ^{3}H-SP binding (data not shown). Thus, the effects of these inhibitors on receptor down-regulation and ligand incorporation could be considered not to reflect any effect of the inhibitors on SP binding to receptors, but to reflect some effect on the processes of SP receptor down-regulation or incorporation. The inhibition of receptor down-regulation and ^{3}H-ligand uptake by metabolic inhibitors has also been observed with opiate receptors (16).

These results suggest that SP receptor down-regulation may be directly associated with the incorporation of SP and suggest the possibility that SP can be incorporated via a receptor-mediated process. Sjödin (8) suggested internalization of SP receptors by guinea pig pancreatic acinar cells using ^{125}I-Bolton–Hunter substance P. In this report we speculate that in rat CNS, SP receptors could be internalized by a process of endocytosis triggered by binding of SP and that the number of receptors on a membrane reflects the net contribution of receptor migration. The physiological significance of this phenomenon remains unclear. Sugiya *et al.* (9) indicate that the homologous desensitization of SP response is involved in the down-regulation of SP receptors in rat parotid gland. Desensitization to SP effects is usually observed even at a 10^{-8} *M* concentration in the peripheral

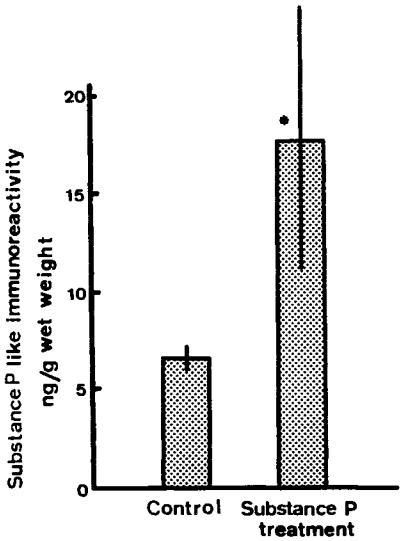

Fig. 5. SP-like immunoreactivity (SPLI) in rat brain slices treated with 10^{-5} *M* SP. Rat brain slices were incubated with 10^{-5} *M* SP for 1 hr at 37°C, then washed with ice-cold 0.2 *M* acetic acid in saline. SPLI in the extract from those slices was determined by a radioimmunoassay as described under Materials and Methods. (*) Significantly different from control, $P < 0.02$.

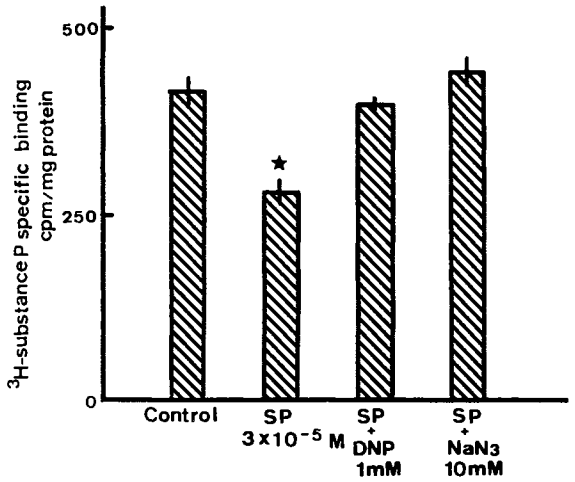

Fig. 6. Effects of metabolic inhibitors on the SP-induced decrease in ^{3}H-SP binding. Rat brain slices were preincubated with 1 m*M* DNP or 10 m*M* NaN_3 for 10 min at 37°C before the addition of 3×10^{-5} *M* SP and the incubation was continued for a further 1 hr at 37°C. Specific binding of ^{3}H-SP (0.7 n*M*) to membranes from those slices was determined as described in the legend to Fig. 1. (*) Significantly different from control, $P < 0.02$.

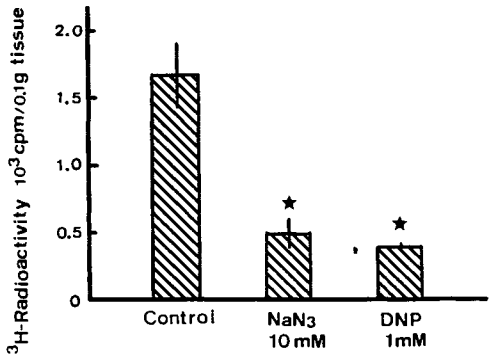

Fig. 7. Effects of metabolic inhibitors on ^{3}H-SP incorporation. Rat brain slices were preincubated with 10 mM NaN_3 or 1 mM DNP for 10 min at 37°C before the addition of 4×10^{-6} M ^{3}H-SP and the incubation was continued for a further 1 hr at 37°C. The radioactivity in the slices washed with ice-cold saline containing 0.2 M acetic acid was measured as described under Materials and Methods. (*) Significantly different from control, $P < 0.01$.

nervous systems (6,7,9), and after desensitization with a high SP concentration most of the SP effects are known to disappear completely. However, in our results, 10^{-8} M SP did not induce receptor down-regulation (Fig. 2), and at 30 μM SP the extent of the down-regulation represents only a 30% loss of binding capacity (Fig. 3). Watson and Downes (17) reported that while a guinea pig ileum SP-induced hydrolysis of inositol phospholipids reached maximum at an SP concentration of 10^{-7} M, in rat hypothalamus slices it reached maximum at 10^{-5} M. Thus, there may be differences in effective concentrations between the central and the peripheral nervous systems. Moreover, desensitization of the SP response could occur at some step distal to the membrane events. For example, physiological responses could be mediated by a second messenger system, e.g., phosphoinositide turnover, after receptor activation. Furthermore, it remains unclear whether receptor activation or only receptor occupation is required for receptor regulation. The elucidation of such a process may shed light on the SP-induced desensitization or the development of tachyphylaxis or tolerance.

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