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Research Report

Dual effects of neurokinin on calcium channel currents and signal pathways in neonatal rat nucleus tractus solitarius

Takayuki Endoh*

Department of Physiology, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan

*Corresponding author. E-mail address: tendoh@tdc.ac.jp (T. Endoh)

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ABSTRACT

Neurokinins, such as substance P (SP), modulates the reflex regulation of cardiovascular and respiratory function in the CNS, particularly in the nucleus tractus solitarius (NTS). There is considerable evidence that the action of SP in the NTS, but the precise effects have not yet been determined. Voltage-dependent Ca^{2+} channels (VDCCs) serve as crucial mediators of membrane excitability and Ca^{2+}-dependent functions such as neurotransmitter release, enzyme activity and gene expression. The purpose of this study was to investigate the effects of neurokinins on VDCCs currents (I_{Ca}) in the NTS using patch-clamp recording methods. In 142 of 282 neurons, an application of [Sar^9, $Met(O_2)^{11}$]-substance P (SSP, NK_1 receptor agonist) caused facilitation of L-type I_{Ba}. Intracellular dialysis of the $G\alpha_{q/11}$-protein antibody attenuated the SSP-induced the facilitation of I_{Ba}. In addition, phospholipase C (PLC) inhibitor, protein kinase C (PKC) inhibitor and PKC activator attenuated the SSP-induced the facilitation of I_{Ba}. In contrast, in 115 of 282 neurons, an application of SSP caused inhibition of N- and P/Q-types I_{Ba}. Intracellular dialysis of the $G\beta\gamma$-protein antibody attenuated the SSP-induced the inhibition of I_{Ba}. These results indicate that NK_1 receptor facilitates L-type VDCCs via $G\alpha_{q/11}$-protein involving PKC

in NTS. On the other hand, NK_1 receptor inhibits N- and P/Q-types VDCCs via $G\alpha_{q/11}$-protein $\beta\gamma$ subunits in NTS.

1. Introduction

Neurokinins such as substance P (SP), neurokinin A (NKA) and neurokinin B (NKB), which are synthesized and released from sensory neurons (Johnson et al., 1998; Marco et al., 1998; Otsuka and Yoshioka, 1993; Zogorodnyuc and Maggi, 1997), contribute to numerous physiological processes, including inflammatory responses (Jia and Seybold, 1996), nociception (Liu and Sandkuhler, 1997), central and peripheral cardiovascular regulation (Thompson et al., 1998), and other autonomic reflexes (Moodley et al., 1999; Serio et al., 1998).

The nucleus tractus solitarius (NTS) is known to play a major role in the regulation of cardiovascular, respiratory, gustatory, hepatic and swallowing functions (Jean, 2001; Lawrence and Jarrott, 1996). The NTS appears not to be a simple 'relay' nucleus, rather it performs complex integration of information from multiple synaptic inputs of both peripheral and central origins.

Neurokinins are important neurotransmitters in the NTS (Otsuka and Yoshioka, 1993). Ljungdahl et al. (1978) identified high concentrations of SP and NKA in the NTS. Three types of neurokinin receptors, NK_1, NK_2 and NK_3, have been cloned and characterized pharmacologically (Regoli et al., 1987) and the endogenous neurokinins, SP, NKA and

NKB can bind to the NK_1, NK_2 and NK_3 receptors, respectively (Lavielle et al., 1990). It has been proposed that all three neurokinin receptors (NK_1, NK_2 and NK_3) are present in the NTS (Mazzone and Geraghty, 2000). However, roles for neurokinin in the NTS remain controversial. More selective neurokinin receptors agonists and antagonists have been developed recently and allow for more precise characterization of the receptors involved in various physiological responses. Neurokinin has produced variable and inconsistent responses in respiration, heart rate and arterial blood pressure. Some authors have indicated that neurokinin in NTS potentiates the vascular response of the baroreceptor reflex (Seagard et al., 2000).

Voltage-dependent Ca^{2+} channels (VDCCs) serve as crucial mediators of membrane excitability and Ca^{2+}-dependent functions such as neurotransmitter release, enzyme activity and gene expression. The modulation of VDCCs is believed to be an important means of regulating Ca^{2+} influx and thus has a direct influence on many Ca^{2+}-dependent processes. Modulation of VDCCs by neurokinin has been described previously in various types of neurons.

Microinjection of NK_1 agonists to the NTS produced potent bradycardia and hypotension (Feldman, 1995) – a pattern of response qualitatively similar to that seen during cardiac vagal receptor stimulation (de Burgh Daly, 1991; Al-Timman et al., 1993;

Paton and Buther, 1998). Microinjection of angiotensin Ⅱ (Ang Ⅱ) to the NTS attenuates both the vagal and cardiac baroreflex (Boscan et al., 2001) via the activation of GABAergic interneurons (Paton et al., 2001). In addition, we previously reported that Ang Ⅱ facilitates VDCCs in NTS (Endoh, 2005). However, the effect of neurokinin on VDCCs in NTS has not yet been clarified, and little is known about signal transduction pathways in NTS.

2. Results

2.1. Neurokinin-induced facilitation of I_{Ba}

An example of [Sar^9, $Met(O_2)^{11}$]-substance P (SSP, NK_1 receptor agonist)-induced facilitation of I_{Ba} is shown in Fig. 1. I_{Ba} was evoked every 20 sec with a 100 msec depolarizing voltage step to 0 mV from a holding potential of −80 mV. As shown in Fig. 1A and B, application of 1 μM SSP rapidly and reversibly facilitates I_{Ba}.

The current-voltage relations measured before and during application of SSP (1 μM) are shown in Fig. 1C. From a holding potential of −80 mV, the I_{Ba} was activated after −30 mV with a peak current amplitude at 0 mV. SSP did not shift the current-voltage

relations (n＝5).

The dose-response relations in the neurokinin-induced facilitation of I_{Ba} is shown in Fig. 1D. Application of 1 nM-10 μM SSP rapidly and reversibly facilitate I_{Ba} without changing current kinetics. For the generation of the concentration-response curve, SSP concentrations were applied randomly, and not all concentrations in a single neuron were tested. Fig. 1D shows that progressive increases in SSP concentration resulted in progressively greater facilitation of I_{Ba}. Neither [βAla^{8}]-Neurokinin A (NK_2 receptor agonist) nor [MePhe^{7}]-Neurokinin B (NK_3 receptor agonist) facilitate I_{Ba}.

To confirm that the agonistic action of SSP was mediated by NK_1 receptors, the effects of SSP on I_{Ba} in neurons treated with specific antagonists were investigated. In this experiment, specific antagonists were applied prior to the SSP. Application of NK_1 receptors antagonist L-732,138 (1 μM for 3 min after assuming the whole-cell configuration) was without effects on basal I_{Ba} but blocked the SSP-induced facilitation of I_{Ba} (p＜0.05, paired t-test). However, both NK_2 receptors antagonist MEN 10,376 and NK_3 receptors antagonist SB 218795 were without effects on SSP-induced facilitation of I_{Ba}. (28.2 ± 5.0% for SSP only, 3.1 ± 1.0% for SSP in neurons treated with L-732,138, 29.5 ± 6.1% for SSP in neurons treated with MEN 10,376 and 27.5 ± 4.5% for SSP in neurons treated with SB 218795, n＝11, 7, 5 and 5, respectively, Fig. 2A). These results

indicate that SSP-induced facilitation of I_{Ba} was mediated by NK_1 receptors in NTS neurons.

2.2. Pharmacological characterization of G-protein subtypes and VDCC subtypes in neurokinin-induced facilitation of I_{Ba}

To characterize the G-protein subtypes in SSP-induced facilitation of I_{Ba}, specific antibody raised against the $G\alpha_i$-, $G\alpha_s$-, $G\alpha_{q/11}$- and $G\beta\gamma$-protein were used. Experiments were performed using a solution in a pipette containing each G-protein antibody. In these experiments, the G-protein antibody (1:50 dilution; the final concentration was approximately 0.5 mg/ml) was dissolved in the internal solution. The tip of the recording pipette was filled with the standard internal solution, and the pipette was then backfilled with solution which containing the G-protein antibody. As controls, antibody was heated at 90 ℃ for 30 min and then used for whole-cell recordings as described above. For six neurons that were tested, intracellular dialysis of the $G\alpha_{q/11}$-protein antibody attenuated the SSP-induced facilitation I_{Ba} (6.1 ± 2.0%, $p < 0.05$, paired *t*-test). In contrast, intracellular dialysis of the $G\alpha_i$-, $G\alpha_s$-, and $G\beta\gamma$-protein antibody did not attenuate the SSP-induced facilitation of I_{Ba}. These results

suggest that the $G\alpha_{q/11}$-protein is involved in the NK_1 receptors-induced facilitation of I_{Ba} in NTS but not $G\alpha_i$-, $G\alpha_s$-, and $G\beta\gamma$-proteins (Fig. 2B).

It has been reported that several different types of VDCCs, such as L-, N-, P-, Q- and R-type VDCCs exist in NTS (Rhim and Miller, 1994). From the functional point of view, VDCCs have been classed into "high" and "low" threshold on the basis of the voltage range at which they are activated. Low voltage-activated (LVA) VDCCs have been also known as T-type, which serve as prolong the duration of the Ca^{2+}-dependent spike burst and of the generation of rhythmic firing (Herrington and Lingle 1992). On the other hand, high voltage-activated (HVA) VDCCs can be grouped into various classes according to their sensitivity to VDCCs blockers and the fact that they have different roles (Endoh 2004). LVA T-type VDCCs currents were isolated by stepping to test potentials of −50 mV to −30 mV from a holding potential of −90 mV. Therefore, LVA T-type VDCCs are inactivated at depolarized test potentials such as −30 mV (Fox et al., 1987). Depolarization more positive than −30 mV evoked only HVA VDCCs. In addition, to ensure that all inward I_{Ba} resulted from HVA VDCCs, i.e. to avoid the possibility of T-type VDCCs contribution, specific HVA VDCCs blockers were sequentially applied in NTS (Endoh 2006). In general, the lack of modulation by G-protein coupled receptors was thought to be a signature of LVA channels (Kostyuk,

1999).

Thus, it was then investigated about which types of the HVA VDCCs were facilitated by SSP. When nifedipine (10 μM, Nif; L-type VDCC blocker) + ω-Aga IVA (1 μM, P/Q-type VDCC blocker) and Nif + ω-CgTx GVIA (1 μM, N-type VDCC blocker) were applied first, the resistant I_{Ba} were not significantly facilitated by a subsequent application of SSP (3.5 ± 1.1 and 2.9 ± 1.0%, n=4 and 4, respectively). On the other hand, when the ω-CgTx GVIA + ω-Aga IVA were applied first, the resistant I_{Ba} were facilitated by a subsequent application of SSP (21.2± 1.9%, n=5, Fig. 3). These results demonstrated that NK_1 receptors facilitated L-type VDCCs, without significantly affecting N- and P/Q-types VDCCs in NTS neurons.

2.3. Characterization of second messengers in neurokinin-induced facilitation of I_{Ba}

In other cells, it has been demonstrated that NK_1 receptors are coupled via phospholipase C (PLC) and synthesis of diacylglycerol (DAG). DAG has been reported to facilitate VDCCs involving activation of protein kinase C (PKC) (Bayguinov et al., 2003).

To evaluate the possible contribution of PLC to the SSP-induced facilitation of I_{Ba}, the

effects of SSP on I_{Ba} in neurons treated with U-73122 (which is a membrane permeable aminosteroid that blocks the phosphatidylinositol-specific PLC) (Bleasdale et al., 1990; Thompson et al., 1991), were investigated. In seven neurons tested, treatment with U-73122 (10 μM for 15 min) attenuated the SSP-induced facilitation of I_{Ba} (8.2 ± 3.1%, $p<0.05$, paired *t*-test, Fig. 4C).

To evaluate the possible contribution of PKC to the SSP-induced facilitation of I_{Ba}, the effects of SSP on I_{Ba} in neurons treated with GF109203X (a selective PKC inhibitor) were investigated. In six neurons tested, treatment with GF109203X (10 μM for 30 min) attenuated the SSP-induced facilitation of I_{Ba} (4.1 ± 2.2%, $p<0.05$, paired *t*-test, Fig. 4A, B and C). To evaluate the possible contribution of PKC to the SSP-induced facilitation of I_{Ba}, the effects of SSP on I_{Ba} in neurons treated with PMA (a selective PKC activator) were also investigated. In four neurons tested, treatment with PMA (0.5 μM for 10 min) attenuated the SSP-induced facilitation of I_{Ba} (5.3 ± 2.3%, $p<0.05$, paired *t*-test, Fig. 4A, B and C).

Phosphoinositide 3-kinase (PI3K) enzyme is involved in the regulation of multiple biological responses including regulation of ionic channel activity (Harvey et al., 2000). Several hormones facilitate L-type VDCCs involving PI3K (Quignard et al., 2001). To evaluate the possible contribution of PI3K to the SSP-induced facilitation of I_{Ba}, the

effects of SSP on I_{Ba} in neurons treated with LY294002 (a selective PI3K inhibitor) were investigated. In five neurons tested, treatment with LY294002 (10 μM for 10 min) did not attenuate the SSP-induced facilitation of I_{Ba} (27.2 ± 4.5%, Fig. 4C).

To evaluate the possible contribution of adenylate cyclase to the SSP-induced facilitation of I_{Ba}, the effects of SSP on I_{Ba} in neurons treated with SQ22536 (an adenylate cyclase inhibitor) were investigated. In five neurons tested, treatment with SQ22536 (10 μM for 30 min) did not attenuate the SSP-induced facilitation of I_{Ba} (26.9 ± 4.4%, Fig. 4C).

To evaluate the possible contribution of protein kinase A (PKA) to the SSP-induced facilitation of I_{Ba}, the effects of SSP on I_{Ba} in the presence of PKI(5-24) (a selective PKA inhibitor) in the recording pipette were investigated. Intracellular application of PKI(5-24) (20 μM for 7 min after assuming the whole-cell configuration) did not attenuate the SSP-induced facilitation of I_{Ba} (28.2 ± 4.9%, Fig. 4C).

To evaluate the possible contribution of mitogen-activated protein kinase (MAPK) tyrosine kinase to the SSP-induced facilitation of I_{Ba}, the effects of SSP on I_{Ba} in neurons treated with PD98,059 (a MAPK tyrosine kinase inhibitor) were investigated. In five neurons tested, treatment with PD98,059 (10 μM for 2 min) did not attenuate the SSP-induced facilitation of I_{Ba} (29.2 ± 5.1%, Fig. 4C).

These results suggest that NK_1 receptors facilitate VDCCs involving PLC and PKC pathways in NTS neurons.

2.4. Neurokinin-induced inhibition of I_{Ba}

In addition to facilitation, SSP-induced inhibition of I_{Ba} could also be observed in 115 of 282 neurons tested. An example of SSP-induced inhibition of I_{Ba} is shown in Fig. 5A. In order to investigate the voltage dependency of inhibition of I_{Ba} by SSP, a double-pulse voltage protocol, as shown in Fig 5A, was used. Paired I_{Ba} were evoked from a holding potential of −80 mV by a 100 msec voltage step to 0 mV at 20 sec intervals. An intervening strong depolarizing voltage prepulse (100 mV, 30 msec) ended 5 msec prior to the second I_{Ba} activation. Application of 1 μM SSP inhibited I_{Ba} without prepulse from −730 pA to −525 pA (28.0% inhibition) in the neuron. On the other hand, 1 μM SSP inhibited I_{Ba} with prepulse from −750 pA to −684 pA (8.8% inhibition) in the same neuron. On average, the inhibition of I_{Ba} was 32.8 ± 6.2% for I_{Ba} without prepulse and 11.1 ± 2.0% for I_{Ba} with prepulse (n=9). These results suggest that an application of a strong depolarizing voltage prepulse attenuates the SSP-induced inhibition of I_{Ba}.

The current-voltage relations measured before and during application of SSP (1 μM) are shown in Fig. 5C. As shown in Fig. 5C, SSP-induced inhibition resulted in a slight, but nonsignificant, in the voltage dependence of the I_{Ba} to more positive potentials (n＝4).

The dose-response relations in the neurokinin-induced inhibition of I_{Ba} is shown in Fig. 5D. Application of 1 nM-10 μM SSP rapidly and reversibly inhibit I_{Ba}. For the generation of the concentration-response curve, SSP concentrations were applied randomly, and not all concentrations in a single neuron were tested. Neither [βAla^{8}]-Neurokinin A (NK_2 receptor agonist) nor [MePhe^{7}]-Neurokinin B (NK_3 receptor agonist) inhibit I_{Ba}.

To confirm that the agonistic action of SSP was mediated by NK_1 receptors, neurokinin receptors agonists and antagonists were tested as well. Application of NK_1 receptors antagonist L-732,138 (1 μM for 3 min after assuming the whole-cell configuration) was without effects on basal I_{Ba} but blocked the SSP-induced inhibition of I_{Ba} ($p<0.05$, paired *t*-test). However, both NK_2 receptors antagonist MEN 10,376 and NK_3 receptors antagonist SB 218795 were without effects on SSP-induced inhibition of I_{Ba}. (32.8 ± 6.2% for SSP only, 4.5 ± 1.1% for SSP in neurons treated with L-732,138, 28.2 ± 4.1% for SSP in neurons treated with MEN 10,376 and 29.1 ± 3.8% for SSP in neurons

treated with SB 218795, n＝9, 5, 4 and 4, respectively, Fig. 6A). These results indicate that SSP-induced inhibition of I_{Ba} was mediated by NK_1 receptors in NTS neurons.

2.5. Pharmacological characterization of G-protein subtypes and VDCC subtypes in neurokinin-induced inhibition of I_{Ba}

To characterize the G-protein subtypes in SSP-induced inhibition of I_{Ba}, specific antibody raised against the $G\alpha_i$-, $G\alpha_{q/11}$-, $G\alpha_s$-, and $G\beta\gamma$-protein were used. For five neurons that were tested, intracellular dialysis of the $G\alpha_{q/11}$-protein antibody attenuated the SSP-induced inhibition I_{Ba} (3.4 ± 1.2%, $p<0.05$, paired *t*-test). For four neurons that were tested, intracellular dialysis of the $G\beta\gamma$-protein antibody also attenuated the SSP-induced inhibition I_{Ba} (5.4 ± 2.1%, $p<0.05$, paired *t*-test). In contrast, intracellular dialysis of the $G\alpha_i$- and $G\alpha_s$-protein antibody did not attenuate the SSP-induced inhibition of I_{Ba}. These results suggest that the $G\alpha_{q/11}$- and $G\beta\gamma$-protein is involved in the NK_1 receptors-induced inhibition of I_{Ba} in NTS but $G\alpha_i$- and $G\alpha_s$-protein is not (Fig. 6B).

It has been demonstrated that NTS possess several types of HVA-VDCCs, i. e., dihydropyridine sensitive component (I_{Ba-L}), ω-CgTx GVIA sensitive component (I_{Ba-N}),

ω-Aga IVA sensitive component ($I_{Ba-P/Q}$) and insensitive to all of these blockers component (I_{Ba-R}). In a previous study, we determined that mean percentages of I_{Ba-L}, I_{Ba-N}, $I_{Ba-P/Q}$ and I_{Ba-R} of total I_{Ba} is 42.2 ± 3.8%, 28.4 ± 3.4%, 19.3 ± 3.2% and 10.1 ± 1.4%, respectively, in NTS (Endoh, 2006). Therefore, it was investigated which types of the VDCCs were inhibited by SSP.

The effect of SSP on the I_{Ba-L} was investigated using a neuron treated with ω-CgTx GVIA (1 μM) and ω-Aga IVA (1 μM). The effect of SSP on the I_{Ba-N} was investigated using a neuron treated with Nif (10 μM) and ω-Aga IVA (1 μM). The effect of SSP on the $I_{Ba-P/Q}$ was investigated using a neuron treated with Nif (10 μM) and ω-CgTx GVIA (1 μM). Each of the I_{Ba} components and the percentage of the inhibition by SSP are summarized in Fig. 7A. Only the inhibition of I_{Ba-N} and $I_{Ba-P/Q}$ was significant. Results shown in Fig. 7A demonstrate that NK_1 receptors inhibited N- and P/Q-type I_{Ba} components in NTS neurons. Pooled data in a previous study was used in the present study.

2.6. Characterization of second messengers in neurokinin-induced inhibition of I_{Ba}

To evaluate the possible contribution of PLC to the SSP-induced inhibition of I_{Ba}, the

effects of SSP on I_{Ba} in neurons treated with U-73122, were investigated. In four neurons tested, treatment with U-73122 (10 μM for 15 min) did not attenuate the SSP-induced inhibition of I_{Ba} (31.0 ± 5.4%, Fig. 7B).

To evaluate the possible contribution of PKC to the SSP-induced inhibition of I_{Ba}, the effects of SSP on I_{Ba} in neurons treated with GF109203X were investigated. In five neurons tested, treatment with GF109203X (10 μM for 30 min) did not attenuate the SSP-induced inhibition of I_{Ba} (29.8 ± 5.2%, Fig. 7B). To evaluate the possible contribution of PKC to the SSP-induced inhibition of I_{Ba}, the effects of SSP on I_{Ba} in neurons treated with PMA were also investigated. In five neurons tested, treatment with PMA (0.5 μM for 10 min) did not attenuate the SSP-induced inhibition of I_{Ba} (31.3 ± 5.5%, Fig. 7B).

To evaluate the possible contribution of PI3K to the SSP-induced inhibition of I_{Ba}, the effects of SSP on I_{Ba} in neurons treated with LY294002 were investigated. In four neurons tested, treatment with LY294002 (10 μM for 10 min) did not attenuate the SSP-induced inhibition of I_{Ba} (29.0 ± 6.0%, Fig. 7B).

To evaluate the possible contribution of adenylate cyclase to the SSP-induced inhibition of I_{Ba}, the effects of SSP on I_{Ba} in neurons treated with SQ22536 were investigated. In five neurons tested, treatment with SQ22536 (10 μM for 30 min) did

not attenuate the SSP-induced inhibition of I_{Ba} (29.2 ± 5.1%, Fig. 7B).

To evaluate the possible contribution of PKA to the SSP-induced inhibition of I_{Ba}, the effects of SSP on I_{Ba} in the presence of PKI(5-24) in the recording pipette were investigated. Intracellular application of PKI(5-24) (20 μM for 7 min after assuming the whole-cell configuration) did not attenuate the SSP-induced inhibition of I_{Ba} (28.9 ± 4.7%, Fig. 7B).

To evaluate the possible contribution of MAPK tyrosine kinase to the SSP-induced inhibition of I_{Ba}, the effects of SSP on I_{Ba} in neurons treated with PD98,059 were investigated. In four neurons tested, treatment with PD98,059 (10 μM for 2 min) did not attenuate the SSP-induced inhibition of I_{Ba} (31.1 ± 5.8%, Fig. 7B).

These results suggest that NK_1 receptors-induced inhibition of VDCCs was not mediated by the above second messengers, i.e., PLC, PKC, PI3K, adenylate cyclase, PKA and MAPK tyrosine kinase in NTS neurons.

3. Discussion

The present study investigated the effects of neurokinin on VDCCs in NTS. This study has shown that NK_1 receptor facilitates L-type VDCCs via $G\alpha_{q/11}$-protein involving

PKC in NTS. On the other hand, NK_1 receptor inhibits N- and P/Q-types VDCCs via G $\alpha_{q/11}$-protein $\beta\gamma$ subunits.

Several studies demonstrated that neurokinin receptors modulate VDCCs in various neurons, such as dorsal root ganglions (DRG) (Sculptoreanu and de Groat, 2003), inner ear spiral ganglion neurons (Sun et al., 2004) and cultured nucleus basalis neurons (Margeta-Mitrovic et al., 1997).

There is evidence for expression of all three types of neurokinin receptors (NK_1, NK_2 and NK_3) in NTS (Mazzone and Geraghty, 2000). In this study, only SSP (NK_1 receptor agonist) modulates I_{Ba} in NTS. Neither [βAla^8]-Neurokinin A (NK_2 receptor agonist) nor [MePhe^7]-Neurokinin B (NK_3 receptor agonist) modulate I_{Ba}. Thus, it can be considered that only NK_1 receptor couples to VDCCs in NTS. SP immunoreactivity is distributed preferentially in cardiac sensory fibers (Papka et al., 1981) and also in vagal afferents impinging on NTS neurons (Sykes et al., 1994) by NK_1 receptors (Nilsson et al., 1991; Regoli et al., 1991; Rusin et al., 1993). In fact, high densities of NK_1 receptors have been identified in the NTS (McRitchie & Tork, 1994; Watson et al. 1995; Maubach and Jones, 1997). Other data indicate that the peptide attenuate the cardiac baroreceptor reflex mediated by NK_1-GABA receptor mechanism in the NTS (Boscan et al., 2002; Pickering et al., 2003). In addition, stimulation of NK_1 receptors with specific

agonists depolarized NTS neurons – an effect which was blocked by selective NK_1 antagonists (Maubach and Jones, 1997). In comparison, selective stimulation of NK_2 or NK_3 receptors in the NTS failed to elicit any changes in cardiovascular variables (Feldman, 1995). Interestingly, SP elicited a potent excitatory effect on some NTS neurons in vitro (Jacquin et al., 1989) and in vivo (Morin-Surun et al., 1984).

This study has shown that NK_1 receptor facilitates L-type VDCCs involving PKC in NTS. Similar observation has been demonstrated in DRG (Sculptoreanu and de Groat, 2003). In spinal dorsal horn neurons, opioid receptor inhibits VDCCs involving PKC (Lee et al., 2004).

There are several mechanisms of VDCCs facilitation (Dolphin, 1996). L-type VDCCs facilitation can result from a strong conditioning depolarization that recruits 'silent channels' (Artelejo et al., 1990). Alternatively, L-type VDCCs can be facilitated by protein kinases. L-type VDCCs possess several consensus PKA and PKC phosphorylation sites and physiological studies have demonstrated channel facilitation by both enzymes (McDonald et al., 1994).

Activation of PKC by DAG, which are metabolically stable activators, leads to a rapid translocation of the enzyme to the plasma membrane. It has been demonstrated that PKC could exert a direct effect on VDCCs possessing PKC phosphorylation sites

(Ahlijanian et al., 1991; Puri et al., 1997). In addition, PKC could indirectly control the activity of VDCCs by inducing phosphorylation of components interacting with VDCCs, as reported for the PKC-dependent up-regulation of channel activity (Barrett and Rittenhouse, 2000; Swartz, 1993; Zamponi and Snutch, 1998).

PKC anchoring proteins called RACKs (receptors for activated C kinases) were shown to localize PKC isoforms in the proximity to ion channels (Mochly-Rosen and Gordon, 1998). There is the possibility that regulation of VDCCs activity by PKC might be dependent on the differential expression of RACKs between different cell types. Alternatively divergent regulatory patterns of VDCCs might be due to one or more of the following factors: (1) presence or absence of specific PKC isoforms; (2) presence of different VDCCs expression; (3) unidentified regulatory protein(s) such as RACKs; and (4) differential expression of as yet to be identified auxiliary subunits of VDCCs.

PKC family is divided into three subgroups: the DAG- and Ca^{2+}-dependent cPKC (α, β and γ), the DAG-dependent but Ca^{2+}-independent nPKC (δ, ε, η and θ) and the DAG- and Ca^{2+}-independent aPKC (ξ and λ). In the present experiments, treatment with PLC inhibitor attenuated the SSP-induced facilitation of VDCCs. Thus, it can be considered that cPKC or nPKC are involved in this cascade.

Since mechanisms of facilitation and inhibition of I_{Ba} are through distinct pathways, it

will be possible to see that the same neuron show both facilitation and inhibition. However, SSP-induced both facilitation and inhibition in the same neuron could not be observed in the present study. The modulating manner, i.e., facilitation or inhibition, did not depend on the experimental conditions. It was impossible to determine the modulating manner of I_{Ba} before SSP application.

What determines the relative expression of the two types of VDCCs responses? As mentioned above, neurokinin in NTS produces both potentiation (Seagard et al., 2000) and attenuation (Boscan et al., 2002; Pickering et al., 2003) of cardiac baroreceptor reflex and vascular response. Neurokinin-induced facilitation of L-type VDCCs demonstrated in this study may lead to potentiation of cardio-vascular responses. In contrast, neurokinin-induced inhibition of N- and P/Q-types VDCCs demonstrated in this study may lead to attenuation of this response. Interestingly, Pickering et al. has demonstrated that neurokinin acted on GABAergic neurons to inhibit the NTS pathways (2003). NTS neurons can be divided into two groups, GABAergic and glutamatergic (Mifflin and Felder, 1990; Brooks et al., 1992). Neurokinin-induced facilitation of VDCCs demonstrated in this study may lead to depolarization of NTS. It can be considered that depolarization of GABAergic NTS enhances GABA release. Enhancement of GABA release may attenuate the cardiac baroreceptor reflex. Similar

results have been demonstrated that NK_1 receptors facilitate GABA release involving PKC through Ca^{2+} influx in NTS by Bailey et al. (2004).

In contrast, neurokinin-induced inhibition of N- and P/Q-types VDCCs in NTS was also observed. N-, P- and Q-types VDCCs are implicated in transmitter release (Reuter, 1996). It can be considered that inhibition of N- and P/Q-types VDCCs may inhibit GABA release. Inhibition of GABA release may potentiate the cardiac baroreceptor reflex. In contrast, a recent report demonstrates that neurokinin depresses glutamate release in NTS (Sekizawa et al., 2003). Inhibition of glutamate release may attenuate the cardiac baroreceptor reflex.

Although there is no evidence showing that the GABAergic or glutamatergic neurons in NTS express different types of VDCCs, it has been reported that these neurons express different types VDCCs presynaptically. Glaum and Miller (1995) demonstrated that GABAergic neurons mainly express P/Q-types VDCCs while glutamatergic neurons express N-types VDCCs presynaptically in NTS. It is important to know which types of VDCCs are expressed in NTS postsynaptically in a further study.

NTS are also divided into subtypes: rostral, medial and caudal NTS. It is important to know what types of NTS neurons express L-type and N-/P-/Q-types VDCCs. It can be considered that the variation is therefore more likely due to differences I expression of

VDCCs subtypes and/or couple the receptors to VDCCs in distinct NTS subtypes. In this study, however, it is difficult to identified distinct NTS subtypes because all obtained brainstem slices were mixed while cell dissociation process.

In this study, experiments were performed in neonatal neurons of rats (postnatal 7 days to 18 days). It has been demonstrated that NK1 receptor density is very high throughout the first 14 postnatal days, and only then it decreases towards adult levels (Rodier et al., 2001). Therefore, it is difficult to predict the function of NTS in adult rats using results on this paper. Neurokinin's functions in NTS must be investigated in adults in a further study.

4. Experimental procedures

4.1. Cell preparation

Experiments were conducted according to international guidelines on the use of animals for experimentation. Young Wistar rats (7-18 days old) were decapitated and their brains were quickly removed and submerged in ice cold artificial cerebrospinal fluid (aCSF) saturated with 95% O_2 and 5% CO_2 of the following composition (in mM): 126

NaCl, 26.2 $NaHCO_3$, 1 NaH_2PO_4, 3 KCl, 1.5 $MgSO_4$, 1.5 $CaCl_2$ and 30 glucose; pH 7.4. Thin transverse slices from brainstems, 400 μm in thickness, were prepared by a tissue slicer (DTK-1000; Dosaka EM Co., Ltd, Kyoto). After being sectioned, 3-5 slices obtained from a single brain were transferred to a holding chamber and stored in oxygenated aCSF at room temperature for at least 40 min before use. Slices were then transferred to a conical tube containing gently bubbled aCSF at 36 ℃ to which 1.8 U/ml dispase (grade Ⅰ; 0.75 ml/slice) was added. After 60 min incubation, slices were rinsed with enzyme-free aCSF. Under a dissecting microscope, the NTS region was micropunched and placed on a poly-l-lysine-coated coverslip. The cells were then dissociated by trituration using progressively smaller diameter pipettes and allowed to settle on a coverslip for 20 min.

4.2. Whole-cell patch-clamp recordings

The methods for the current recordings and data analyses were described in a previous study (Endoh, 2005a). In brief, voltage-clamp recordings were conducted using the whole-cell configuration of the patch-clamp technique (Hamill et al. 1981). Fabricated recording pipettes (2-3 MΩ) were filled with the internal solution of the following

composition (in mM): 100 CsCl, 1 $MgCl_2$, 10 HEPES, 10 BAPTA, 3.6 MgATP, 14 $Tris_2$phosphocreatine (CP), 0.1 GTP, and 50 U/ml creatine phosphokinase (CPK). The pH was adjusted to 7.2 with CsOH. The inclusion of CP and CPK effectively reduced "rundown" of current. No data were included in the analysis where unable to reduce run-down. After the formation of a giga seal, in order to record I_{Ca} carried by Ba^{2+} (I_{Ba}), the external solution was replaced from Krebs solution to a solution containing the following (in mM): 151 tetraethylammonium (TEA) chloride, 5 $BaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose. The pH was adjusted to 7.4 with TEA-OH. All drugs except G-protein antibodies and PKI(5-24) were applied by superfusion; time to reach equilibrium concentrations in the bath was about 90 sec. In order to obtain the effect of antibodies and PKI(5-24), neurokinin agonists and antagonists were applied 7 min after assuming the whole-cell configuration. Command voltage protocols were generated with a computer software pCLAMP version 8 (Axon Instruments, Union City, CA, USA) and transformed to an analogue signal using a DigiData 1200 interface (Axon Instruments, Union City, CA, USA). DigiData 1200 interface was used to record and digitize current. The command pulses were applied to cells through an L/M-EPC7 amplifier (HEKA Elektronik, Lambrecht, Germany).

4.3. Materials

[Sar^{9}, Met(O$_2$)^{11}]-substance P, L-732,138 and SB 218796 were purchased from Tocris. [βAla^{8}]-Neurokinin A, MEN 10,376, nifedipine, PMA and PD98,059 were purchased from Sigma. [MePhe^{7}]-Neurokinin B, 2-[1-(3-Dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide (GF109203X) and LY294002 were purchased from Calbiochem (La Jolla, CA, USA). Anti-Gα_i antibodies, anti-Gα_s antibodies, anti-G$\alpha_{q/11}$ antibodies and anti-G$\beta\gamma$ antibodies were purchased from Upstate biotechnology (Lake Placid, NY, USA). Each antibodies were from rabbits immunized with a synthetic peptide corresponding to the COOH-terminal sequence of the human Gα_i, Gα_s, G$\alpha_{q/11}$ and G$\beta\gamma$ subunit, respectively. ω-conotoxin GVIA (ω-CgTx GVIA) and ω-agatoxin IVA (ω-Aga IVA) were purchased from Peptide Institute. U-73122 was purchased from Wako Pure Chemical Industries (Osaka, Japan). SQ22536 and PKI(5-24) were purchased from Biomol Research Laboratories (Plymouth, PA, USA).

4.4. Analysis and statistics

All data analysis were performed using pCLAMP 8.0 acquisition system. Values in text and figures are expressed as mean ± SEM. Statistical analysis was made by student t-test for comparisons between pairs of groups and by one-way analysis of variance (ANOVA) followed by Dunnett's test. Probability (p) values of less than 0.05 were considered significant.

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Figure 1. Typical example of SSP-induced facilitation of I_{Ba}

A, Typical superimposed I_{Ba} traces at the times indicated in the time course graph *B*. I_{Ba} were evoked from a holding potential of −80 mV by a 100 msec voltage step to 0 mV at 20 sec intervals. *B*, Typical time course of SSP-induced facilitation of I_{Ba}. SSP (1 μM) was bath-applied during the time indicated by the filled bar. *C*, Current-voltage relations of I_{Ba} evoked by a series of voltage steps from a holding potential of −80 mV to test potentials between −80 and +40 mV in +10 mV increments in the absence (opened points) and presence (filled points) of 1 μM SSP. Values of I_{Ba} are the averages of five neurons. *D*, Dose dependence of neurokinin receptor agonists-induced facilitation of I_{Ba}. Each neuron was tested with only one drug concentration and only 1st application of drug was analyzed. Numbers in parentheses indicate the number of neurons tested.

Figure 2. SSP-induced facilitation of I_{Ba} in various conditions

A, Antagonism of SSP-induced facilitation of I_{Ba}. The histogram demonstrates the degree of I_{Ba} facilitation by 1 μM SSP, SSP after L-732,138 (NK_1 receptor antagonist, 1 μM), SSP after MEN 10,376 (NK_2 receptor antagonist, 1 μM) and SSP after SB 218795 (NK_3 receptor antagonist, 1 μM). *B*, G-protein selectivity of SSP-induced facilitation of I_{Ba}. The histogram demonstrates the degree of I_{Ba} facilitation by 1 μM SSP in control (recording pipette was filled with GTP), intracellular dialysis with anti-G α_i antibody, intracellular dialysis with anti-G α_s antibody, intracellular dialysis with anti-G $\alpha_{q/11}$ antibody, intracellular dialysis with boiled anti-G $\alpha_{q/11}$ antibody (90 ℃ for 30 min) and intracellular dialysis with anti-G $\beta\gamma$ antibody. Numbers in parentheses indicate the number of neurons tested. *＜0.05 compared with control, ANOVA.

Figure 3. SSP-induced facilitation of I_{Ba} of distinct types of VDCCs

Histogram demonstrating the degree of I_{Ba} facilitation by 1 μM SSP on L + R-types (after treatments with ω-CgTx GVIA + ω-Aga IVA), N + R-types (after treatments with Nif + ω-Aga IVA) and P/Q + R-types (after treatments with Nif +ω-CgTx G VIA) VDCCs. Numbers in parentheses indicate the number of neurons tested.

Figure 4. SSP-induced facilitation of I_{Ba} in neurons treated with PKC inhibitor

A, Typical superimposed I_{Ba} traces at the times indicated in the time course graph *B*. *B*, Typical time course of SSP-induced facilitation of I_{Ba} in neurons treated with PKC inhibitor GF109203X (10 μM for 30 min). SSP (1 μM) was bath-applied during the time indicated by the filled bar. *C*, Histogram demonstrating the degree of I_{Ba} facilitation by SSP in control (untreated neurons), after U-73122 (a PLC inhibitor), after GF109203X, after PMA (PKC activator), after LY294002 (a PI3K inhibitor), after SQ22536 (an adenylate cyclase inhibitor), intracellular dialysis with PKI(5-24) (a PKA inhibitor) and after PD98,059 (a MAPK tyrosine kinase inhibitor). Numbers in parentheses indicate the number of neurons tested. *$<$0.05 compared with control, ANOVA.

Figure 5. Typical example of SSP-induced inhibition of I_{Ba}

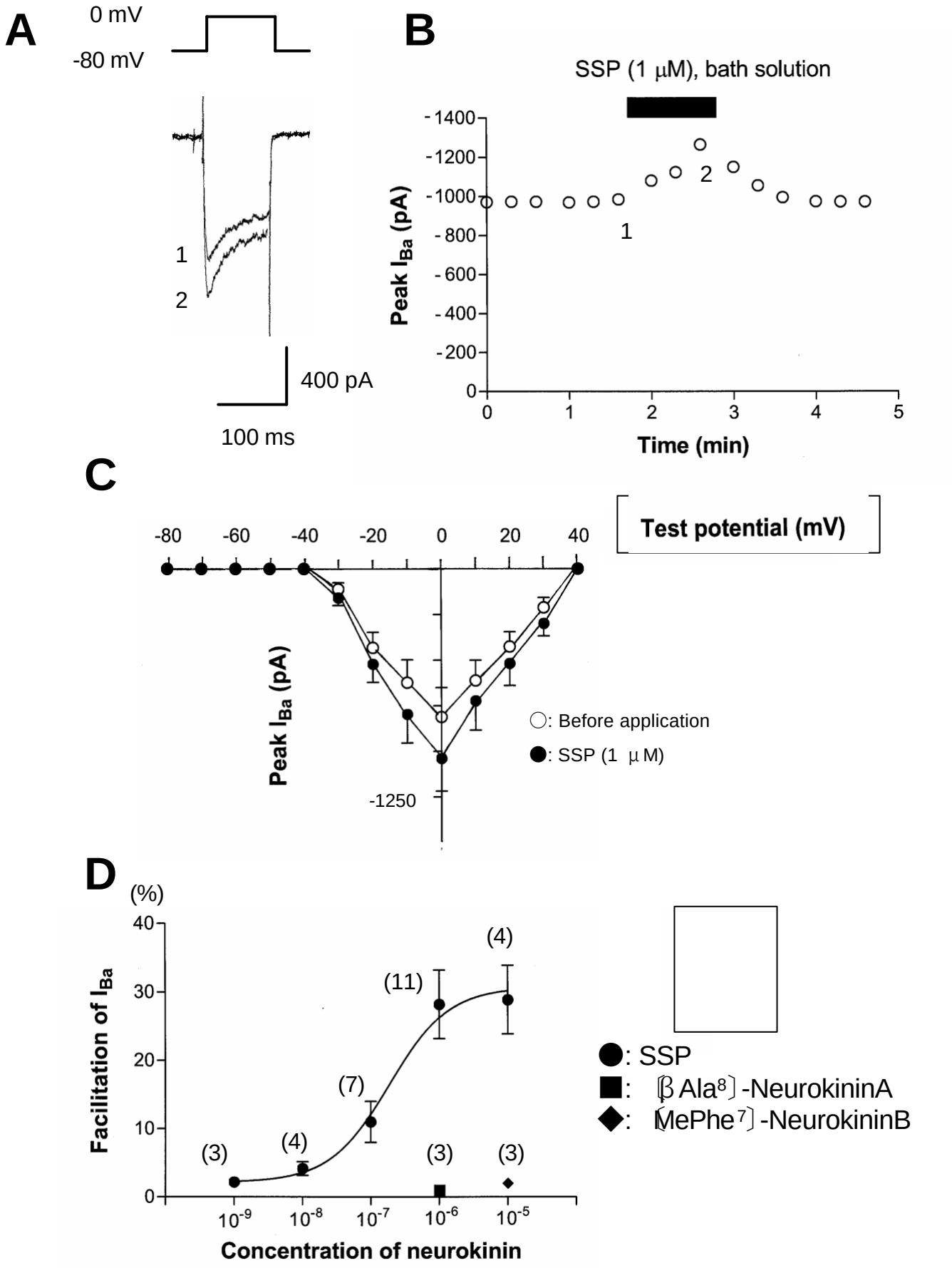
A, Typical superimposed I_{Ba} traces recorded using a double-pulse voltage protocol at the times indicated in the time course graph *B*. Paired I_{Ba} were evoked from a holding potential of −80 mV by a 100 msec voltage step to 0 mV at 20 sec intervals. An intervening strong depolarizing prepulse (100 mV, 30 msec) ended 5 msec prior to the second I_{Ba} activation. *B*, Typical time course of SSP-induced inhibition of I_{Ba}. SSP (1 μM) was bath-applied during the time indicated by the filled bar. *C*, Current-voltage relations of I_{Ba} evoked by a series of voltage steps from a holding potential of −80 mV to test potentials between −80 and +40 mV in +10 mV increments in the absence (opened points) and presence (filled points) of 1 μM SSP. Values of I_{Ba} are the averages of five neurons. *D*, Dose dependence of neurokinin receptor agonists-induced inhibition of I_{Ba}. Each neuron was tested with only one drug concentration and only 1st application of drug was analyzed. Numbers in parentheses indicate the number of neurons tested.

Figure 6. SSP-induced inhibition of I_{Ba} in various conditions

A, Antagonism of SSP-induced inhibition of I_{Ba}. The histogram demonstrates the degree of I_{Ba} inhibition by 1 μM SSP, SSP after L-732,138 (NK_1 receptor antagonist, 1 μM), SSP after MEN 10,376 (NK_2 receptor antagonist, 1 μM) and SSP after SB 218795 (NK_3 receptor antagonist, 1 μM). *B*, G-protein selectivity of SSP-induced inhibition of I_{Ba}. The histogram demonstrates the degree of I_{Ba} inhibition by 1 μM SSP in control (recording pipette was filled with GTP), intracellular dialysis with anti-Gα_i antibody, intracellular dialysis with anti-Gα_s antibody, intracellular dialysis with anti-G$\alpha_{q/11}$ antibody, intracellular dialysis with boiled anti-G$\alpha_{q/11}$ antibody (90 ℃ for 30 min) and intracellular dialysis with anti-G$\beta\gamma$ antibody. Numbers in parentheses indicate the number of neurons tested. *＜0.05 compared with control, ANOVA.

Figure 7. VDCCs subtypes and second messenger in SSP-induced inhibition of I_{Ba}

A, Fractional components of L-, N-, P/Q- and R-types I_{Ba} and those inhibited by SSP (1 μM). The total height of the bars (open and hatched) represents the mean ± SEM contribution of the indicated VDCCs type to the total I_{Ba}. The closed bars represent the mean ± SEM inhibition by SSP (1 μM) of the corresponding VDCCs type. *B*, Histogram demonstrating the degree of I_{Ba} inhibition by SSP in control (untreated neurons), after U-73122 (a PLC inhibitor), after GF109203X, after PMA (PKC activator), after LY294002 (a PI3K inhibitor), after SQ22536 (an adenylate cyclase inhibitor), intracellular dialysis with PKI(5-24) (a PKA inhibitor) and after PD98,059 (a MAPK tyrosine kinase inhibitor). Numbers in parentheses indicate the number of neurons tested.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Facilitation of I_{Ba}
Concentration of neurokinin
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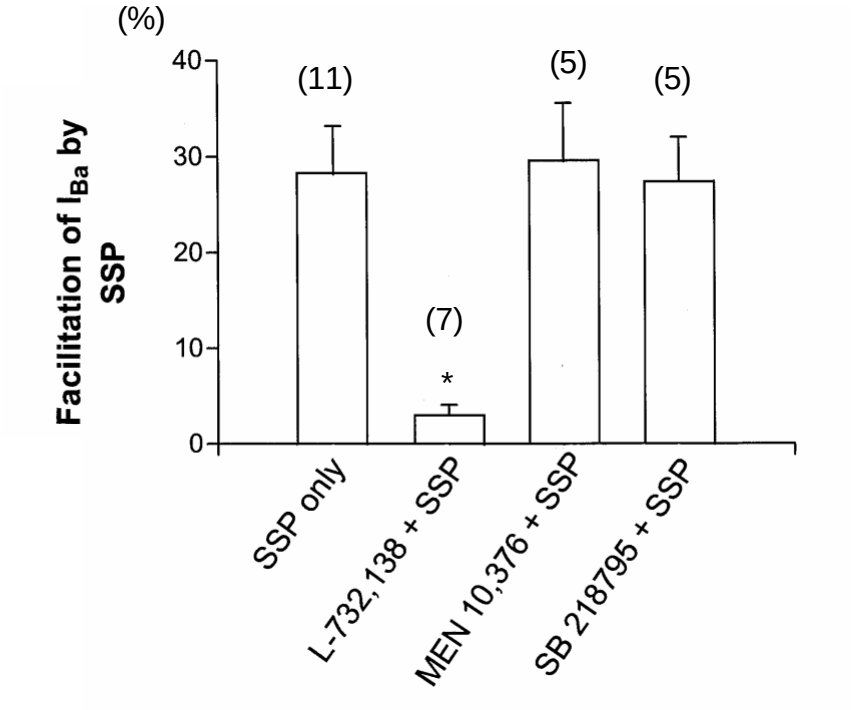
■: [βAla8]-NeurokininA
◆: [MePhe7]-NeurokininB

A


(%)
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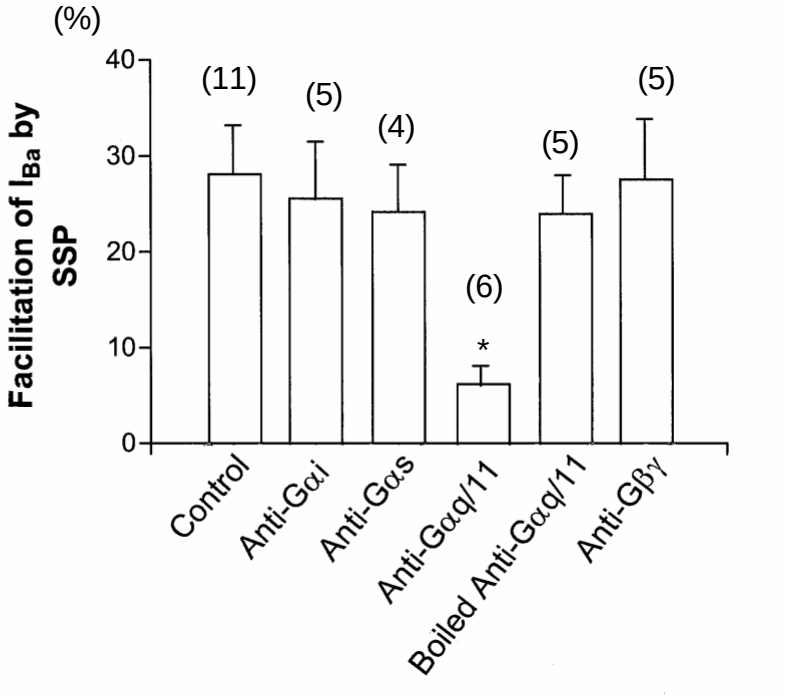
SSP only
L-732,138 + SSP
MEN 10,376 + SSP
SB 218795 + SSP

B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Facilitation of I_{Ba} by SSP
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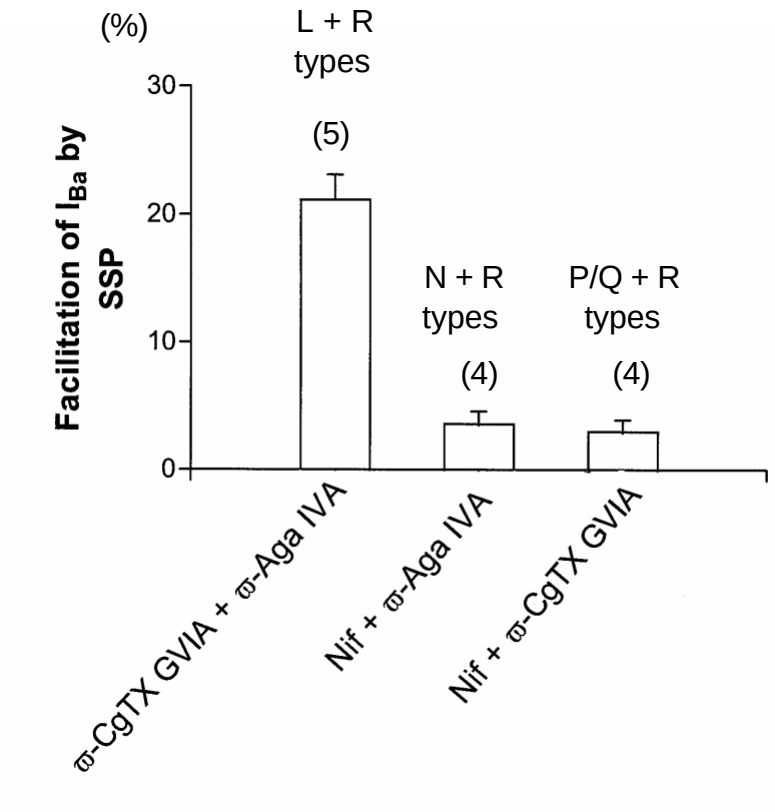
Anti-Gαq/11
Boiled Anti-Gαq/11
Anti-Gβγ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N + R types
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P/Q + R types
(4)
Facilitation of I_{Ba} by SSP
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10
0
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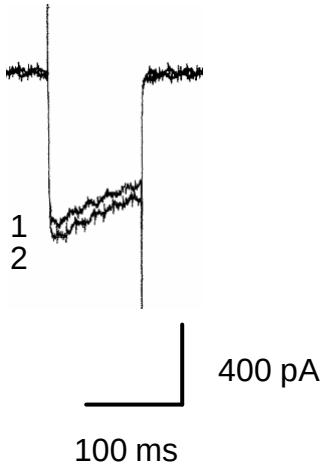
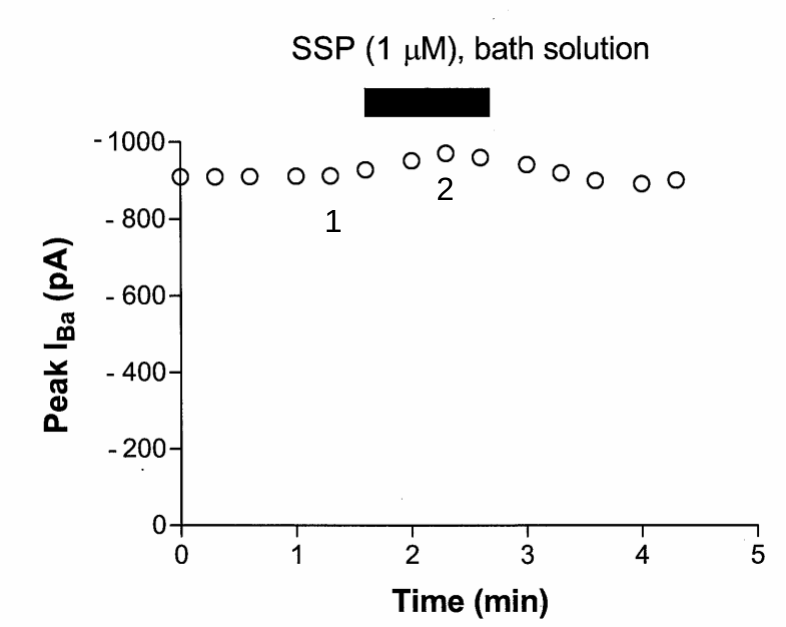
Nif + ω-Aga IVA
Nif + ω-CgTX GVIA

A

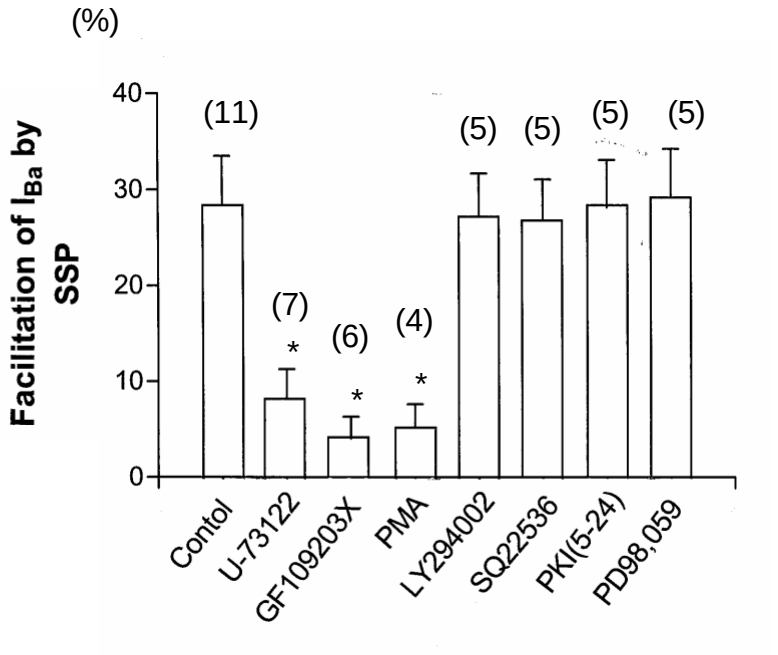


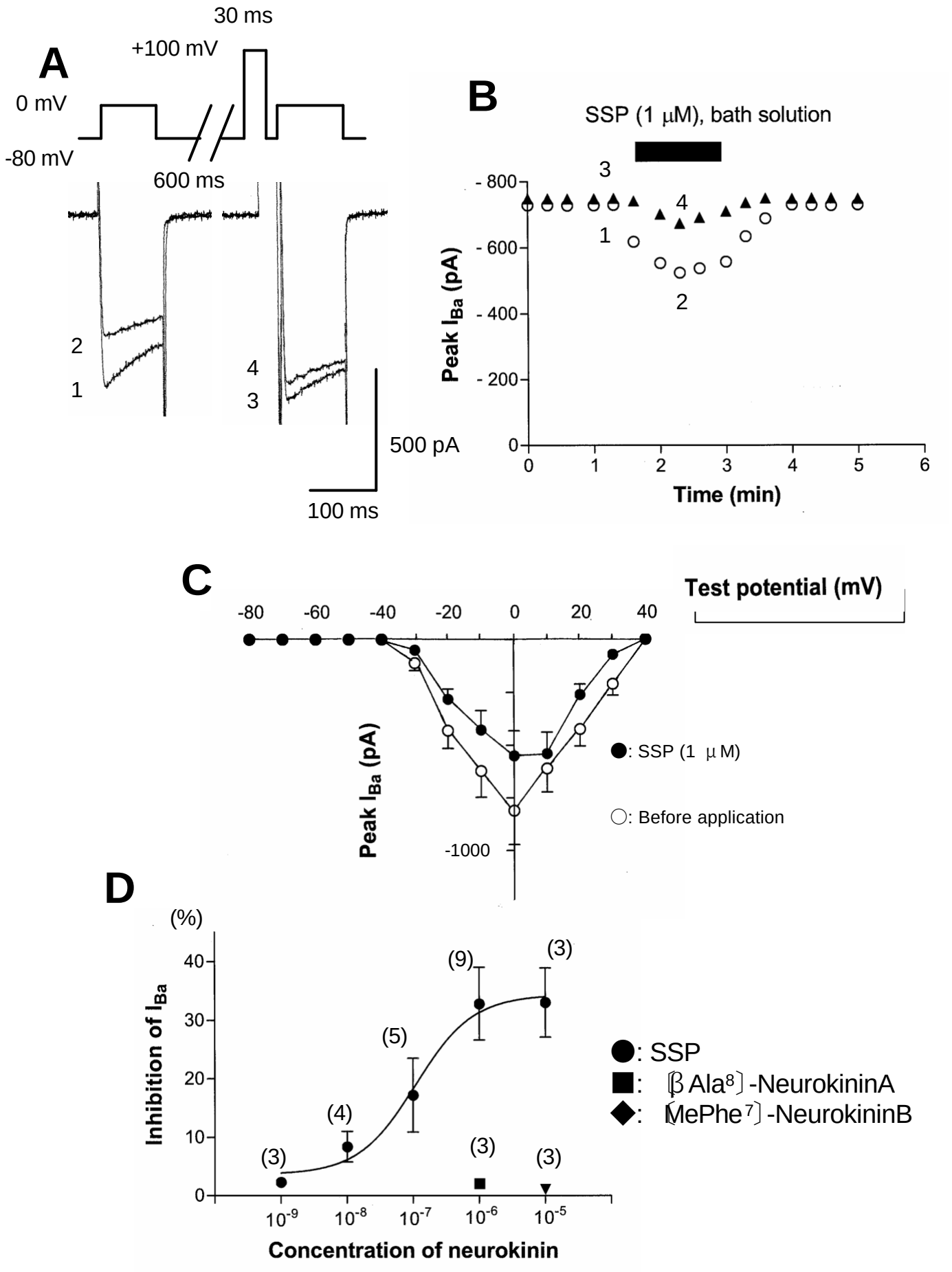
B

In neuron treated with GF109203X 10 μM for 30min before patch clamp experiments

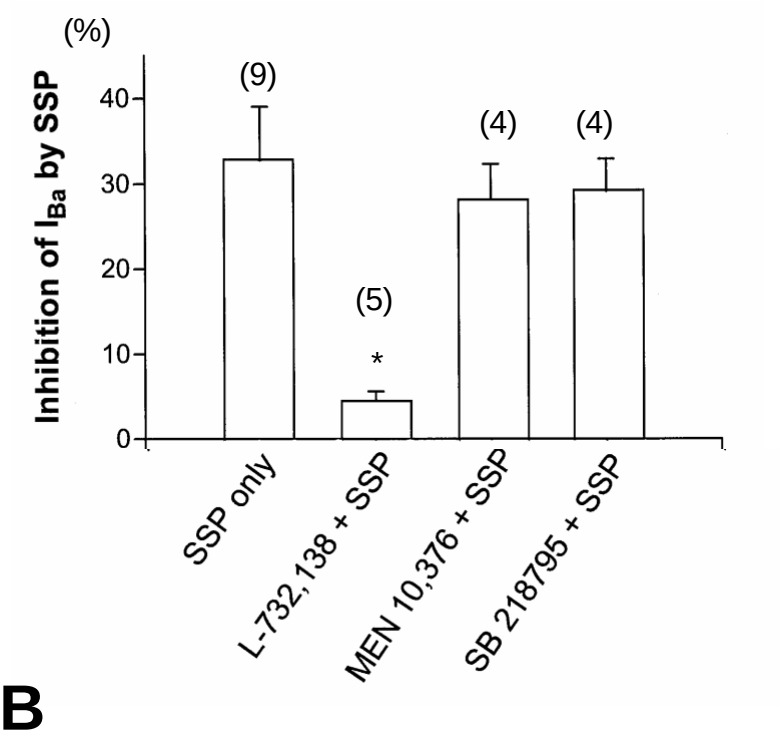


C

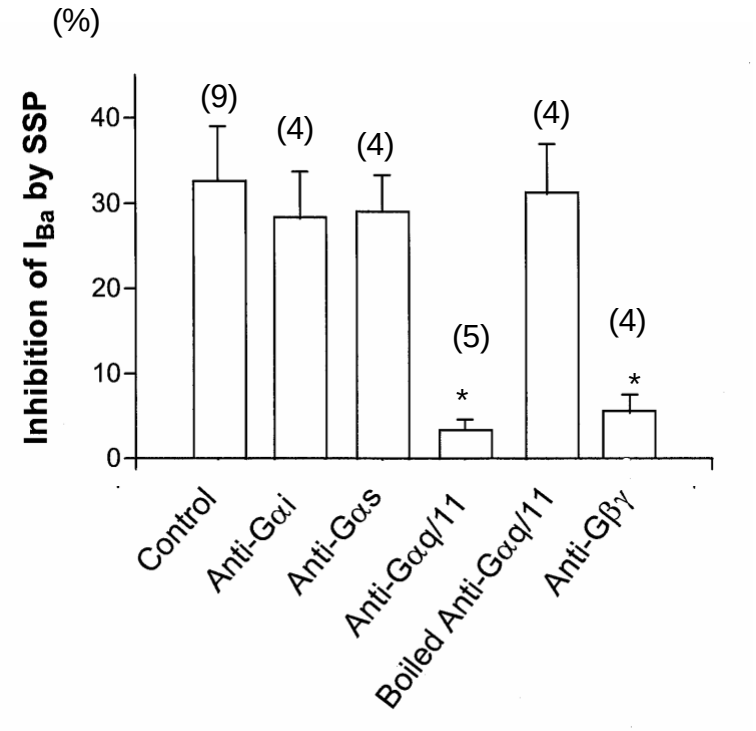



A
30 ms
+100 mV
0 mV
-80 mV
600 ms
2
1
4
3
500 pA
100 ms
B
SSP (1 μM), bath solution
3
4
1
2
- 800
- 600
- 400
- 200
0
Peak I_{Ba} (pA)
0
1
2
3
4
5
6
Time (min)
C
Test potential (mV)
-80
-60
-40
-20
0
20
40
Peak I_{Ba} (pA)
-1000
●: SSP (1 μM)
○: Before application
D
(%)
40
30
20
10
0
Inhibition of I_{Ba}
(3)
(4)
(5)
(9)
(3)
(3)
(3)
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Concentration of neurokinin
●: SSP
■: [βAla8]-NeurokininA
◆: [MePhe7]-NeurokininB

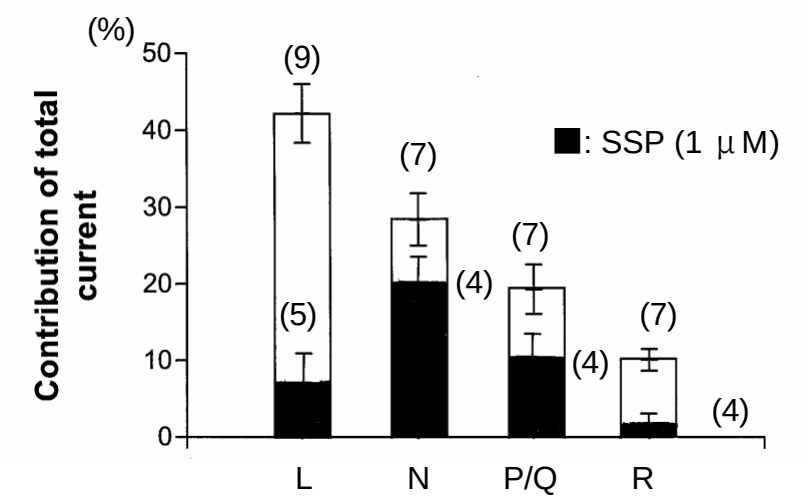
A


(%)
Inhibition of I_{Ba} by SSP
0
10
20
30
40
(9)
(5)
(4)
(4)
*
SSP only
L-732,138 + SSP
MEN 10,376 + SSP
SB 218795 + SSP

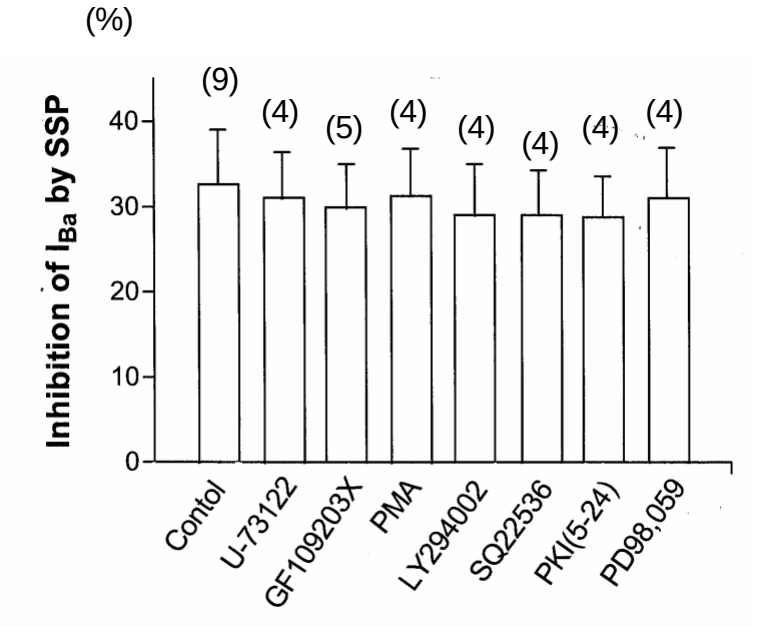
B


(%)
Inhibition of I_{Ba} by SSP
0
10
20
30
40
(9)
(4)
(4)
(5)
(4)
(4)
*
*
Control
Anti-Gαi
Anti-Gαs
Anti-Gαq/11
Boiled Anti-Gαq/11
Anti-Gβγ

A


(%)
Contribution of total current
50
40
30
20
10
0
(9)
(5)
(7)
(4)
(7)
(4)
(7)
(4)
■: SSP (1 μM)
L
N
P/Q
R

B


(%)
Inhibition of I_{Ba} by SSP
40
30
20
10
0
(9)
(4)
(5)
(4)
(4)
(4)
(4)
(4)
Contol
U-73122
GF109203X
PMA
LY294002
SQ22536
PKI(5-24)
PD98,059