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# Mechanisms of the Modulation of Pacemaker Activity by GnRH Peptides in the Terminal Nerve-GnRH Neurons

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**ABSTRACT**—According to our working hypothesis, the terminal nerve (TN)-gonadotropin releasing hormone (GnRH) system functions as a neuromodulatory system that regulates many long-lasting changes in animal behaviors. We have already shown by using *in vitro* whole brain preparations of a small fish (dwarf gourami) that the pacemaker activities of TN-GnRH neurons are modulated biphasically by salmon GnRH, which is the same molecular species of GnRH produced by TN-GnRH neurons themselves; the modulation consists of initial transient decrease and late increase of firing frequency. In the present study, we investigated the possible involvement of Ca<sup>2+</sup> release from intracellular store and voltage dependent Ca<sup>2+</sup> currents in the modulation of pacemaker activities. Pharmacological blockade of Ca<sup>2+</sup> release from intracellular stores or apamin-sensitive Ca<sup>2+</sup>-activated K<sup>+</sup> current inhibited the initial transient decrease of firing frequency by sGnRH. On the other hand, bath application of Ca<sup>2+</sup> channel blockers Ni<sup>2+</sup> or La<sup>3+</sup> slowed down the pacemaker frequency and attenuated the rate of the late increase of pacemaker frequency by GnRH. Furthermore, voltage-clamp experiments suggested that low-voltage-activated (LVA) Ca<sup>2+</sup> current and high-voltage-activated (HVA) Ca<sup>2+</sup> current were present in the TN-GnRH neurons, and bath application of GnRH shifted the activation threshold of HVA Ca<sup>2+</sup> current to more negative potentials. These results suggest that (1) sGnRH induces Ca<sup>2+</sup> release from intracellular stores and activates apamin-sensitive Ca<sup>2+</sup>-activated K<sup>+</sup> current so that it decreases the frequency of pacemaker activity in the initial phase, (2) some kinds of Ca<sup>2+</sup> currents contribute to the generation and modulation of pacemaker activities, and (3) HVA Ca<sup>2+</sup> current is facilitated by sGnRH so that it increases the frequency of pacemaker activity in the late phase.

**Key words:** GnRH, peptide, pacemaker, neuromodulation, electrophysiology

## INTRODUCTION

The gonadotropin-releasing hormone (GnRH) was originally identified as a hypophysiotropic decapeptide hormone that is produced in the preoptic area, transported to the median eminence, and facilitates the release of gonadotropins from the pituitary. However, GnRH neurons and their fibers have been found not only in the preoptic area but also in several brain areas outside the hypothalamic area. Such 'extrahypothalamic' GnRH systems have been found mainly in the terminal nerve (TN) and midbrain (Schwanzel-Fukuda and Silverman, 1990; Parhar and Iwata, 1994; Yamamoto *et al.*, 1995), and they project widely in the brain instead of the

median eminence or the pituitary.

By taking advantage of anatomical feature of the *in vitro* whole brain preparation of the dwarf gourami, we have been studying the electrophysiological characteristics of the TN-GnRH system (reviewed by Oka, 1997; Oka and Abe, 2002). We have previously shown that (1) individual cells of TN-GnRH system project widely in the entire brain from the olfactory bulb to the spinal cord (Oka and Matsushima, 1993), and (2) single TN-GnRH neurons show spontaneous pacemaker activity (Oka and Matsushima, 1993) which consists of the depolarizing phase produced by I<sub>Na(slow)</sub> (Oka, 1995, 1996) and the repolarizing phase mainly produced by TEA-sensitive persistent potassium current, I<sub>K(v)</sub> (Abe and Oka, 1999). Moreover, the frequency of pacemaker activity seems to vary according to the physiological conditions of the animal, and the firing frequency and/or firing mode may

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affect the efficacy of exocytosis of GnRH peptides from the GnRH neurons (Peng and Horn, 1991). On the other hand, a growing body of evidence suggests that GnRH peptide modulates the function of ion channels such as Na<sup>+</sup> channels (Eisthen *et al.*, 2000), K<sup>+</sup> channels (Adams and Brown, 1980) and Ca<sup>2+</sup> channels (Elmslie *et al.*, 1990) and thus may regulate the excitability or neurotransmitter release of target neurons. Thus, it has been suspected that the TN-GnRH system may function as a neuromodulatory system that is involved in the regulation of long-lasting changes in the animal's behavior, e.g., motivational or arousal states (Oka and Matsushima, 1993; Oka, 1997). Therefore, the study of nature and mechanisms of modulation of the pacemaker activity of TN-GnRH neurons by hormones or transmitters will give us invaluable information about the control mechanism of neuromodulatory GnRH system.

We have previously indicated that the frequency of pacemaker activity of TN-GnRH neurons was biphasically modulated by sGnRH in a dose dependent and paracrine/autocrine manner (Abe and Oka, 2000, 2002). This biphasic modulation consisted of the transient decrease (early phase) and subsequent increase (late phase) of firing frequency. Furthermore, it was suggested that the G-protein coupled GnRH receptor in the cell membrane triggers signal transduction pathway that ended up modulating the frequency of pacemaker activity of TN-GnRH neurons. This biphasic modulation of pacemaker activity may contribute to a synchronized facilitation of pacemaker activities of neighboring TN-GnRH neurons.

In other types of neurons, it has been proposed that G-protein coupled receptors can modulate the gating properties of the ion channels. Both direct effects of G-proteins and indirect effects via diffusible second messengers have been implicated in these modulations (Hille, 1994). Generally, it has been suggested that GnRH receptors are coupled to the G<sub>q/11</sub> type G-proteins, and the G<sub>q/11</sub> type G-proteins enhance inositol phosphate formation and subsequent increase in [Ca<sup>2+</sup>]<sub>i</sub> (Naor *et al.*, 1998; Stojilkovic *et al.*, 1994a, b; Stojilkovic and Catt, 1995a, b). In the pituitary gonadotrophs, it has been accepted that GnRH-induced activation of phospholipase C is the major signal transduction pathway of GnRH receptor-coupled processes, and the subsequent mobilization of [Ca<sup>2+</sup>]<sub>i</sub> and the activation of protein kinase C (PKC) are the key elements in the control of gonadotropin secretion by pituitary gonadotrophs (Stojilkovic and Catt, 1995a, b).

On the other hand, the dependence on the extracellular Ca<sup>2+</sup> of episodic release of GnRH from perfused hypothalamic neurons and GT1-7 cells suggests that GnRH secretion is controlled by Ca<sup>2+</sup> entry through the plasma membrane Ca<sup>2+</sup> channels (Krsmanovic *et al.*, 1992). Electrophysiological studies have demonstrated the expression of several types of plasma membrane Ca<sup>2+</sup> channels in the embryonic GnRH neurons (Kusano *et al.*, 1995) and GT1 cells (Bosma, 1993; Costantin and Charles, 1999; Javors *et al.*, 1995; Van Goor *et al.*, 1999b), including transient and

sustained voltage-dependent Ca<sup>2+</sup> channels.

Therefore, in the present paper, we examined the possible involvement of [Ca<sup>2+</sup>]<sub>i</sub> mobilization from the intracellular stores and the plasma membrane Ca<sup>2+</sup> channels in the biphasic modulations of pacemaker activity in the TN-GnRH neurons.

## MATERIAL AND METHODS

### Preparations

Adult male and female dwarf gourami (*Colisa lalia*), ~4 cm in standard length, were purchased from a local dealer and kept at 22 ~ 27 °C until used. The whole brain *in vitro* preparation was made according to the procedures described in the previous papers (Oka, 1995, 1996; Abe and Oka, 1999, 2000).

### Electrophysiology

The *in vitro* whole brain preparation was continuously superfused with an oxygenated Ringer solution containing (in mM) 124 NaCl, 5 KCl, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 2.4 CaCl<sub>2</sub>, 1.3 MgSO<sub>4</sub>, 26 NaHCO<sub>3</sub>, and 10 glucose (pH 7.4 adjusted with NaOH) in the silicone elastomer (Shin-Etsu Silicone No.KE-106, Shin-Etsu Chemical Co., Ltd., Japan) base of a small recording chamber. Whole cell voltage- and current-clamp recordings were carried out with the use of CEZ-2300 amplifier (Nihon Kohden, Japan) and pCLAMP software (Axon instruments). Pipette resistance was ~8 MΩ, and seal resistance was >10GΩ. Series resistance was compensated as much as possible. The patch pipette was visually guided to the cluster of TN-GnRH neurons exposed on the ventral surface of the brain under a dissecting microscope (Oka and Matsushima, 1993). After gigaohm seal formation and "break in" for the whole cell recording mode, characteristic spontaneous pacemaker activity was confirmed in the current-clamp mode (see Oka and Matsushima, 1993; Abe and Oka, 2000).

For current-clamp experiments, the patch pipettes contained (in mM) 110 K-gluconate, 3 MgCl<sub>2</sub>, 40 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 0.3 ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 2 Na<sub>2</sub>ATP, and 0.2 Na<sub>2</sub>GTP (pH 7.4 adjusted with NaOH), NiCl<sub>2</sub> (1 mM), LaCl<sub>3</sub> (5 μM), Apamin (100 nM; Alomone Labs.) and salmon GnRH (sGnRH, 200 ~ 300 nM; RBI) were dissolved directly in the Ringer solution. Ruthenium red (50 μM; Sigma) and heparin (300 μg / ml; Sigma) were dissolved in the pipette solution. Pacemaker activities were digitized (2 kHz), displayed on-line with Axotape software or pClamp8 (Axon Instruments), and stored on a computer.

For voltage-clamp recording of Ca<sup>2+</sup> currents, the extracellular solution contained (in mM) 95 choline Cl, 40 tetraethylammonium chloride (TEACl), 2.4 CaCl<sub>2</sub>, 1.3 MgCl<sub>2</sub>, 5 Glucose, 5 4-aminopyridine (4AP), and 10 HEPES (pH 7.4 adjusted with NaOH). In addition, 0.75 μM tetrodotoxin (TTX) was added to the extracellular solution. Patch pipettes were filled with a solution consisting of (mM) 90 CsCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 20 TEACl, 10 EGTA, 10 HEPES, 2 Na<sub>2</sub>ATP, and 0.2 Na<sub>2</sub>GTP (pH 7.4 adjusted with CsOH). For voltage-clamp recording of tentative Ca<sup>2+</sup>-activated K<sup>+</sup> currents, the extracellular solution contained (in mM) 115 choline Cl, 20 TEACl, 5 KCl, 2.4 CaCl<sub>2</sub>, 1.3 MgCl<sub>2</sub>, 5 glucose, and 10 HEPES (pH 7.4 adjusted with NaOH). 0.75 μM TTX was also added to the extracellular solution. Patch pipettes were filled with a solution consisting of (mM) 110 KCl, 3 MgCl<sub>2</sub>, 40 HEPES, 0.3 EGTA, and 2Na<sub>2</sub>ATP (pH 7.4 adjusted with NaOH). The linear leakage currents were digitally subtracted, either automatically with the use of the P/4 protocol, or manually, after measuring ohmic resistance in response to hyperpolarizing command pulses. The data were not corrected for the liquid junction potentials.

### Data analysis

Data analysis, fitting, averaging and presentation were carried out using a combination of pCLAMP6 and 8, Axograph (Axon Instruments), Microsoft Excel (Microsoft), DeltaGraph (Polaroid), and Canvas (Deneba software) softwares. All data are present as means $\pm$ SE.

## RESULTS

Most of the TN-GnRH neurons showed regular spontaneous pacemaker activity, and it was biphasically modulated by bath application of sGnRH, as we have reported previously (Abe and Oka, 2000; also see Fig. 3Ba~d).

### Involvement in the control of pacemaker activity of Apamin-sensitive Ca<sup>2+</sup>-activated K<sup>+</sup> currents induced by Ca<sup>2+</sup> released from the intracellular store

First, in order to examine the possible involvement of Ca<sup>2+</sup> released from the intracellular store in the control of pacemaker activity of TN-GnRH neuron, the cells were dialyzed with ruthenium red, an inhibitor of Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release, and heparin, that of IP<sub>3</sub>-induced Ca<sup>2+</sup> release, respectively, by including them in the patch pipette solution. After that, the effects of bath application of sGnRH on the pacemaker activity were examined. To ensure the diffusion of ruthenium red and heparin into the cytoplasm of the TN-GnRH neuron, the data collection was started 10 min after the whole-cell recording was established; this time period was determined on the basis of the results of intracellular application of QX-314 (see Abe and Oka, 2000). Fig. 1 shows the pacemaker activity of a cell recorded with patch pipette containing ruthenium red (50  $\mu$ M) and heparin (300  $\mu$ g / ml) in the pipette solution. In Ringer solution, the cells showed slightly irregular beating discharge pattern (Fig. 1Ba). Many cells tended to show such a firing pattern by intracellular application of the blockers of Ca<sup>2+</sup> release from the intracellular store. In these experiments, we further applied La<sup>3+</sup> (5  $\mu$ M) to the bath solution in order to block some Ca<sup>2+</sup> channels (see below). Bath application of La<sup>3+</sup> slowed down the frequency of pacemaker activity (Fig. 1Bb). Further bath application of sGnRH (1  $\mu$ M) failed to evoke transient decrease of firing frequency (Fig. 1Bc) but evoked subsequent increase of firing frequency (Fig. 1Bd). Figure 1Bc and Bd (20 and 120 s after the onset of sGnRH perfusion, respectively) were recorded during time periods similar to that of transient decrease and subsequent increase of firing frequency by sGnRH. Fig. 1Bf shows the time course of these changes before and after the application of sGnRH (during the period corresponding to Fig. 1Bb~d).

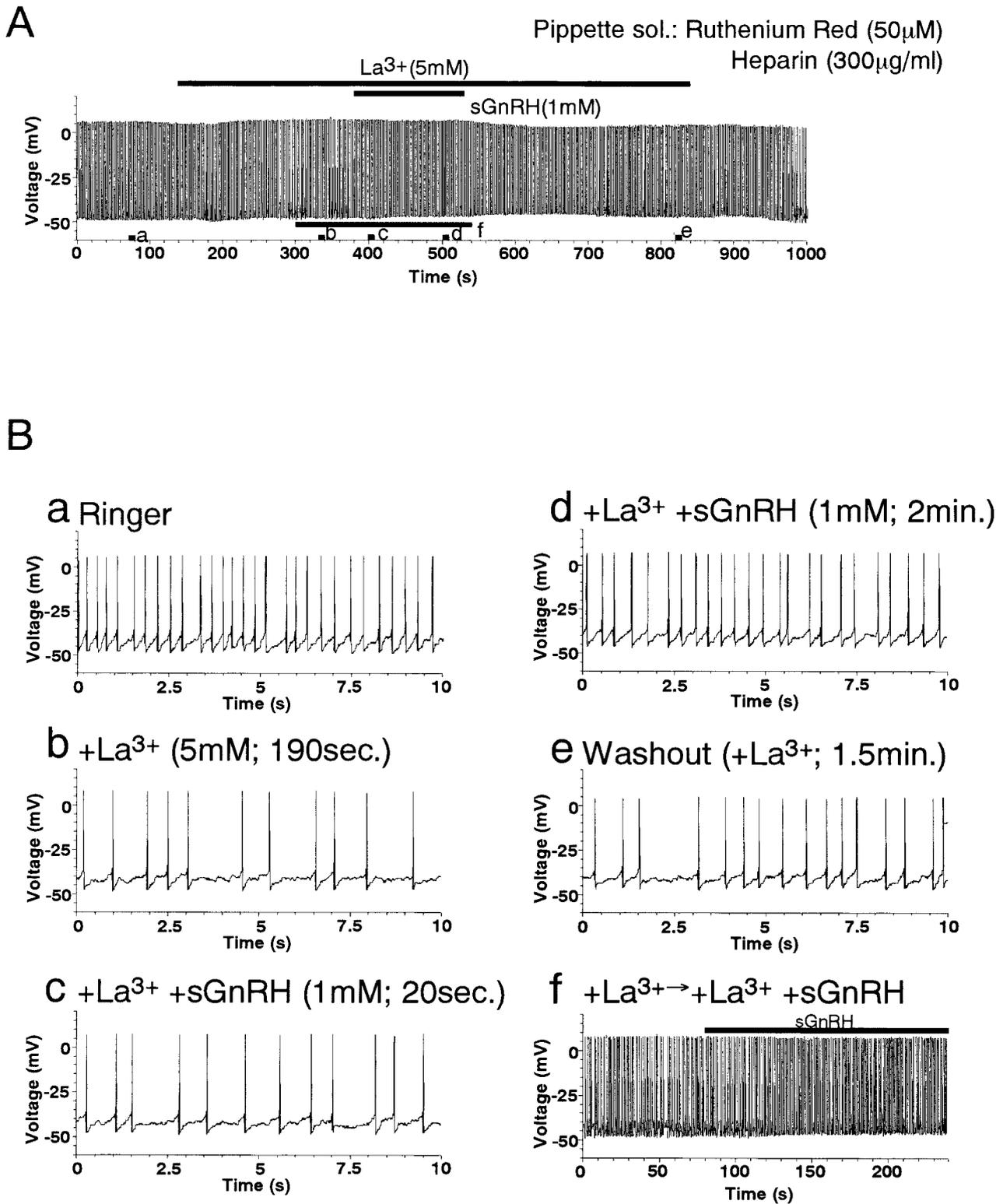
We compared the degree of transient decrease of firing frequency in the presence and absence of the intracellular Ca<sup>2+</sup> mobilization blockers. Each effect of the blockers was measured during a 20 s period that was 30 ~ 50 s after the onset of sGnRH perfusion, which corresponded to the early transient phase of pacemaker frequency decrease, and was normalized to the frequency in Ringer solution. The transient decrease that was induced by sGnRH (0.88 $\pm$ 0.06; n=16)

was nullified by heparin (1.01 $\pm$ 0.05; n=5). Similarly, intracellular application of ruthenium red alone or both ruthenium red and heparin nullified the transient decrease or rather increased slightly the firing frequency (1.11 $\pm$ 0.14; n=7 and 1.16 $\pm$ 0.11; n=4, respectively). However, we could not find statistically significant nullifying effects of blockers of Ca<sup>2+</sup> release from the intracellular store due to the large SE. Anyway, these results suggest that the Ca<sup>2+</sup> release from the intracellular store may be involved in the transient decrease of firing frequency that was induced by sGnRH.

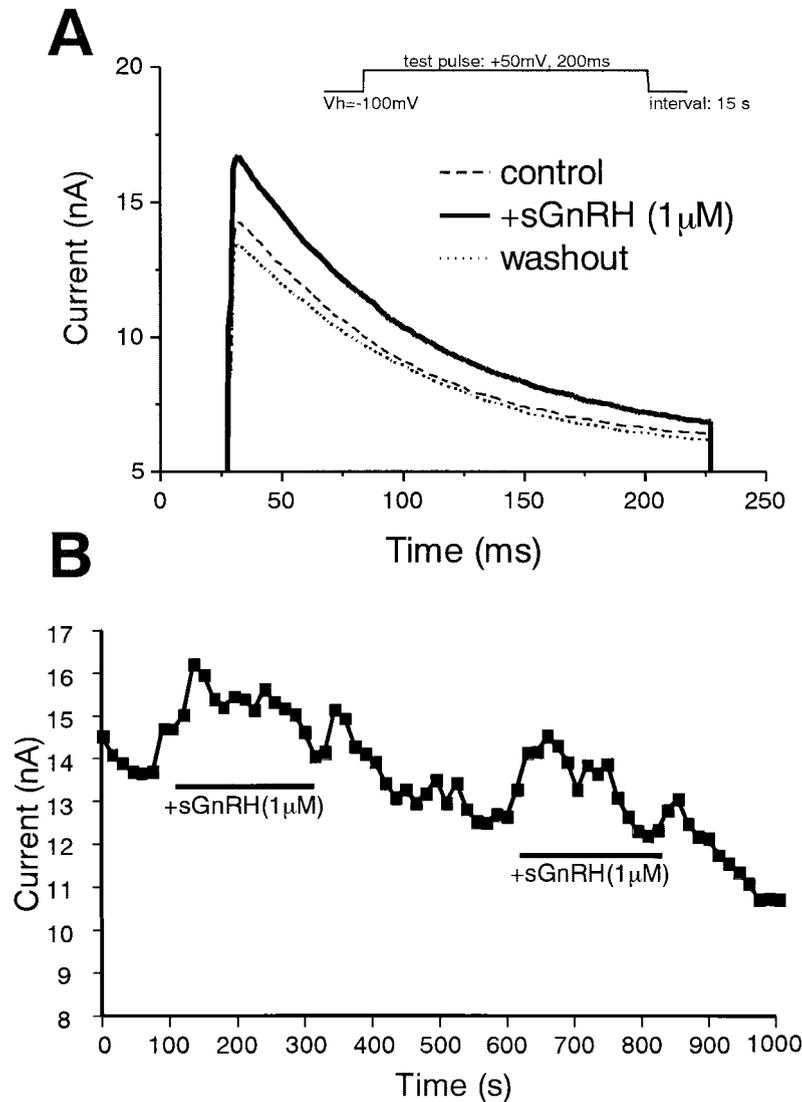
In our previous studies of voltage-dependent K<sup>+</sup> currents (Abe and Oka, 1999), we found that a transient and 4AP-sensitive, but not TEA- or charybdotoxin-sensitive Ca<sup>2+</sup>-dependent outward current component was present in TN-GnRH neurons. Because this current could be observed only when Ca<sup>2+</sup> was present in the external solution, we defined this 4AP-sensitive transient outward current that was dependent on the presence of extracellular Ca<sup>2+</sup> ions as tentative Ca<sup>2+</sup>-activated K<sup>+</sup> currents. When the current responses were measured in Ca<sup>2+</sup>-containing extracellular solutions containing 0.75  $\mu$ M TTX and 20 mM TEA, a mixture of large transient currents and smaller persistent outward currents could be recorded in response to depolarizing pulses from a holding potential of -100 mV. The activation and steady-state inactivation curves of the mixture current containing tentative Ca<sup>2+</sup>-activated K<sup>+</sup> current are shifted to more depolarized potentials compared with those of "4AP-sensitive transient current" (data not shown). These tentative Ca<sup>2+</sup>-activated K<sup>+</sup> currents are only partially inactivated at membrane potentials of -60 to -40 mV, which correspond to the base membrane potentials of the pacemaker activity of TN-GnRH neurons. Thus, it is reasonable to think that tentative Ca<sup>2+</sup>-activated K<sup>+</sup> current can be activated in the pacemaker range of TN-GnRH neurons.

Next, we examined the effect of sGnRH on the tentative Ca<sup>2+</sup>-activated K<sup>+</sup> current. Fig. 2A shows the superimposed traces of the tentative Ca<sup>2+</sup>-activated K<sup>+</sup> currents before, during, and after sGnRH applications elicited during a series of +50 mV test pulses from a holding potential of -100 mV. In this case, bath application of sGnRH (1  $\mu$ M) facilitated current amplitude about 18% (Fig. 2A). The current amplitude recovered to the control level after washout by Ringer solution. Fig. 2B shows the time course of the tentative Ca<sup>2+</sup>-activated K<sup>+</sup> current before, during, and after sGnRH applications. Test pulses were applied at 15 s intervals. The current amplitude showed a tendency to gradually decrease due to the rundown. However, it was rapidly increased by bath application of sGnRH (within 15 to 90 s; Fig. 2B, solid bars). This time course corresponded to the onset of the transient decrease of firing frequency of pacemaker activity induced by sGnRH. Averaged increase of the amplitude of tentative Ca<sup>2+</sup>-activated K<sup>+</sup> current was about 9% (2 to 19%, n=4).

Finally, we examined the effects of bath application of apamin, a specific SK-type Ca<sup>2+</sup>-activated K<sup>+</sup> channel blocker. Fig. 3 shows the effect of bath application of



**Fig. 1.** Inhibition of Ca<sup>2+</sup> release from the intracellular store disrupts the transient decrease of pacemaker frequency after GnRH application. (A) Continuous recording of the pacemaker activity. Traces indicated by bars a-f are shown in (B) on enlarged time scales. Similar conventions are used in Figs. 3, 5, and 6. Intracellular application of Ruthenium Red (50  $\mu$ M), which inhibits the Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release, and heparin (300  $\mu$ g/ml), which inhibits IP<sub>3</sub>-induced Ca<sup>2+</sup> release, blocked transient decrease (Bc) but not subsequent increase in the frequency of pacemaker activities (Bd), both of which should be induced by sGnRH. Compare with normal changes in the frequency of pacemaker activities that are induced by sGnRH shown in Fig. 3Ba-d.



**Fig. 2.** Tentative  $\text{Ca}^{2+}$ -activated transient  $\text{K}^+$  current is facilitated by sGnRH. (A) Voltage clamp recordings of tentative  $\text{Ca}^{2+}$ -activated transient  $\text{K}^+$  currents. Bath application of 1 mM sGnRH increased the amplitude of tentative  $\text{Ca}^{2+}$ -activated transient  $\text{K}^+$  current. Current traces before, during, and after sGnRH application are shown. (B) shows the time course of the peak amplitude of the tentative  $\text{Ca}^{2+}$ -activated transient  $\text{K}^+$  current during sGnRH applications (bars).

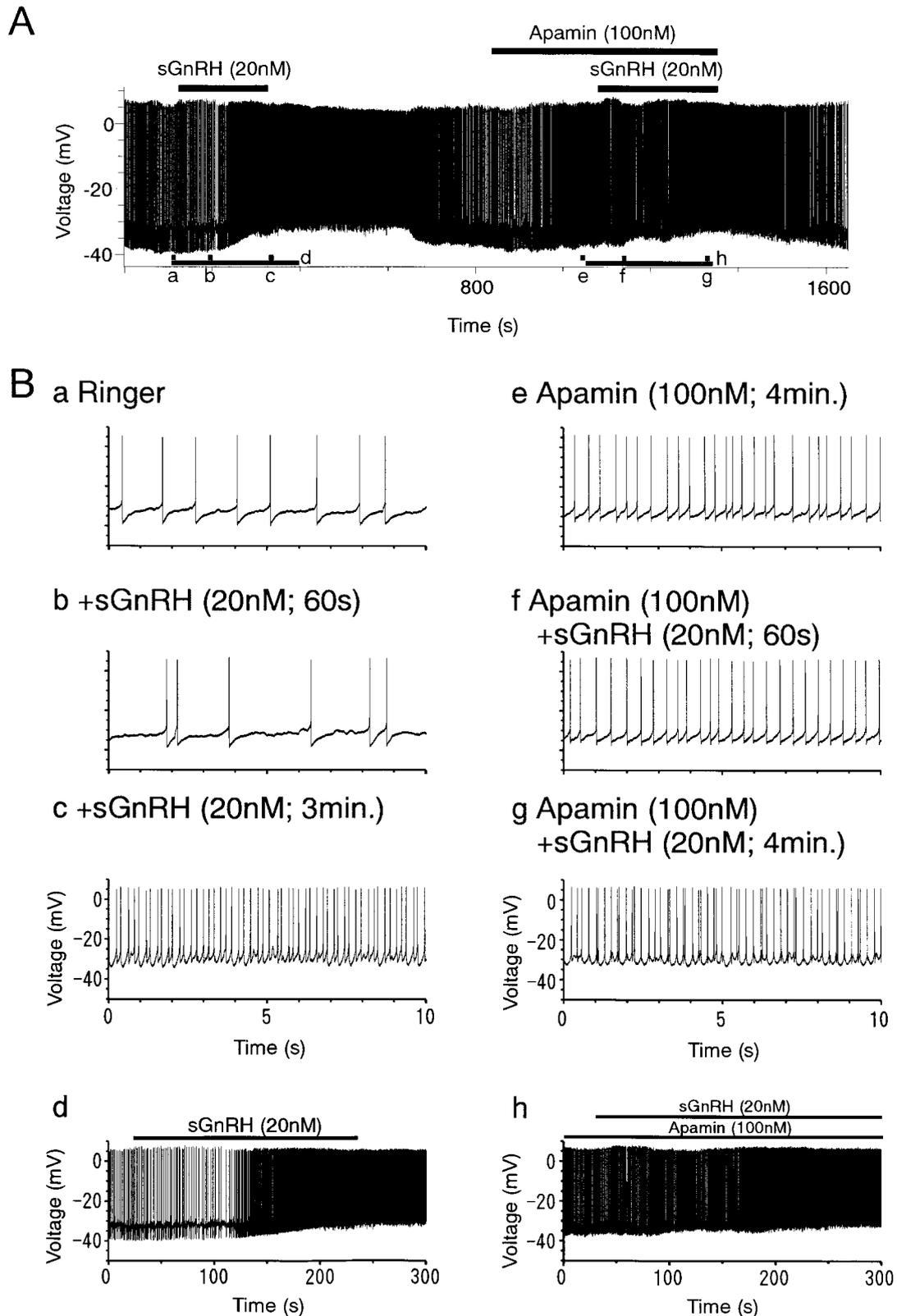
apamin. In Ringer, TN-GnRH neurons showed regular beating discharge ( $3.02 \pm 0.86$  Hz;  $n=4$ ). Bath application of sGnRH (20 nM) transiently decreased ( $2.74 \pm 0.83$  Hz; Fig. 3Bb) and subsequently increased ( $4.98 \pm 0.60$  Hz; Fig. 3Bc) firing frequency of pacemaker activity. After washout of sGnRH, bath application of apamin (100 nM) facilitated the frequency of pacemaker activity ( $3.60 \pm 0.68$  Hz; Fig. 3Be). However, further bath application of sGnRH (20 nM) failed to evoke transient decrease of firing frequency ( $3.70 \pm 0.74$  Hz; Fig. 3Bf) but evoked subsequent increase of firing frequency ( $6.59 \pm 1.15$ ; Fig. 3Bg). Fig. 3Bd and Bh show the time course of these changes before and after the application of sGnRH. We also compared quantitatively the normalized transient decrease of firing frequency in the presence and absence of apamin. The transient decrease that was induced by sGnRH ( $0.92 \pm 0.07$ ;  $n=4$ ) was nullified by apamin

( $1.02 \pm 0.03$ ;  $n=4$ ).

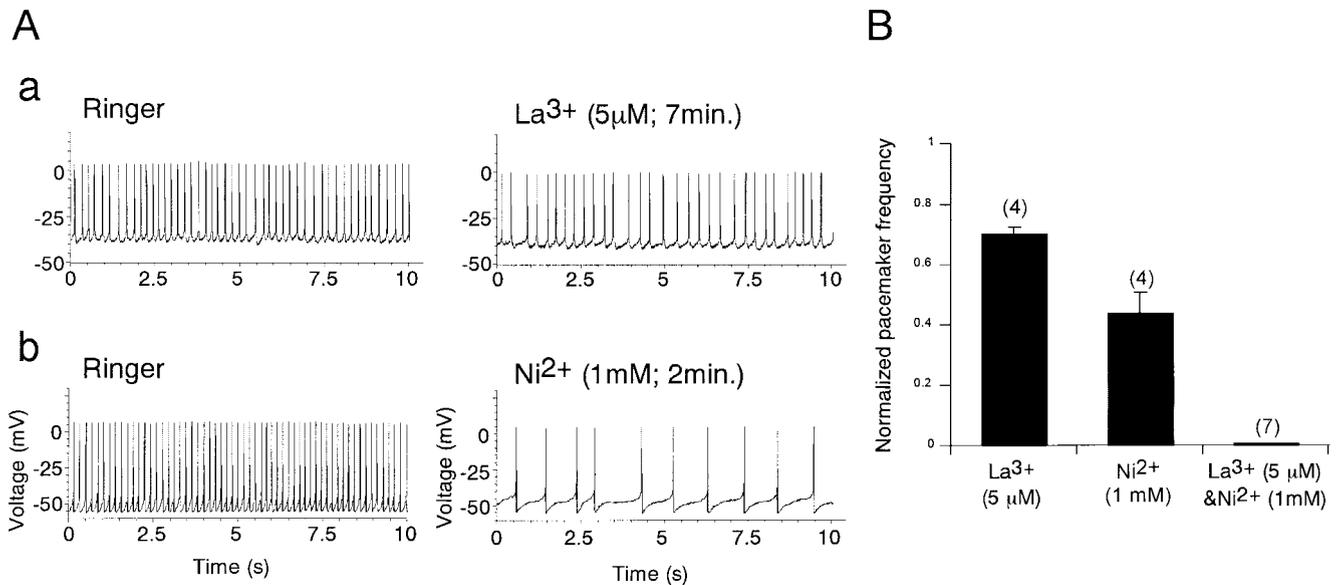
From these data, it is suggested that sGnRH triggers the  $\text{Ca}^{2+}$  release from the intracellular store and activates apamin-sensitive  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  currents, which leads to the transient decrease of the frequency of pacemaker activity.

#### Involvement of $\text{Ca}^{2+}$ currents in the pacemaker activity

Because the preliminary experiments that examined the effect of sGnRH upon  $I_{\text{Na}(\text{slow})}$  and/or  $I_{\text{K}(\text{V})}$  did not show any noticeable modulation, we investigated the possible involvement of  $\text{Ca}^{2+}$  currents in the modulation of pacemaker activity. Fig. 4 shows the effects of  $\text{Ca}^{2+}$  channel blockers on the pacemaker frequency of TN-GnRH neurons. Bath application of various  $\text{Ca}^{2+}$  channel blockers slowed down the pacemaker frequency of TN-GnRH neurons. Fig. 4Aa shows



**Fig. 3.** Inhibition of SK-type  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel disrupts the transient decrease of pacemaker frequency after GnRH application. (A) Continuous recording of the pacemaker activity. In Ringer solution, bath application of sGnRH (20 nM) transiently decreased (Bb) and subsequently increased (Bc) the frequency of pacemaker activity (the overall time course of these changes is shown in Bd). By bath application of apamin (100 nM), which inhibits the SK type  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel, the frequency of pacemaker activity was increased (Be). Furthermore, bath application of apamin blocked transient decrease (Bf) but not subsequent increase in the frequency of pacemaker activities by sGnRH (Bg)(the overall time course of these changes is shown in Bh). Following washout by Ringer solution, the firing frequency decreased again.



**Fig. 4.** Blockade of  $\text{Ca}^{2+}$  currents decreases the pacemaker frequency. Aa, b: Effects of blockers of  $\text{Ca}^{2+}$  currents,  $\text{La}^{3+}$  and  $\text{Ni}^{2+}$ , respectively. Both of these treatments decreased the frequency of pacemaker activities. Following washout, the frequency of pacemaker activities recovered. B: Comparison of the effects of  $\text{Ca}^{2+}$  channel blockers on the pacemaker activity. The numbers in parentheses represent the numbers of cells tested for each blocker. The ordinate indicates the relative pacemaker frequencies after each treatment normalized to those before the treatment; (Frequency of pacemaker activity in Ringer solution containing  $\text{Ca}^{2+}$  channel blocker) / (Frequency of pacemaker activity in Ringer solution). Bath application of  $\text{Ca}^{2+}$  channel blockers decreased the pacemaker frequency. It should be noted that simultaneous bath application of both  $\text{La}^{3+}$  and  $\text{Ni}^{2+}$  (the right column in B) had an additive effect of blocking the frequency of pacemaker activity (compare with the left and middle columns in B).

the effects of the blocker of high-voltage-activated  $\text{Ca}^{2+}$  currents,  $\text{La}^{3+}$  (5  $\mu\text{M}$ ,  $n=4$ ), and Fig. 4Ab shows those of the blockers of low-voltage-activated  $\text{Ca}^{2+}$  current and store-operated  $\text{Ca}^{2+}$  current,  $\text{Ni}^{2+}$  (1 mM,  $n=4$ ). The frequencies of pacemaker activities before and after these treatments were  $3.7 \pm 0.8$  and  $2.6 \pm 0.5$  Hz for  $\text{La}^{3+}$  ( $P < 0.05$ , Student's two-tailed paired  $t$  test) and  $4.1 \pm 0.7$  and  $1.7 \pm 0.2$  Hz for  $\text{Ni}^{2+}$  ( $P < 0.05$ , Student's two-tailed paired  $t$  test). Furthermore, when the bath application of the combination of  $\text{La}^{3+}$  (5  $\mu\text{M}$ ) and  $\text{Ni}^{2+}$  (1 mM) was examined, it completely blocked the generation of pacemaker activity (in 7/8 cells, Fig. 6Ba, c and d). The results are presented in Fig. 4B as the relative pacemaker frequencies after each treatment normalized to those before the treatment, which were defined as the ratio (firing frequency in Ringer solution containing  $\text{Ca}^{2+}$  channel blockers) / (firing frequency in Ringer solution). These results indicate that some kinds of  $\text{Ca}^{2+}$  currents are present in TN-GnRH neurons and contribute to the generation of pacemaker activity.

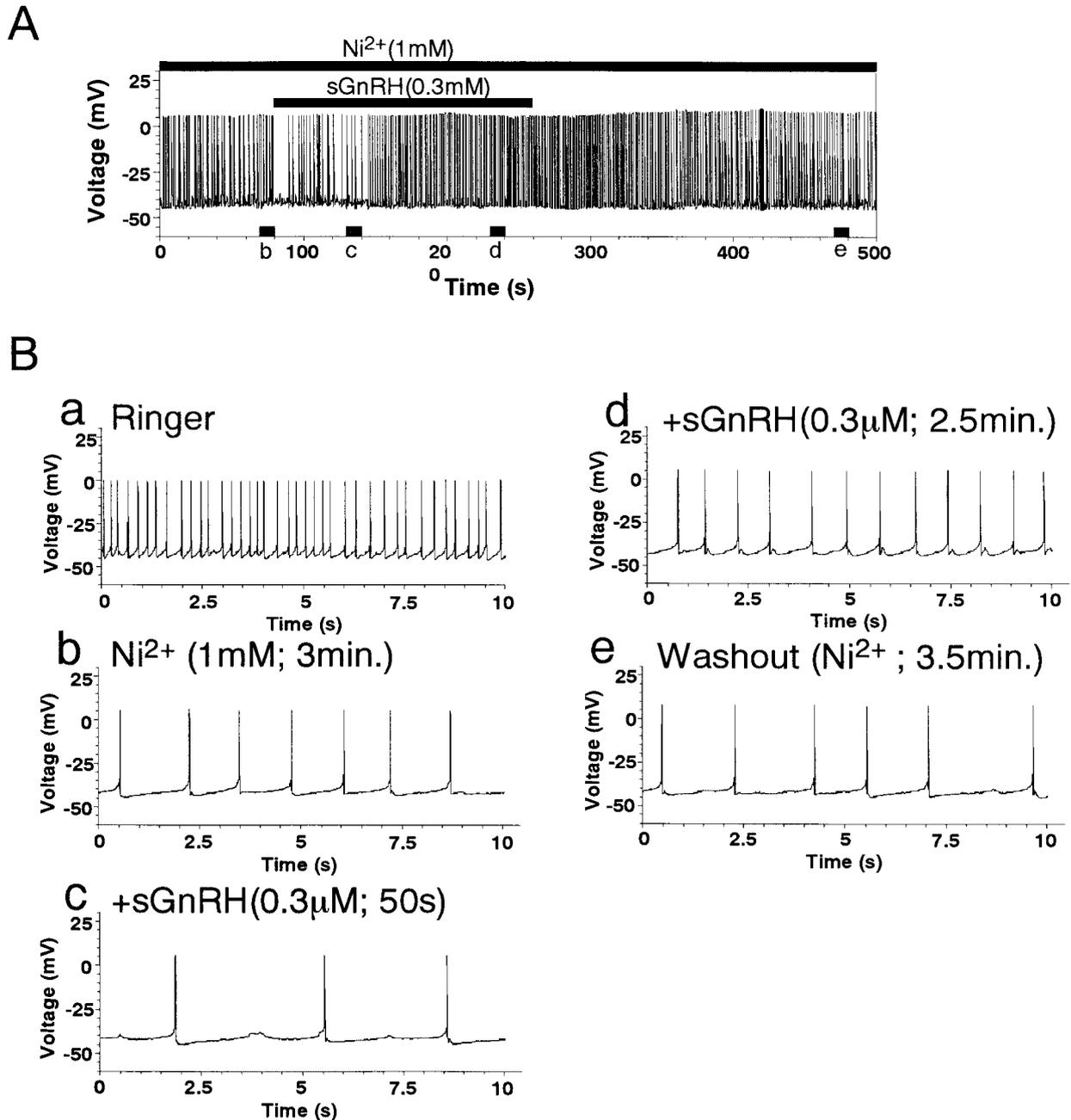
Next, we examined the effects of  $\text{Ca}^{2+}$  channel blockers on the modulation of pacemaker activity by sGnRH. Fig. 5 shows the effects of  $\text{Ni}^{2+}$  on the modulation of pacemaker activity by sGnRH. In Ringer solution, TN-GnRH neuron showed regular beating discharge ( $3.5 \pm 0.5$  Hz; Fig. 5Ba). Bath application of  $\text{Ni}^{2+}$  (1 mM) decreased the firing frequency of pacemaker activity ( $1.8 \pm 0.2$  Hz; Fig. 5Bb). Subsequent bath application of sGnRH (300 nM) transiently decreased ( $1.5 \pm 0.2$  Hz; Fig. 5Bc) and then increased ( $2.4 \pm 0.8$  Hz; Fig. 5Bd) the firing frequency of pacemaker

activity in a qualitatively similar manner with sGnRH application in Ringer. However, the late-phase increase of pacemaker frequency was less pronounced compared with sGnRH application in Ringer (Fig. 7; compare the left and right columns). Similarly, the late-phase increase of pacemaker frequency was less pronounced under  $\text{La}^{3+}$  treatment compared with sGnRH application in Ringer (Fig. 7; compare the left and middle columns). During the simultaneous bath application of  $\text{La}^{3+}$  (5  $\mu\text{M}$ ) and  $\text{Ni}^{2+}$  (1 mM) (Fig. 6), the pacemaker activity was stopped (Fig. 6Bb, Bc), and further bath application of sGnRH (300 nM, in 3/4 cells, Fig. 6Bd) or depolarizing DC current injection (in 2/2 cells, data not shown) did not reinstate the pacemaker activities.

Taken together, these data indicate that some kind(s) of  $\text{Ca}^{2+}$  channels present in the TN-GnRH neurons may be the target for the modulation by sGnRH, which somehow contributes to the late phase increase of pacemaker frequency.

#### Voltage-clamp recordings of voltage-dependent $\text{Ca}^{2+}$ currents

To reveal the quantitative contribution of  $\text{Ca}^{2+}$  currents to the generation and modulation of pacemaker activity, it is important to determine the  $\text{Ca}^{2+}$  channel subtypes and kinetics. Depolarizing steps from a holding potential of  $-100$  mV elicited a mixture of transient and sustained inward current components (Fig. 8Aa). They showed the characteristics of combined low voltage-activated (LVA) and high voltage-activated (HVA)  $\text{Ca}^{2+}$  currents. The peak inward currents (measured at peak current amplitudes during depo-

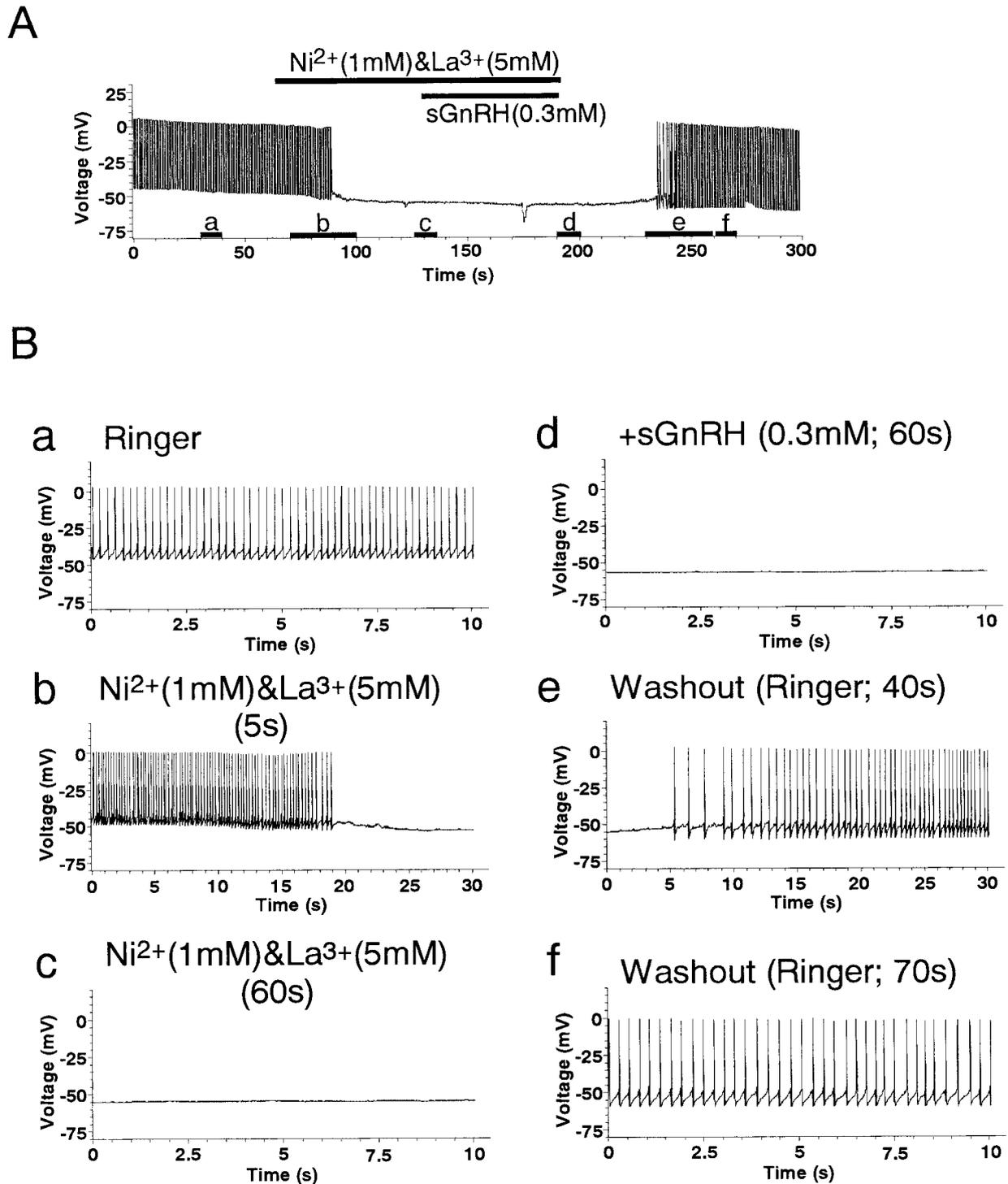


**Fig. 5.** The late-phase increase of pacemaker frequency by sGnRH is less pronounced under  $\text{Ni}^{2+}$  treatment. Bath application of  $\text{Ni}^{2+}$  (1 mM) decreased the frequency of pacemaker activity (Bb) but did not qualitatively affect either transient decrease (Bc) or subsequent increase of firing frequency (Bd) by sGnRH. However, the late-phase increase was less pronounced compared with sGnRH application in Ringer (see Fig. 7). Following washout by  $\text{Ni}^{2+}$ -containing Ringer solution, the firing frequency decreased again (Be).

larizing pulses) began to appear more positive than  $-60$  mV and reached a maximum near  $-20$  mV. The sustained currents (measured at 200 ms after the onset of the test pulse) began to appear more positive than  $-20$  mV and reached a maximum near 0 mV. The currents that were elicited by depolarizing steps from a holding potential of  $-60$  mV are shown in Fig. 8Ab. The transient inward current almost disappeared, and sustained current(s) mainly remained. At this holding potential, the activation of both peak and sustained currents required depolarization more positive than  $-20$  mV,

and the maximum inward current was obtained near  $-10$  mV. In the currents recorded from a different cell, however, the sustained current began to appear more positive than  $-40$  mV and rapidly reached its maximum at  $-30$  mV, when a holding potential was  $-60$  mV (see below).

Fig. 8B shows the averaged current-voltage relationship of transient and sustained  $\text{Ca}^{2+}$  currents ( $n=40$ ). We defined the current component that was obtained by subtraction of sustained current from peak current, as the 'transient'  $\text{Ca}^{2+}$  current. On the average, the transient and sustained cur-

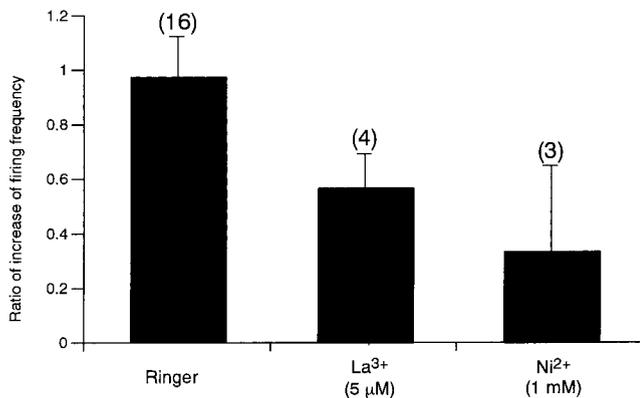


**Fig. 6.** Simultaneous application of  $\text{La}^{3+}$  and  $\text{Ni}^{2+}$  stops the pacemaker activity. Bath application of  $\text{La}^{3+}$  (5 mM) and  $\text{Ni}^{2+}$  (1 mM) blocked the generation of pacemaker activities (Bb, c). Furthermore, bath application of sGnRH did not induce pacemaker activities of TN-GnRH neurons (Bd). Pacemaker activities recovered after washout by Ringer solution (Bf).

rents measured at  $-10$  mV from a holding potential of  $-100$  mV was  $-1.41 \pm 0.26$  and  $-0.87 \pm 0.12$  nA, respectively (Fig. 8B and ). However, the averaged current-voltage relationship of sustained current elicited from a holding potential of  $-60$  mV showed an activation threshold of  $-40$  mV but also had a shoulder of current at  $-20$  mV (Fig. 8B, arrows).

This shoulder of current may reflect the difference of activation threshold and voltage dependence of sustained currents among different cells. The sustained current measured at  $-10$  mV from a holding potential of  $-60$  mV was  $-0.80 \pm 0.13$  nA ( $n=18$ ; Fig. 8B ).

These results suggest that a transient inward current



**Fig. 7.** The late-phase increase of pacemaker frequency by sGnRH is less pronounced in the presence of Ca<sup>2+</sup> channel blockers. Normalized increase of firing frequency by sGnRH in the presence or absence of Ca<sup>2+</sup> channel blockers was defined as

$$\frac{\text{Frequency}_{(\text{sGnRH})} - \text{Frequency}_{(\text{Ringer})}}{\text{Frequency}_{(\text{Ringer})}}$$

and was calculated from the data such as those shown in Fig. 5. Normalized increase of pacemaker frequencies was less pronounced under Ni<sup>2+</sup> or La<sup>3+</sup> treatment. The numbers in parentheses represent the numbers of cells tested for each Ca<sup>2+</sup> channel blocker.

component that begins to activate near  $-60$  mV and is largely inactivated when the holding potential is  $-60$  mV represents the LVA Ca<sup>2+</sup> current. On the other hand, HVA Ca<sup>2+</sup> current component that are not inactivated at holding potentials of  $-60$  mV consist of two types of sustained currents. The one current begins to activate from  $-20$  mV and reaches its maximum amplitude near  $-10$  mV. The other current begins to activate near  $-40$  mV and rapidly reaches maximum at  $-30$  mV.

### Actions of GnRH on Ca<sup>2+</sup> currents

Previous preliminary studies suggested that neither  $I_{\text{Na}(\text{slow})}$  nor  $I_{\text{K}(\text{v})}$  is significantly modulated by sGnRH applications (our unpublished observations). Therefore, we suspected that the Ca<sup>2+</sup> currents may be modulated by sGnRH. Fig. 9 shows an example of the effects of sGnRH on the Ca<sup>2+</sup> currents. The modulation was examined in 10 cells held at  $-100$  mV. In this case, when  $1 \mu\text{M}$  sGnRH was added to the bath solution, the current amplitude and activation threshold of transient current were not changed (Fig. 9Ba and ). However, the amplitude of sustained current increased ( $-0.3$  to  $-1.3$  nA) in a wide voltage range. Furthermore, the activation threshold and maximum amplitude of sustained current were shifted to more negative potentials (Fig. 9Bb and ). Responses of Ca<sup>2+</sup> currents to GnRH were variable among the recorded cells. In another case, the amplitudes of both transient and sustained Ca<sup>2+</sup> currents did not increase. However, the activation threshold and the peak amplitude of sustained Ca<sup>2+</sup> current were shifted to more negative potentials (data not shown). On the average, the amplitude of the transient Ca<sup>2+</sup> current measured at its maximum was decreased from  $-1.4 \text{ nA} \pm 0.3$  to  $-1.1 \pm 0.4$  nA ( $P > 0.05$ , Student's two-tailed paired t-test; Fig. 10A and

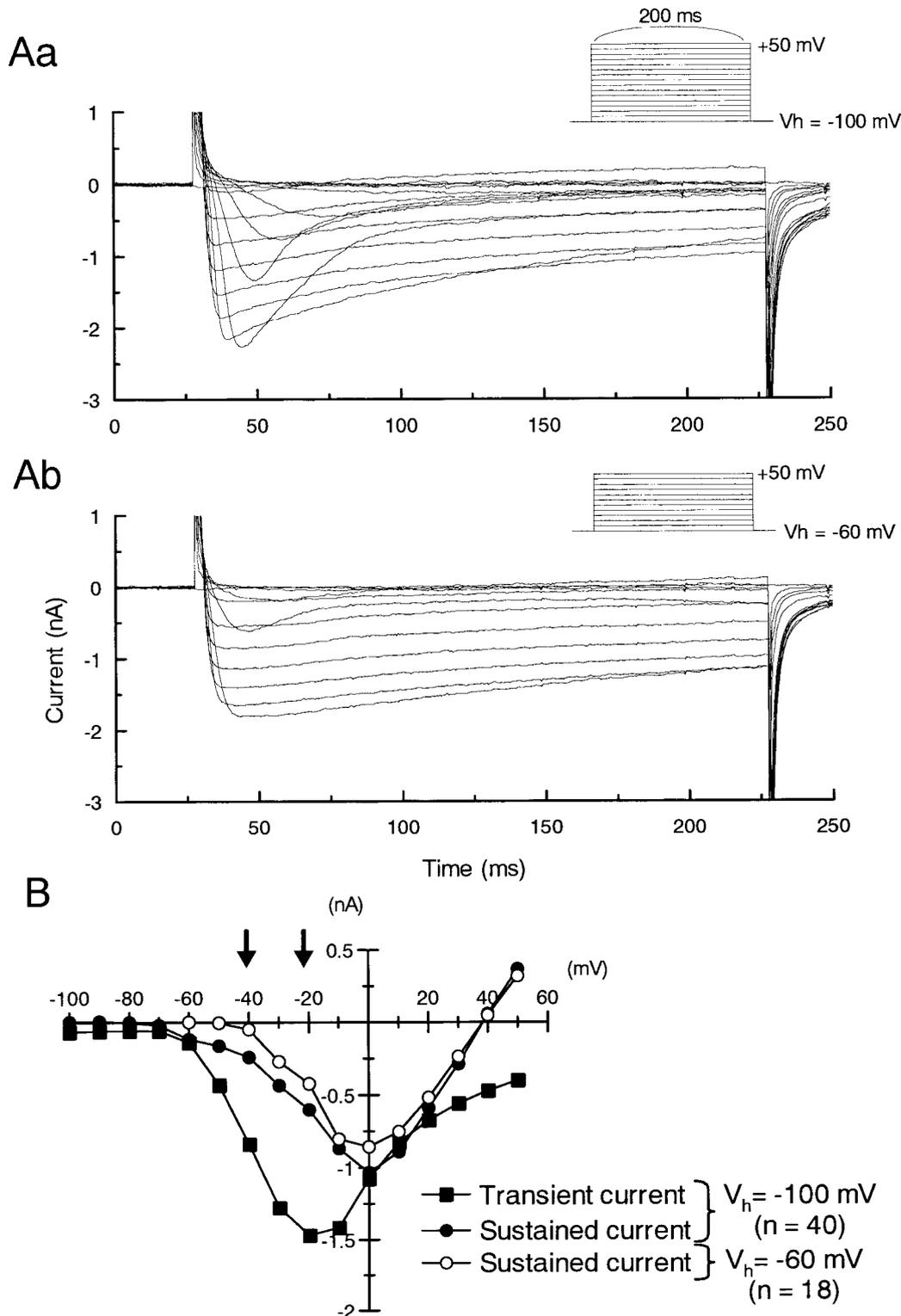
), and those of the sustained Ca<sup>2+</sup> current was increased from  $-1.6 \pm 0.4$  to  $-1.9 \pm 0.4$  ( $P < 0.05$ , Student's two-tailed paired *t* test; Fig. 10B and ), before and after bath application of  $1 \mu\text{M}$  sGnRH, respectively ( $n=10$ ). Moreover, the activation threshold and the voltage that evokes the maximum amplitude of the sustained Ca<sup>2+</sup> currents were shifted to more negative potentials (Fig. 10B and ), while those of transient Ca<sup>2+</sup> current were not changed. These results indicate that the sustained HVA Ca<sup>2+</sup> current is facilitated by sGnRH, i.e., the relative contribution of HVA Ca<sup>2+</sup> current to the pacemaker activity may be increased by sGnRH. It is then suggested that this increased availability of HVA Ca<sup>2+</sup>-current increases the frequency of pacemaker activity.

## DISCUSSION

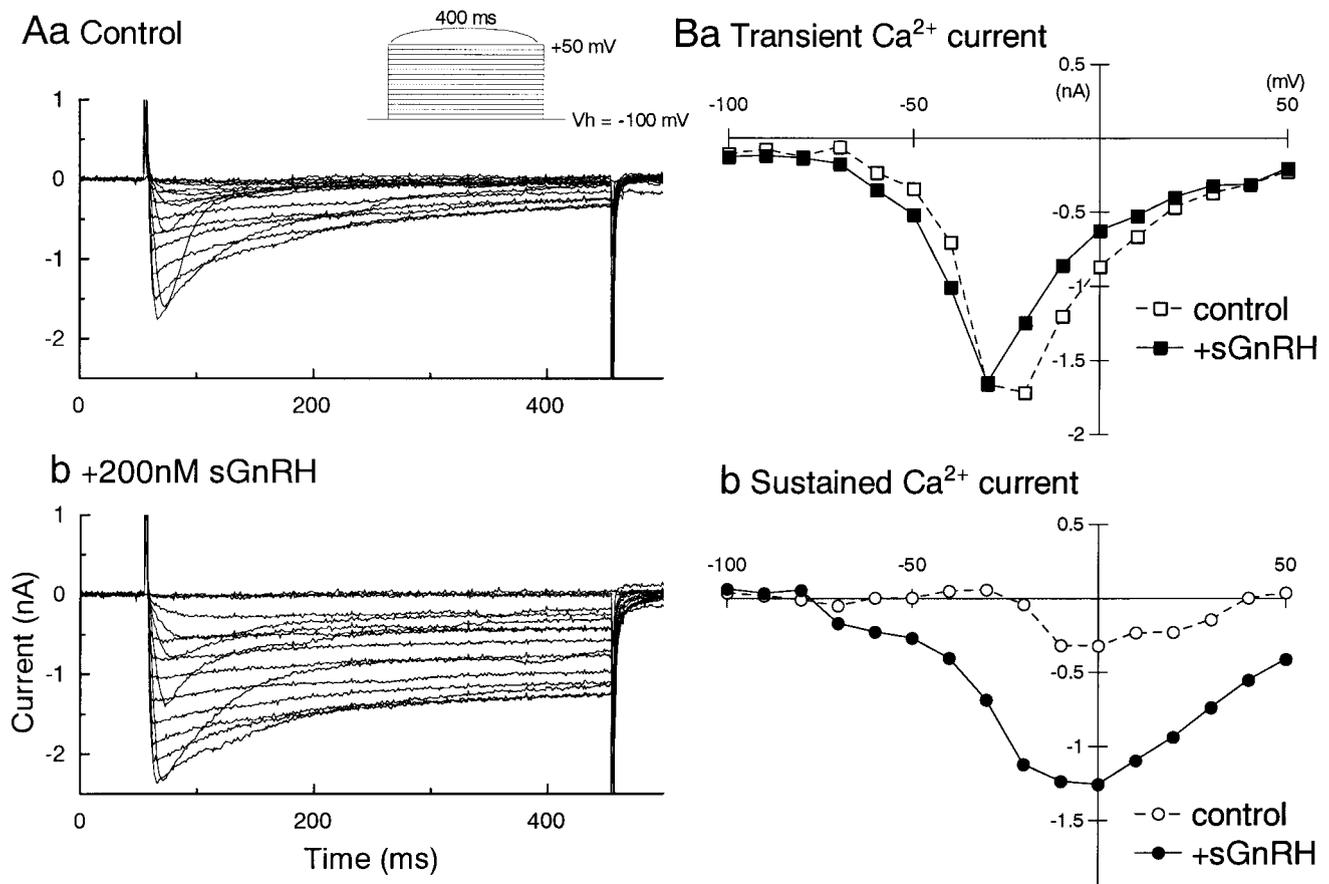
### Involvement of Ca<sup>2+</sup> release from intracellular store and apamin-sensitive K<sup>+</sup> current in the transient decrease of firing frequency of TN-GnRH neurons

Intracellular application of ruthenium red and heparin inhibited the transient decrease but not late-phase increase of the pacemaker frequency, both of which were induced by bath application of sGnRH. This result suggests that  $[\text{Ca}^{2+}]_i$  mobilization is involved in the transient decrease but not the late-phase increase of firing frequency of pacemaker activity. Then, the presence of tentative Ca<sup>2+</sup>-activated transient K<sup>+</sup> current was suggested from the result of present voltage-clamp experiments. This current was TEA-insensitive but 4AP-sensitive, and could be only evoked when the Ca<sup>2+</sup> is present in the extracellular bath solutions. However, the activation and steady-state inactivation curves of this current were shifted to more depolarized potentials compared with the 4AP-sensitive A-like currents of TN-GnRH neurons (Abe and Oka, 1999), and the two currents are considered to be different. The amplitude of the tentative Ca<sup>2+</sup>-activated transient K<sup>+</sup> current was rapidly increased by bath application of sGnRH. Furthermore, bath application of apamin inhibited the transient decrease but not the late-phase increase of the pacemaker frequency by sGnRH. These results suggest that the apamin-sensitive Ca<sup>2+</sup>-activated transient K<sup>+</sup> current was activated by Ca<sup>2+</sup> released from the intracellular store, which had been induced by sGnRH. This may explain the early-phase transient decrease of pacemaker activity by sGnRH.

It has been reported that the Ca<sup>2+</sup>-activated K<sup>+</sup> current exist in the embryonic GnRH neurons (Kusano *et al.*, 1995) and GT1-7 cells (Spergel *et al.*, 1996; Van Goor *et al.*, 1999). The transient decrease of firing frequency by GnRH, which was induced by the activation of SK type Ca<sup>2+</sup>-activated K<sup>+</sup> current, has been reported in the GT1-7 cells (Van Goor *et al.*, 1999). It has been generally accepted that the activation of G<sub>q/11</sub>-coupled GnRH receptor increases the intracellular Ca<sup>2+</sup> concentration via phosphoinositide signaling pathway (Naor, 1990; Naor *et al.*, 1998; Stojilkovic *et al.*, 1994b), and the increased  $[\text{Ca}^{2+}]_i$  may open Ca<sup>2+</sup>-activated K<sup>+</sup> current (Sah, 1996). All of these reports are in favor of



**Fig. 8.** Voltage clamp recording reveals the presence of one transient LVA and two types of sustained HVA  $\text{Ca}^{2+}$  current components. A: Currents evoked during 200 ms voltage steps from  $-100$  to  $+50$  mV (holding potential= $-100$  mV)(a) and from  $-60$  to  $+50$  mV (holding potential= $-60$  mV)(b). B: Current-voltage relations of the  $\text{Ca}^{2+}$  currents averaged from 40 ( $V_h=-100$  mV) and 18 ( $V_h=-60$  mV) TN-GnRH neurons. I/V curves were constructed by plotting the transient current amplitudes (filled squares) obtained by subtracting sustained current from peak current, and sustained current amplitudes (filled circles) measured at the end of 200 ms test pulses. The transient current probably correspond to the low-voltage activated (LVA) T type  $\text{Ca}^{2+}$  current. The I/V curve of the sustained currents evoked from a holding potential of  $-60$  mV (open circles) had a shoulder at  $-20$  mV in addition to the activation threshold of  $-40$  mV (arrows). These may correspond to the activation thresholds of two HVA  $\text{Ca}^{2+}$  currents.



**Fig. 9.** sGnRH modulates the Ca<sup>2+</sup> current components. A: Current traces from a holding potential of -100 mV before (a) and after the addition of 200 nM sGnRH to the bath solutions (b). B: The current-voltage relations of the transient current (a), and the sustained current measured at the end of 400 ms test pulse (b), which were constructed from the traces in A. Symbols are defined in the inset. In this cell, the current amplitude of sustained current was augmented by sGnRH, and the activation threshold of sustained current was shifted to more hyperpolarized potentials.

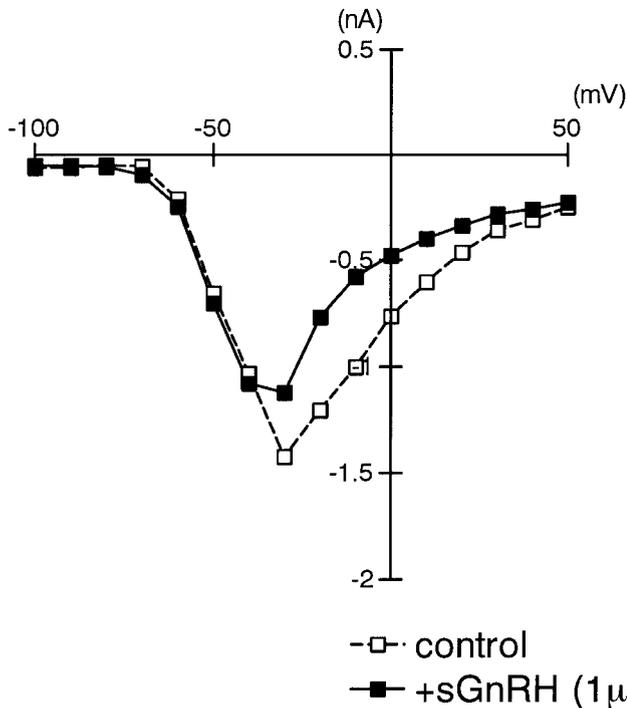
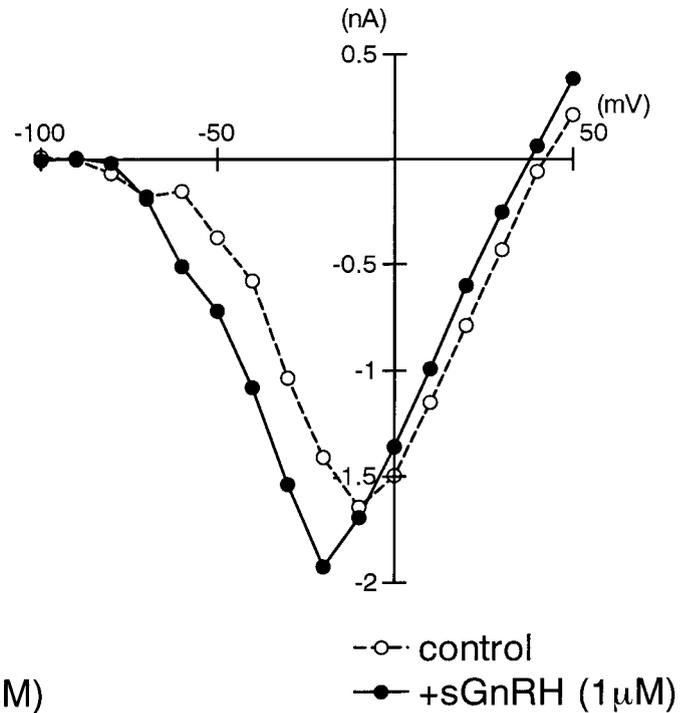
the above-mentioned mechanisms for the transient decrease of pacemaker activity of TN-GnRH neurons by sGnRH.

#### Ca<sup>2+</sup> current component is involved in the pacemaker activity

We have shown that some kind(s) of Ca<sup>2+</sup> current contribute(s) to the modulation of the pacemaker activity of TN-GnRH neurons. Furthermore, the result that simultaneous bath application of 5  $\mu$ M La<sup>3+</sup> and 1 mM Ni<sup>2+</sup> completely inhibited the generation of pacemaker activity and its modulation by sGnRH, suggests that Ca<sup>2+</sup> current components that were blocked by simultaneous application of La<sup>3+</sup> and Ni<sup>2+</sup> may be somehow involved in the generation of pacemaker potentials and may be the target of modulation by sGnRH.

The present results may appear to be partly inconsistent with those of the previous study that the Ca<sup>2+</sup> currents are not essential for the generation of pacemaker potentials (Oka, 1995). One possible explanation may be the difference in the recording methods, intracellular recording (Oka, 1995) vs. whole-cell patch-clamp recording (present study).

It may be possible that the intracellular dialysis of EGTA, which was introduced by the patch pipette solution, may prevent Ca<sup>2+</sup> channels from Ca<sup>2+</sup>-dependent inactivation (Hille, 2001). Thus, a relatively large proportion of Ca<sup>2+</sup> channel may be available for the generation of the pacemaker activity in the whole-cell patch-clamp recording. Second possibility is the presence of diffusion barrier. While the ventral meningeal membrane of the brain was not removed in the previous study, it is always completely removed in the patch-clamp experiments. The meningeal membrane may have served as a diffusion barrier for the drug delivery. Thirdly, the bath application of Ca<sup>2+</sup> channel blockers may have simultaneously blocked other currents (for example, I<sub>Na(slow)</sub>). Preliminary experiments suggest that Ni<sup>2+</sup> may inhibit I<sub>Na(slow)</sub> (data not shown). It has also been reported that Ni<sup>2+</sup> and La<sup>3+</sup> also blocks Na<sup>+</sup>/Ca<sup>2+</sup> exchanger, store-operated Ca<sup>2+</sup> current, and other voltage-gated ion channels (Nowycky, 1991; Taylor and Brond 1998). In the present study, we used supramaximum concentrations of these Ca<sup>2+</sup> channel blockers to block the Ca<sup>2+</sup> influx completely. Especially, the concentrations of Ni<sup>2+</sup> (1 mM) and La<sup>3+</sup> (5  $\mu$ M) used here are sufficient to block store-operated Ca<sup>2+</sup> cur-

A Transient  $\text{Ca}^{2+}$  currentB Sustained  $\text{Ca}^{2+}$  current

**Fig. 10.** Averaged I/V relations also show the modulation of  $\text{Ca}^{2+}$  current by sGnRH. I/V curves were constructed by plotting the averaged current amplitudes ( $n=10$ ) evoked from holding potentials of  $-100$  mV before and after the addition of  $200$  nM sGnRH to the bath solution. The current-voltage relations of transient current (A) and sustained current (B) are shown.

rents (Skryma *et al.*, 2000). Thus, it may be possible that these  $\text{Ca}^{2+}$  channel blockers blocked not only voltage-gated  $\text{Ca}^{2+}$  channels, but also store-operated  $\text{Ca}^{2+}$  channels, other channels, and/or exchangers. In fact, it has been reported in GT 1-7 cell-lines that store-operated  $\text{Ca}^{2+}$  current contributes to the modulation of firing activity by GnRH (Van Goor *et al.*, 1999a). Further studies using more specific  $\text{Ca}^{2+}$  channel blockers are necessary to clarify the quantitative contributions of voltage-dependent  $\text{Ca}^{2+}$  current and store-operated  $\text{Ca}^{2+}$  current.

### Both LVA and HVA $\text{Ca}^{2+}$ currents are present in the TN-GnRH neurons

Voltage-clamp experiments of whole-cell patch-clamp recording suggested that TN-GnRH neuron has at least one LVA  $\text{Ca}^{2+}$  current component and two HVA  $\text{Ca}^{2+}$  current components, and the relative prevalence of each current component seems to be different among different TN-GnRH neurons. LVA  $\text{Ca}^{2+}$  current was a transient current evoked from membrane potentials more positive than  $-60$  mV and reached its maximum at about  $-20$  mV (Figs. 8; Transient current). This current was almost inactivated at a holding potential of  $-60$  mV. It most probably corresponds to the T-type  $\text{Ca}^{2+}$  current (Nowicky *et al.*, 1985). In contrast, both of the two HVA  $\text{Ca}^{2+}$  currents inactivated slowly and could be observed at holding potentials of  $-100$  mV and  $-60$  mV. One of the HVA  $\text{Ca}^{2+}$  current activated at potentials more

positive than  $-40$  mV. The other HVA  $\text{Ca}^{2+}$  current activated at potentials more positive than  $-20$  mV. Thus, the averaged I/V curve of the sustained current had a shoulder and reached its maximum around  $0$  mV (Fig. 8B). Preliminary experiments showed that the amplitude of HVA  $\text{Ca}^{2+}$  current was reduced (= was not completely blocked) by bath application of nifedipine (data not shown). Therefore, at least one of the HVA  $\text{Ca}^{2+}$  current may correspond to the L-type  $\text{Ca}^{2+}$  current.

It has been demonstrated that embryonic GnRH neurons (Kusano *et al.*, 1995) and GT1 cells (Bosma, 1993; Hales *et al.*, 1994; Javors *et al.*, 1995; Costantin and Charles, 1999) express several types of plasma membrane  $\text{Ca}^{2+}$  channels, including transient and sustained voltage-dependent  $\text{Ca}^{2+}$  channels. Kusano *et al.* (1995) reported that embryonic GnRH neurons possess T- and L-type  $\text{Ca}^{2+}$  channels, and GT1 cells possess T-, N-, and L-type  $\text{Ca}^{2+}$  channels. On the other hand, Costantin and Charles (1999) and Van Goor *et al.* (1999b) reported that GT1 cells possess T- and L-type  $\text{Ca}^{2+}$  channels. The present study used for the first time the adult authentic GnRH neurons, and the results basically agreed well with these reports. However, pharmacological isolation and detailed analysis of kinetic properties of each  $\text{Ca}^{2+}$  current component have not yet been done in the present study mainly due to the poor space-clamp of the *in vitro* whole-brain preparations of TN-GnRH neurons. Therefore, the use of dissociated TN-GnRH neurons may be

necessary to evaluate quantitatively the contribution of each  $\text{Ca}^{2+}$  current component to the control of pacemaker and secretory activities of TN-GnRH neurons.

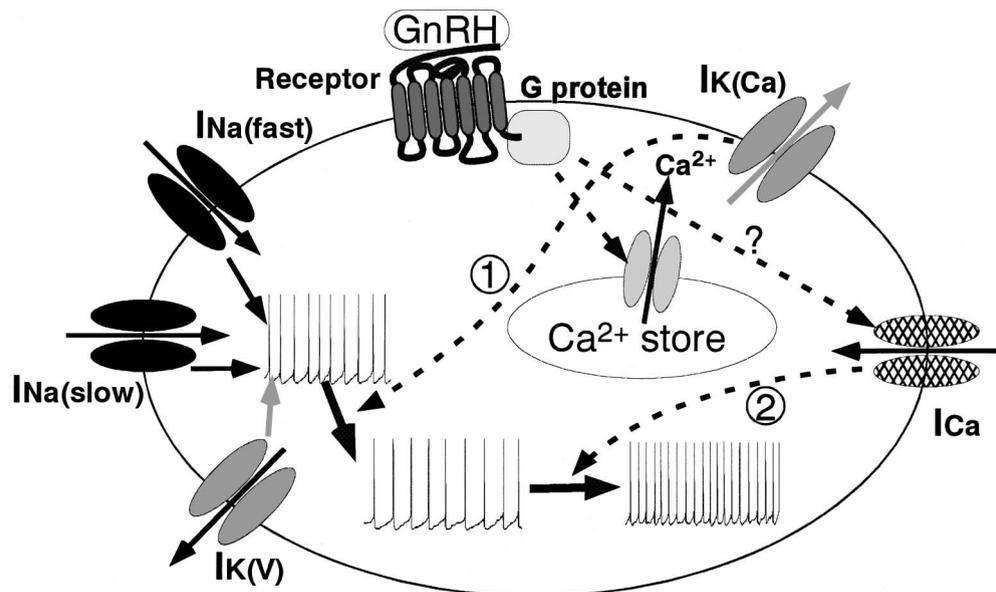
### Modulation of HVA type $\text{Ca}^{2+}$ channels by GnRH

Bath application of sGnRH shifted the activation threshold of sustained  $\text{Ca}^{2+}$  current component to more hyperpolarized potentials. Moreover, the increase of sustained current amplitude was observed in some recordings, although complete recovery of  $\text{Ca}^{2+}$  current amplitude by washout could not be obtained due to the rundown of  $\text{Ca}^{2+}$  currents. It is generally accepted that GnRH receptors are coupled to  $\text{G}_{q/11}$  type G-proteins, and  $\text{G}_{q/11}$  type G-proteins are coupled to phosphoinositide-mediated signaling pathways (Stojilkovic *et al.*, 1994a,b). It has also been reported that  $\text{Ca}^{2+}$  channels (especially, N- and L-type  $\text{Ca}^{2+}$  channel) are positively modulated by PKC (Bourinet *et al.*, 1992; Dolphin, 1998; Meir *et al.*, 1999; Stea *et al.*, 1995; Swartz, 1993; Yang and Tsien, 1993; Zamponi *et al.*, 1997; Zhu and Ikeda, 1994). Bosma and Hille (1992) reported that immortalized gonadotrope (aT3-1 cell), which also has GnRH receptors, express  $\text{Ca}^{2+}$  channels, and these  $\text{Ca}^{2+}$  channel were augmented by application of GnRH or phorbol ester.

From these observations and present results, it is highly possible that certain type(s) of  $\text{Ca}^{2+}$  currents that contribute to the generation of pacemaker activity are modulated by sGnRH and are involved in the late-phase increase of the pacemaker frequency.

### Mechanisms of the generation and modulation of pacemaker activity and possible physiological functions

From the results of the present study, we present a model about the generation and modulation mechanisms of pacemaker activity in the TN-GnRH neuron (Fig. 11). In the intact brain, TN-GnRH neurons show regular beating pacemaker activity. The  $I_{\text{Na}(\text{slow})}$ ,  $I_{\text{Na}(\text{fast})}$ , and  $I_{\text{K}(\text{V})}$  interact in the following manner to generate the pacemaker potentials. The  $I_{\text{Na}(\text{slow})}$ , which is persistently active in the subthreshold membrane potential range, always supplies the persistent depolarizing drive and gradually depolarizes the membrane potentials. When the membrane potential reaches the activation threshold for  $I_{\text{K}(\text{V})}$ , outward current develops, and the net flux of current reverses to outward. Then, the membrane potential becomes hyperpolarized and deactivates the  $\text{K}^+$  current, and the next cycle begins. This is the subthreshold pacemaker activity, and when the membrane potential reaches the activation threshold for the  $I_{\text{Na}(\text{fast})}$ , the spiking pacemaker activities ensues. In addition, some kind(s) of  $\text{Ca}^{2+}$  currents and apamin-sensitive  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  current may be also involved in the generation of pacemaker potentials. Although  $I_{\text{Na}(\text{slow})}$  and  $I_{\text{K}(\text{V})}$  are essential for the generation of the basic rhythm of pacemaker activities, they do not seem to be modulated by the process described below. When the GnRH peptide binds to the GnRH receptor located on the cell surface of TN-GnRH neurons,  $\text{G}_{q/11}$  protein-coupled process starts and activates phospholipase C, which leads to the production of  $\text{IP}_3$  and diacylglycerol.  $\text{IP}_3$  stimulates the release of  $\text{Ca}^{2+}$  from the  $\text{IP}_3$ -sensitive intracellular  $\text{Ca}^{2+}$  store, and the increased  $[\text{Ca}^{2+}]_i$  then opens



**Fig. 11.** Model of the biphasic modulation of pacemaker activity of TN-GnRH neuron. The GnRH peptide, which binds to its receptor located at the cell membrane of TN-GnRH neurons, induces biphasic modulation of the pacemaker activities; (1) Facilitates  $\text{Ca}^{2+}$  release from the intracellular  $\text{Ca}^{2+}$  store. The increased  $[\text{Ca}^{2+}]_i$  activates apamin-sensitive  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  current,  $I_{\text{K}(\text{Ca})}$ . This, in turn, decreases the frequency of pacemaker activities transiently. (2) Up-regulates the  $\text{Ca}^{2+}$  current(s),  $I_{\text{Ca}}$ , and increases the frequency of pacemaker activities in the late phase.  $I_{\text{Na}(\text{slow})}$ ,  $I_{\text{K}(\text{V})}$ , and  $I_{\text{Na}(\text{fast})}$  are involved in the generation of basic pattern of pacemaker activities but are not directly modulated by GnRH.

apamin-sensitive  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel. Thus, the pacemaker frequency of TN-GnRH neuron is transiently decreased. On the other hand, diacylglycerol activates protein kinase C, and protein kinase C then phosphorylates and modulates the  $\text{Ca}^{2+}$  current. Thus, the pacemaker frequency of TN-GnRH neurons is increased in the late phase.

What is the physiological significance of the pacemaker activity of TN-GnRH neurons and its modulation? Unfortunately, the functional link between the electrical activities of peptidergic neurons and the peptide release has not been firmly established thus far. A recent amperometric and RIA studies showed that the membrane depolarization triggers the secretion of GnRH from the TN-GnRH neuron in the teleost brain-pituitary slice preparation (Ishizaki and Oka, 2001 and in preparation). In the intact brain, the pacemaker activity of TN-GnRH neurons is characterized by low frequency (<10 Hz) regular beating discharge. The depolarization that is produced by single action potential of pacemaker activity may not be strong enough to induce GnRH release from the dense cored vesicles. However, it has been reported that such low frequency firing activity enables vasopressin release from rat neurohypophysis (Bondy *et al.*, 1987), substance P or thyrotropin releasing hormone release from rat ventral spinal cord (Iverfeldt *et al.*, 1989), and GnRH release from preganglionic C-neurons of the bullfrog (Peng and Horn, 1991). Furthermore, increase of firing frequency potentiated peptide release from these cells. Thus, it may be possible to think that the frequency of beating pacemaker activity of TN-GnRH neurons may affect the release of GnRH.

What then is the physiological significance of the modulation by sGnRH of pacemaker activity of TN-GnRH neurons? TN-GnRH neurons of the dwarf gourami make tight cell clusters with no intervening glial cells (Oka and Ichikawa, 1991; Oka and Matsushima, 1993; Oka, 1997), and the possibility of active exocytotic release from the cell body and its vicinity has also been suggested (Oka and Ichikawa, 1991). Other studies have shown that GnRH receptors are widely distributed throughout the brain (Jennes *et al.*, 1997; Stojilkovic *et al.*, 1994b). In addition, considerable overlap of the brain areas that contain GnRH-producing cells and GnRH receptor mRNA-expressing cells has been reported (Jennes *et al.*, 1996). Also, cultured hypothalamic GnRH neurons have GnRH receptors (Krsmanovic *et al.*, 1999). Furthermore, it has been reported using an *in vivo* analysis of multiunit activities in ovariectomized rats that GnRH injected in and around the median eminence is able to cause a population of GnRH neurons to fire synchronously (Hiruma and Kimura, 1995). From these observations and the present results, it is suggested that GnRH released from GnRH neurons facilitates the activities of their own (autocrine) and/or neighboring GnRH neurons (paracrine) and may cause synchronized positive feedback facilitation of multiple GnRH neurons. It is well known that in oxytocin neurons the release of oxytocin from single neuron into the brain environment stimulates its own activity and

thus further release (Freund-Mercier and Richard, 1984; Moos *et al.*, 1984). A similar effect has been reported for insulin-stimulated insulin release in pancreatic  $\beta$  cells (Aspinwall *et al.*, 1999). Therefore, this mechanism is probably common to all neurosecretory neurons or secretory cells, whose synchronized facilitation of firing leads to facilitated release.

### Comparison with the autoregulation in the other putative GnRH neurons

Possible autoregulation mechanisms of TN-GnRH cells suggested in the present study are discussed here in relation to references that described studies on putative GnRH neurons. The concept of an ultrashort feedback mechanism in the control of neurosecretion was first suggested by Hyyppa *et al.* (1971) in studies on the control of FSH secretion. Similarly, it was revealed from *in vivo* (Valenca *et al.*, 1987) and *in vitro* studies (DePaolo *et al.*, 1987) that GnRH release from hypothalamic GnRH system was negatively autoregulated. It was postulated that negative feedback could be mediated via axo-dendritic/axo-somatic synapses on adjacent GnRH or other types of neurons (DePaolo *et al.*, 1987), and histological evidence for such connections has been reported (Leranth *et al.*, 1985; Witkin and Silverman, 1985).

However, the results using recording of multiunit activity (MUA) in the hypothalamus, which are considered to reflect the secretory activity of GnRH, have been very complicated. Hiruma and Kimura (1995) reported that intravenous injection of GnRH or microinjection of GnRH into the median eminence immediately evoked a MUA volley of the rat. However microinjection of GnRH into the medial preoptic area did not cause these effects. Moreover, intravenous or intracerebroventricular injections of GnRH did not affect MUA volleys of the rhesus monkeys (Kesner *et al.*, 1986; Ordog *et al.*, 1997). Unfortunately, the neuronal elements responsible for the MUA volley, i.e., whether the MUA volley represent the activity of GnRH neurons themselves or other neuronal elements, have not been determined. Therefore, it is difficult to directly compare the results of the present paper with the results of MUA studies.

Exposure of perfused GT1-7 cells to a GnRH agonist (analog) caused a transient elevation of GnRH release and subsequent suppression of the basal pulsatile secretion. During further continuous exposure to the agonist, the cells recovered from inhibition and exhibited infrequent but increasingly prominent peaks, with a net increase in GnRH release (Krsmanovic *et al.*, 1993). Recently, similar autoregulation of GnRH secretion has been reported in hypothalamic culture (Krsmanovic *et al.*, 1999). However, the time courses of such autoregulation are much slower than those of TN-GnRH neurons. The autoregulation of GnRH release in GT1-7 cell and hypothalamic culture takes dozens of minutes to several hours to occur. However, the biphasic autoregulation of pacemaker activity of TN-GnRH neurons took only a few minutes. The former has been suggested to

result from the internalization of receptor molecules and downregulation of the expression of GnRH receptor gene that was induced by GnRH (Park, 1998; Stojilkovic *et al.*, 1994a). On the other hand, the biphasic autoregulation of pacemaker activity of TN-GnRH neurons is considered to be due to the modulations of ionic channels induced by the activation of cell signaling pathways downstream of GnRH receptor activation. Thus, the two phenomena are considered to be based on quite different mechanisms.

Recently, Van Goor *et al.* (1999a, b) reported that the membrane excitability of GT1 cells was modulated by GnRH peptide. In this modulation of membrane excitability, they suggested that the activation of GnRH receptor induces  $[Ca^{2+}]_i$  mobilization, and this  $[Ca^{2+}]_i$  mobilization activates SK-type  $Ca^{2+}$ -activated  $K^+$  channel and activates store-operated  $Ca^{2+}$ -channel, which transiently hyperpolarizes and then persistently depolarizes membrane potentials, respectively. They further suggested that this sustained membrane depolarization is explained by complex interplay of  $Na^+$  channel,  $K^+$  channel, store-operated  $Ca^{2+}$  channel, and L-type  $Ca^{2+}$  channel (Van Goor *et al.*, 2000). Furthermore, LeBeau *et al.* (2000) showed the contribution of cAMP-operated inward current to this modulation in GT1 cells. The mechanism of the transient decrease of firing frequency in TN-GnRH neurons (the present study) basically agrees well with these reports. However, detailed mechanism of the sustained increase of firing frequency seems to differ from each other. To further understand this mechanism, more detailed kinetic and pharmacological investigation of the control of pacemaker activities of TN-GnRH neurons are under way.

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