Modulation of Pacemaker Activity by Salmon Gonadotropin-Releasing Hormone (sGnRH) in Terminal Nerve (TN)-GnRH Neurons

HIDEKI ABE AND YOSHITAKA OKA
Misaki Marine Biological Station, Graduate School of Science, University of Tokyo, Kanagawa 238-0225, Japan

Abe, Hideki and Yoshitaka Oka. Modulation of pacemaker activity by salmon gonadotropin-releasing hormone (sGnRH) in terminal nerve (TN)-GnRH neurons. J. Neurophysiol. 83: 3196–3200, 2000. The terminal nerve (TN)-gonadotropin-releasing hormone (GnRH) neurons project widely in the brain instead of the pituitary and show endogenous pacemaker activity that is dependent on the physiological conditions of the animal. We suggest that the TN-GnRH system may act as a putative neuromodulator that is involved in the regulation of many long-lasting changes in the animal’s behavior (e.g., motivational or arousal states). As to the mechanisms of the generation of the pacemaker activity, we have already shown that whole-cell patch clamp recordings that the tetrodotoxin-resistant persistent sodium current, $I_{Na,t}$, and the tetraethylammonium-sensitive potassium current, $I_{K,TA}$, contribute to the depolarizing and repolarizing phase of the pacemaker potentials, respectively (Abe and Oka 1999; Oka 1995, 1996). Here, we show that the pacemaker activity of TN-GnRH neurons is modulated by salmon GnRH (sGnRH), which is the same molecular species of GnRH peptide produced by TN-GnRH neurons themselves. Thus the pacemaker activity may be modulated by GnRH peptide that is produced by TN-GnRH neurons, and the TN-GnRH system may be a good model system to study the modulatory effect of GnRH peptides on the target neurons as well.

METHODS
Adult male and female dwarf gourami (Colisa lalia, a freshwater tropical fish), ~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 previously published protocols (Abe and Oka 1999; Oka 1995). This whole brain preparation was continuously superfused with an oxygenated Ringer solution containing (in mM): 124 NaCl, 5 KCl, 1.2 KH2PO4, 2.4 CaCl2, 1.3 MgSO4, 26 NaHCO3, and 10 glucose (pH 7.4 adjusted with NaOH). Patch pipette contained (in mM): 110 K-gluconate, 3 MgCl2, 40 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 0.3 ethylene glycol-bis (β-aminoethyether)-N,N′,N′,N′-tetraacetic acid (EGTA), 2 Na3ATP, and 0.2 Na2GTP (pH 7.4 adjusted with NaOH). 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 gigahm 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). The voltage was not corrected for liquid junction potentials, which were of the order of 5 mV. Pacemaker activities were digitized (2 kHz), displayed on-line with Axotape software (Axon Instruments), and stored on a computer. Thereafter, the data were analyzed off-line by using Axograph software (Axon Instruments). Drugs that were used are as follows: salmon GnRH (0.2–200 nM, RBI), mammalian GnRH (mGnRH, 20 or 50 nM, Peptide Institute, Inc.), GnRH antagonist ([Ac-pCL-DPhe1,2,DTrp3, DArg6, DAla10] luteinizing hormone-releasing hormone (LH-RH) (American Peptide Company, Inc.), GDP-β-S (1 mM, Sigma), guanosine 5′-0-(3-thiotri-phosphate) (GTP-γ-S) (0.1 mM, Sigma). All data in this report are presented as means ± SE.
RESULTS

Most of the TN-GnRH neurons showed regular spontaneous beating discharge pattern (Oka and Matsushima 1993). The pacemaker activities of TN-GnRH neurons were modulated by bath application of sGnRH. Figure 1 shows an example of the changes in pacemaker activity by sGnRH (20 nM). In Ringer, TN-GnRH neuron showed slow regular beating discharge (3.06 ± 0.20 Hz, n = 53, range 0.8–9.0 Hz; Fig. 1Ba). During the bath application of sGnRH, the firing frequency of pacemaker activity was transiently decreased (early phase; Fig. 1Bb shows 70 s after the onset of bath application), and subsequently increased (late phase; Fig. 1Bc shows 140 s after the onset of bath application). Figure 1 shows a typical example of this “biphasic” modulation, and it is clearly seen in a trace on a slower time scale (Fig. 1A). It should be noted that this modulation occurred without detectable membrane potential changes in most cases, which precludes the possibility that the modulation of pacemaker activity, decrease and then increase of frequency, may be simply caused by hyperpolarization and depolarization of the membrane, respectively. The time course of this biphasic modulation of the frequency of pacemaker activity is plotted in Fig. 1C. The latency of the transient decrease in firing frequency was about 20–90 s, but this varied from neuron to neuron even at the same concentration of sGnRH. This relatively long latency may be partly due to the delivery time during which the adequate quantity of perfusion solution reaches TN-GnRH neurons and partly due to the biochemical nature of GnRH responses (see DISCUSSION). The duration of the transient decrease in firing frequency was 34.7 ± 3.2 s for 200 nM sGnRH (n = 16). It increased steadily with decreasing sGnRH concentration, reaching about 87.8 ± 11.8 s at 2 nM (n = 9). Furthermore, when the sGnRH concentration was 0.2 nM, this decrease in firing frequency became persistent and the late-phase frequency increase was not observed (n = 7/11). This suggests that the onset of the late-phase increase of the pacemaker potential may be dependent on the sGnRH concentration and the early-phase decrease less dependent on it, although it is difficult to assess quantitatively. The early-phase transient decrease in firing frequency was 11.7 ± 2.6% (n = 52) on the average and was not significantly influenced by the sGnRH concentrations used in the present study. On the other hand, the late-phase increase in the firing frequency showed a clear dose-dependence (Fig. 1D).

Normalized increase of the firing frequency of pacemaker activity by 10.2 ± 0.3 on October 20, 2017 http://jn.physiology.org/ Downloaded from

**FIG. 1.** Effects of bath application of salmon gonadotropin-releasing hormone (sGnRH). A: in a current-clamp whole cell recording from a terminal nerve (TN)-GnRH neuron, bath application of sGnRH, the same molecular species of GnRH peptide produced by TN-GnRH neurons themselves, biphasically modulated their pacemaker activity. B: bath application of sGnRH transiently decreased (b) and subsequently increased the frequency of pacemaker activity (c). After washout, the firing frequency of pacemaker activity recovered (d). C: frequency of pacemaker activity plotted against the time course. D: dose-response relationships between the sGnRH concentration and the normalized increase in firing frequency. Numbers in parentheses by the filled squares represent the numbers of cells tested for each sGnRH concentration. The smooth curve was drawn assuming a one-to-one binding relationship with an EC50 = 7.18 nM.
activity was plotted against concentration of sGnRH. Normalized increase in firing frequency was defined as:

$$R = \frac{1}{1 + \frac{[\text{EC}_{50}]/(\text{sGnRH})]}$$

where $R$ is the normalized increase of the firing frequency. The $\text{EC}_{50}$ was 7.18 nM. Finally, the frequency of the pacemaker activity recovered to the control level within 5 min of washout in normal Ringer (Fig. 1D). Such biphasic modulation of pacemaker activity was also shown by bath application of mGnRH (20 or 50 nM; data not shown). In addition, we also examined whether GnRH antagonist affects this biphasic modulation of pacemaker activity. Bath application of GnRH antagonist (100 or 200 nM) alone did not evoke any modulation of pacemaker activity ($n = 11$). However, pre- and coperfused GnRH antagonist inhibited the modulation of pacemaker activity by bath application of sGnRH (20 nM; data not shown).

To elucidate possible mechanisms underlying this modulation of pacemaker activity, we next examined whether a GTP-binding protein (G-protein) process was involved in this modulation. To examine this possibility, we dialyzed the cell with GDP-\(\beta\)-S, a GDP derivative which is a competitive inhibitor of many G-protein-mediated processes, by including it in the patch pipette solution. After that, we examined the effect of bath application of sGnRH under the same conditions as in the control. To ensure the diffusion of GDP-\(\beta\)-S into the cytoplasm of the TN-GnRH neuron, we first calibrated the likely diffusion time by dialyzing the cell with a pipette solution containing QX-314, a sodium channel blocker which is effective from inside of the cell. Under these conditions, the action potentials were blocked within 5 min (data not shown), although subthreshold pacemaker potentials remained because of the presence of a TTX-resistant persistent sodium current ($I_{\text{Na(slow)}}$) (Oka 1996). On the basis of this result, we started data collection 10 min after the whole-cell recording was established. Figure 2 shows the pacemaker activity of a cell recorded with a large-tip patch pipette containing 1 mM GDP-\(\beta\)-S in the pipette solution. In the Ringer solution, this cell showed a slightly irregular beating discharge pattern (Fig. 2Ba). Many cells tended to show such a firing pattern by intracellular application of GDP-\(\beta\)-S, but the firing frequency did not change significantly under these conditions. After the diffusion of GDP-\(\beta\)-S into the TN-GnRH neuron, we first calibrated the likely diffusion time by dialyzing the cell with a pipette solution containing GDP-\(\beta\)-S (60 and 120 s after the onset of sGnRH perfusion, respectively) was recorded during similar time periods of transient decrease and subsequent increase of firing frequency (corresponding to Fig. 1, Bb and Bc, respectively). The time course of the frequency of pacemaker activity is plotted in Fig. 2C. The normalized increase in firing frequency was significantly blocked by the intracellular application of GDP-\(\beta\)-S to the TN-GnRH neurons [Fig. 2D; 0.77 ± 0.1 (control) versus 0.16 ± 0.1 (+GDP-\(\beta\)-S), $n = 17, P < 0.0001$, two-tailed alternate Welch $t$-test]. Similarly, when the pipette solution contained GTP-\(\gamma\)-S and dialyzed the cell well, bath application of sGnRH did not show any modulation of the firing frequency of pacemaker activity (data not shown). Under these conditions, we would expect that intracellular application of GTP-\(\gamma\)-S alone increases the frequency of pacemaker activity after the rupture of the membrane patch. However, we could not observe such changes because the pacemaker activity became unstable immediately after the rupture of cell membrane. Presumably, GTP-\(\gamma\)-S modulated the pacemaker activity maximally soon after the rupture and occluded the effects of sGnRH. From these results, we suggest that G-protein coupled process mediates this biphasic modulation of the pacemaker activity by sGnRH in TN-GnRH neurons.

**Discussion**

In this study, we have demonstrated that the pacemaker activity of TN-GnRH neurons was biphasically modulated in a dose-dependent manner by sGnRH, which is the same molecular species of GnRH peptide that is probably produced by TN-GnRH neurons themselves (Yamamoto et al. 1995). This biphasic modulation of pacemaker activity was also evoked by bath application of another kind of GnRH peptide (mGnRH) but was not evoked by inactive GnRH analogue (GnRH antagonist alone) and was inhibited or attenuated by GnRH antagonist. The present results strongly suggest that modulation by GnRH peptide of pacemaker activity of TN-GnRH neurons is caused by GnRH receptor activation, although there does not seem to be a selectivity of different molecular species of GnRH for the receptor activation. This is in agreement with the report that GnRH receptors of nonmammalian species respond to any types of GnRH peptides (King and Millar 1997). This does not, however, mean that GnRH released from the GnRH neurons that belong to the other GnRH systems (other molecular species of GnRH) activates the TN-GnRH neurons, because immunoactive fibers of the other GnRH systems are not distributed near the TN-GnRH neurons (Yamamoto et al. 1995).

The biphasic modulation consists of the transient decrease and subsequent increase in the firing frequency of pacemaker activity. Such biphasic modulations of the electrical activities have been reported for the changes in membrane potentials of clonal GH3 cell lines induced by TRH (Ozawa and Sand 1986), and those of gonadotropes and immortalized GnRH cell line (GT1-7 cell) induced by GnRH (Zheng et al. 1997). In such cases, it has been suggested that a transient hyperpolarization arises from the activation of \(\text{Ca}^{2+}\)-activated \(\text{K}^+\) currents induced by the elevation of \([\text{Ca}^{2+}]_i\). In fact, a similar mechanism may exist in TN-GnRH neurons because we have preliminary data indicating that the transient decrease in firing frequency of pacemaker activity is blocked by intracellular application of heparin, an inhibitor of the \(\text{IP}_3\)-induced calcium release (Abe and Oka, in preparation). Future studies should analyze the changes of \([\text{Ca}^{2+}]_i\) induced by GnRH application and determine the target ion channel(s) modulated by the GnRH-induced signaling pathway.

There are, however, alternative possible mechanisms of such biphasic modulations of the frequency of pacemaker activity of
TN-GnRH neurons. First, GnRH receptors may exist on the cell surface of TN-GnRH neurons, and the pacemaker activity of TN-GnRH neurons may be directly modulated by the downstream cell signaling pathways. Second, GnRH receptors may exist on the cell surface of non-GnRH neurons, and the pacemaker activity of TN-GnRH neurons may be indirectly modulated by these neurons. In the present study, the biphasic modulations of the frequency of pacemaker activity were blocked by intracellular application of GDP-β-S contained in the patch pipette solution. It has been already established that the GnRH receptors are members of the G-protein-coupled receptors (Stojilkovic et al. 1994b). Also, it has been reported that GT1–7 cells express GnRH receptors (Krsmanovic et al. 1993; Stojilkovic et al. 1994a,b). Moreover, GnRH neurons of hypothalamic culture have been shown by double immunostaining to coexpress GnRH and GnRH receptors (Krsmanovic et al. 1999). Taken together, it is highly possible that the GnRH receptor exists on the cell surface of TN-GnRH neurons and plays a triggering role in modulating the ion channel(s) underlying the pacemaker activity via G-protein-mediated signaling pathways. To confirm this possibility, we are now trying to identify the molecular nature of the GnRH receptors expressed on the cell surface of TN-GnRH neurons by using in situ hybridization and patch-RT-PCR methods.

What then is the physiological significance of such modulations of pacemaker activity by sGnRH on TN-GnRH neurons? TN-GnRH neurons of the dwarf gourami make tight cell clusters with no intervening glial cells (Oka 1997; Oka and Ichikawa 1991; Oka and Matsushima 1993), 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 those that exhibit expression of GnRH receptor mRNA has been reported (Jennes et al.)
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Address reprint requests: Y. Oka, Misaki Marine Biological Station, Graduate School of Science, University of Tokyo, Misaki, Miura, Kanagawa 238-0225, Japan.

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