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Characterization of Voltage-Gated Calcium Currents in Gonadotropin-Releasing Hormone Neurons Tagged with Green Fluorescent Protein in Rats

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GnRH neurons play an essential role in reproductive neuroendocrine regulation. Despite the importance of GnRH neurons, functional analysis of these neurons is limited. This is mainly due to difficulty in identifying GnRH neurons in electrophysiological experiments. Recently, transgenic mice were produced for specific labeling of GnRH neurons with enhanced green fluorescence protein (EGFP) (1–3), which has facilitated the cell physiological study of GnRH neurons. Firing patterns of GnRH neurons were studied in whole cell patch-clamp recordings (2, 4, 5), and the basic membrane properties were studied in neurons examined. These results indicate that GnRH neurons functionally express L-, N-, P/Q-, R-, and T-type channels. Expressions of P/Q- and T-type channels are developmentally regulated. (Endocrinology 144: 5118–5125, 2003)

Materials and Methods

All experiments were performed with the approval of Nippon Medical School animal care committee.

Transgenic rats

The rat GnRH promoter (~3026 to +116; a gift from Dr. M. E. Wierman, University of Colorado Health Science Center, Denver, CO) (8) was used to express a transgene consisting of the intron of rabbit β-globin (640 bp; a gift from Dr. J. Miyazaki, Osaka University, Osaka, Japan), the coding sequence for EGFP (739 bp; CLONTECH Laboratories, Inc., Tokyo, Japan), and the polyadenylation signal. The excised transgene was injected into the pronucleus of fertilized oocytes obtained from Wistar rats (YS New Technology, Tochigi, Japan). Six transgenic founders were identified through Southern blot analysis of DNA harvested from tail snips of 112 pups with a 32P-labeled EGFP probe. The offspring of these 6 transgenic lines were cytologically examined, and one transgenic line, which had high and specific expression of EGFP in GnRH neurons, was selected for physiological experiments. The other five lines were not used because they had weak EGFP fluorescence. For cytological observation, brains were fixed with 4% paraformaldehyde. Forty-micrometer frozen sections of the fixed brain were cut and immunostained with antisera to GnRH (a gift from Dr. K. Inoue, Saitama

Abbreviations: Aga-IVA, ω-Agatoxin IVA; APW, action potential waveform; EGFP, enhanced green fluorescence protein; GVIA, ω-conotoxin GVIA; MVIC, ω-conotoxin MVIC; OVLt, organum vasculosum of the lamina terminalis.
University, Saitama, Japan) and Cy3-labeled second antibody (Jackson ImmunoResearch Laboratories, West Grove, PA).

**Primary culture**

The brains were excised from either 1- to 7-d-old pups or 35- to 40-d-old rats under ether anesthesia. The former were used to prepare neonatal neurons, and the latter were used for the neurons around puberty. The latter could include prepubertal animals because we did not check for the onset of puberty. Medial septum, diagonal band of Broca, organum vasculosum of the lamina terminalis (OVLT), and medial preoptic area were cut out with a razor and surgical blades. The sections were minced and treated with papain (21 U/ml; Funakoshi, Tokyo, Japan) for 30–60 min at 30°C with gentle agitation. The tissues were triturated with a 5-ml plastic pipette after several washes with MEM (Life Technologies, Inc., Tokyo, Japan). The cell suspension was applied to discontinuous Percoll density gradient centrifugation to remove debris. The cells were obtained from the middle layer of the density gradient centrifugation composed of 1.0, 1.023, and 1.078 g/ml layers and were plated on poly-lysine-coated coverslips and incubated overnight in Neurobasal-A medium (Life Technologies, Inc.) supplemented with 0.5 mM 1-glutamine and B-27 (Life Technologies, Inc.) at 37°C. Most of the dissociated GnRH neurons were round, but some were spindle-shaped. These neurons did not change their shape during the overnight culture.

**Electrophysiology**

The List EPC-9 patch-clamp system (Physio-Tech, Tokyo, Japan) was used for electrophysiological recordings and data analysis. Whole cell currents were measured by the perforated patch-clamp technique (9) at room temperature (25°C). The final concentration of amphotericin B (Seikagaku Corp., Tokyo, Japan) in the pipette solution was 0.05 mg/ml. The pipette solution consisted of 95 mM cesium aspartate, 47.5 mM CsCl, 1.0 mM MgCl₂, 0.1 mM EGTA, and 10 mM HEPES (pH 7.2), and the osmolality was adjusted to 270 mosmol. The extracellular solution consisted of 95 mM cesium aspartate, 47.5 mM CsCl, 1.0 mM MgCl₂, 0.6 mM NaCl, 0.8 mM MgCl₂, 0.6 mM NaHCO₃, 10 mM glucose, 20 mM HEPES (pH 7.4), 0.1% BSA (fraction V, Sigma-Aldrich Corp., St. Louis, MO), and 0.3 mM TTX (Seikagaku Corp.), and the osmolality was adjusted to 300 mosmol. Pipettes were fabricated with borosilicate glass capillaries and had a resistance of 7–9 MΩ. The pipettes were targeted to GnRH neurons in the extracellular solution without BSA. After touching the cell, slight negative pressure was applied to the pipette, which made a seal resistance of 5–10 GΩ. Perforation with amphotericin B was achieved within 5–10 min after gigaseal formation. Currents were filtered at 2.3 kHz, digitized at 10 kHz, and recorded. Series resistance was 70% electronically compensated. Data were taken when the series resistance was stable and less than 30 MΩ. Capacitative and leak currents were subtracted by the p/4 protocol, and the liquid junction potential was not compensated. Cell capacitances were 9.2 ± 2.2 pF (n = 46) in males and 9.8 ± 2.4 pF (n = 34) in females in neonates, and 12.8 ± 2.6 pF (n = 13) in males and 10.8 ± 2.7 pF (n = 11) in females around puberty. The input resistance of the cells ranged from 1–5 GΩ. Cells with a peak Ca²⁺ current less than −100 pA were excluded from the analysis, because it is difficult to obtain a reliable subtracted current with such small currents. To confirm the perforated patch configuration, we examined the capacitative current and its change by rupturing the patch membrane at the end of the recording. Data are expressed as the mean ± so unless otherwise stated. The Kruskal-Wallis test and paired t test were used for statistical analysis. The significance level was set at P < 0.05.

**Chemicals**

Nimodipine and nifedipine were obtained from Wako Junyaku (Osaka, Japan). ω-Conotoxin GVIA (GVIA), ω-conotoxin MVIIIC (MVIIIC), ω-agatoxin IVA (AgA-IVA), and SNX-482 were purchased from Peptide Institute, Inc. (Osaka, Japan).

**Results**

In the transgenic rats, EGFP fluorescence was observed only in GnRH-immunoreactive neurons, approximately one third of which had strong EGFP fluorescence (Fig. 1). The fluorescence was observed not only in soma, but also in processes including axons in the median eminence (data not shown). GnRH neurons were also identified with EGFP in a dissociated culture (Fig. 1, D–F).

**Ca²⁺ currents in neonatal GnRH neurons**

In neonatal GnRH neurons Ca²⁺ currents were activated by 100-msec voltage steps from −60 to 60 mV in 10-mV increments from a holding potential of −80 mV at 0.2 Hz (Fig. 2). The maximum amplitudes were −57.8 ± 20.7 pA/pF (n = 13) in males and −46.6 ± 12.2 pA/pF (n = 14) in females. The maximum current was activated at 20–20 mV and showed a rapid activation and a relatively slow inactivation.

The effects of several Ca²⁺ channel blockers on the maximum currents are shown in Fig. 3. The maximum currents were elicited by 100-msec voltage pulses to 0 or 10 mV from the holding potential of −80 mV at 0.2 Hz. After the control currents were recorded, 10 μM nifedipine, 1 μM GVIA, 200 nM Aga-IVA, and 100 nM SNX-482 were successively applied (Fig. 3, A and B). The initial peak currents and late sustained currents were examined. In the initial peak currents, nifedipine and GVIA each attenuated the currents by approximately 20%, and SNX-482 reduced the currents by about 55% in both sexes. A similar inhibition by SNX-482 (60 ± 7%; n = 4) was observed when SNX-482 was applied without prior application of the other Ca²⁺ channel blockers. Inhibition by Aga-IVA was small and negligible in both sexes. Aga-IVA exerted 7.5 ± 3.3% inhibition in 4 of 11 male cells examined and 3% inhibition in 1 of 14 female cells examined. No inhibition was observed in other cells. After combined application of all the above drugs, 6–7% of the control current remained. To examine the presence of T-type Ca²⁺ current, the membrane potential was held at −100 mV, and the voltage steps to −70, −60, and −50 mV were given at 0.2 Hz. In this voltage protocol the current density of −1 pA/pF was activated at −50 mV in 2 cells among 10 male cells examined, and that of −1.4 pA/pF was activated at −50 mV in 3 cells among 9 female cells examined (Fig. 7C). No current was activated at −50 mV in the other cells. In the late sustained currents, the inhibition caused by GVIA was approximately 30%, and that by SNX-482 was 33% in males and 47% in females. The proportion of SNX-482-sensitive currents was smaller in the late sustained currents than in the initial peak currents. This is probably due to inactivation of the SNX-482-sensitive currents. The inhibition by each blocker was significant (P < 0.01), except for that by Aga-IVA.

The action potential waveform (APW) was used for activation of the Ca²⁺ currents (Fig. 4). The half-amplitude width of APW was set at 2.5 msec, because that of the GnRH neuron ranged from 2.5–3 msec at room temperature (data not shown). In this voltage protocol, the inhibitory effect of nifedipine was small (9.4%), and the inhibition caused by GVIA and Aga-IVA was 29%, whereas that by SNX-482 was 45%, so that the contribution of the nifedipine-sensitive current was smaller in the APW than in the current activated by the square pulse.

The voltage-dependent activation of R-type current was studied by measuring tail currents at −80 mV after 10-msec
prepulses of −60 to 60 mV in 10-mV increments from the holding potential of −80 mV at 0.2 Hz in the presence of nimodipine, GVIA, and Aga-IVA (Fig. 5). The activation started at a prepulse of −40 mV and reached full activation at 30−40 mV. The half-activation voltage was 0 mV (two male and two female neurons). Steady state inactivation was also studied in the R-type Ca$^{2+}$ current (Fig. 5). The holding potential varied from −100 to 0 mV in 10-mV increments, and a 100-ms test pulse was applied at 0.2 Hz. The inactivation started from the holding potential of −80 mV and reached almost complete inactivation at 0 mV. The half-inactivation voltage was −39 mV (seven male and three female neurons).

Ca$^{2+}$ currents in GnRH neurons around puberty

Ca$^{2+}$ currents were activated by 100-msec voltage steps from −60 to 60 mV in 10-mV increments from the holding potential of −80 mV at 0.2 Hz. The activation started at −40 mV and reached maximum amplitude around 0 mV. The maximum amplitudes were −49.5 ± 15.1 pA/pF (n = 13) in males and −43.9 ± 26.8 pA/pF (n = 10) in females. The
comparisons were made in four groups according to developmental stage and sex. There was no significant difference in the control maximum current densities among these four groups. Nifedipine, GVIA, and SNX-482 exerted similar inhibitory effects to that in the neonatal GnRH neurons (Fig. 6). The inhibitory effect of Aga-IVA was stronger and clearer than that in the neonatal GnRH neurons. The overall inhibitions were 8% in males and 5% in females. The number of cells in which Aga-IVA attenuated the peak current more than 5 pA was eight in 13 male cells and four in 10 female cells. A similar inhibition was exerted by 2 μM MVIIC (P/Q-type Ca²⁺ channel blocker) in four male and six female neurons examined (6.6 ± 9%). The proportions of the remaining currents after treatment with all of the above drugs were 12.2 ± 7.3% (n = 13) in males and 16.5 ± 18.9% (n =
10) in females. These remaining currents were further attenuated by 50 μM Ni²⁺ to 6.5 ± 4.5% and 3.8 ± 4.7%, respectively, which are comparable to the remaining currents in neonatal GnRH neurons without application of Ni²⁺ (Fig. 3). These Ni²⁺-sensitive currents were clearly seen at −30 mV in all cells examined (Fig. 7). The remaining current densities at −30 mV were −3.2 ± 2.2 pA/pF (n = 8) in males and −6.4 ± 4.3 pA/pF (n = 9) in females. These currents were inhibited by about 90% with 50 μM Ni²⁺. Similar currents were activated by a voltage step to −50 mV from a holding potential of −100 mV at 0.2 Hz in all cells examined (Fig. 7C). The values were −2.2 ± 1.8 pA/pF (n = 6) in males and −4.4 ± 2.5 pA/pF (n = 6) in females. The inhibition by each blocker was statistically significant, except for that by Aga-IVA in females.

Discussion

In the present study we used isolated cells instead of cells in acute slice preparations because we could obtain much better and more reliable recordings of the Ca²⁺ currents in isolated cells. Moreover, the isolated cells may retain their original cellular characteristics to a certain extent even after overnight culture. It should be noted, however, that the cells used in the present experiments lacked both dendrites and axons, so that the currents originated in the cell soma.

We revealed an expression profile of the voltage-gated Ca²⁺ currents in GnRH neurons by using specific blockers for the voltage-gated Ca²⁺ currents. In neonatal GnRH neurons, L-, N-, and R-type Ca²⁺ currents were clearly observed in all cells examined, but P/Q- and T-type Ca²⁺ currents were small and were seen in less than 50% of the cells examined. In the GnRH neurons around puberty, besides L-, N-, and R-type Ca²⁺ currents, a P/Q-type Ca²⁺ current was observed in 62% of male cells examined and 40% of female cells, whereas a T-type Ca²⁺ current was clearly observed in all
cells examined, so that the expression of P/Q- and T-type Ca\(^{2+}\) currents was developmentally regulated. There was no substantial sex difference in the profile of expression of the voltage-gated Ca\(^{2+}\) currents in GnRH neurons either in neonates or around puberty. To date, the presence of L- and T-type Ca\(^{2+}\) currents has been reported in mouse GnRH neurons in explant culture of olfactory pit (7) and GT1 cells (10, 11). No other types of Ca\(^{2+}\) current were examined in these reports.

We identified an R-type current by two criteria. One was a current resistant to specific blockers for L-, N-, and P/Q-type Ca\(^{2+}\) channels in high voltage-activated Ca\(^{2+}\) currents in GnRH neurons either in neonates or around puberty. To date, the presence of L- and T-type Ca\(^{2+}\) currents has been reported in mouse GnRH neurons in explant culture of olfactory pit (7) and GT1 cells (10, 11). No other types of Ca\(^{2+}\) current were examined in these reports.

It should be noted that the proportion of R-type current was surprisingly big both in neonates (55%) and around puberty (40%) compared with approximately 20% in magnocellular and unidentified hypothalamic neurons (18–21) and neocortical and neostriatal neurons (22, 23). This means that the R-type Ca\(^{2+}\) current greatly contributes to intracellular Ca\(^{2+}\) regulations in GnRH neurons in these developmental stages, but in adult GnRH neurons the proportion of R-type current was approximately 30% (our preliminary results). The half-inactivation voltage was −40 mV in 10 mM Ca\(^{2+}\) in the extracellular solution (Fig. 5). This value would be −50 mV in a normal Ca\(^{2+}\) concentration (2.5 mM). If we take −60 mV as the resting potential value, the contribution of R-type Ca\(^{2+}\) current would be more than 30% of the total Ca\(^{2+}\) current activated by the action potential. In fact, the contribution of the R-type current was 45% in our APW experiment (Fig. 4). Cytochemistry revealed a wide distribution of the prime candidate of R-type channel α1E (17) in the brain in both mice (13) and rats (24), including the OVLT and medial preoptic area. These findings suggest that R-type Ca\(^{2+}\) channels must be expressed at least in the somadendritic region of GnRH neurons and contribute to Ca\(^{2+}\) dependent regulation in GnRH neurons. The R-type Ca\(^{2+}\) channels might be involved in GnRH release at nerve endings, because the R-type channels are reported to contribute transmitter release at a rat calyx synapse (25), oxytocin release from the nerve endings (26, 27), and exocytosis in mouse adrenal chromaffin cells (28).

We used the dihydropyridine antagonists nifedipine and nimodipine to block L-type current (29, 30). An L-type current was observed both in neonates and around puberty as approximately 20% of total Ca\(^{2+}\) currents. Kusano et al. (7) reported a high voltage-activated Ca\(^{2+}\) current sensitive to 100 μM Cd\(^{2+}\) and 1 μM nifedipine expressed in mouse GnRH neurons in explant culture of the olfactory pit, suggesting the presence of an L-type current in these neurons. A similar type of current has been reported in GT1 cells (10, 11). The L-type
current is well known to contribute hormone release in a variety of neuroendocrine cells, including pancreatic β cells (31) and pituitary somatotrophs (32). In physiological conditions, an L-type current may be activated by slow depolarization, such as by an excitatory postsynaptic potential, rather than by an action potential (33). Moreover, L-type currents become prominent in slow depolarization because the inactivation process eliminates some other Ca2⁺ currents, such as the R-type to a certain extent. Taken together with preferential expression of L-type Ca2⁺ channels in the somadendritic region of central neurons (34), L-type currents may regulate Ca2⁺-dependent functions, such as protein phosphorylation (33), enzyme activity, and gene expression, in GnRH neurons in a different manner from that of the R-type current.

The peptide antagonist GVIA is widely used to identify the N-type Ca2⁺ current in physiological studies (35, 36). We used 1 μM GVIA and found that the proportion of N-type Ca2⁺ current was 15–20% of the total Ca2⁺ currents. N-Type Ca2⁺ channels could be involved in GnRH release at nerve endings, because the N-type channel is known to be involved in vasopressin release (20), oxytocin release (26), and synaptic transmission in cultured hypothalamic neurons (37) and several central synapses (38). Immunostaining of N-type Ca2⁺ channel subunit α1B revealed the presence of the N-type channel not only at nerve terminals, but also in the soma-dendritic region of central neurons (39), so that N-type channels in the GnRH neuron may play some roles in the soma-dendritic region besides at nerve terminals.

In the present study we did not separately identify P-type and Q-type Ca2⁺ currents, but treated them as P/Q-type Ca2⁺ currents by using a high concentration (200 nM) of Aga-IVA that does not distinguish between P- and Q-type channels (14). This was further confirmed with another P/Q-type channel blocker, MVIC (2 μM). The P/Q-type Ca2⁺ current was small, but clearly observed around puberty in 40–62% of GnRH neurons examined. This developmental change in the expression of P/Q-type Ca2⁺ current may have functional significance. For example, a P/Q-type channel might be involved in GnRH release from nerve terminals at the median eminence, which changes dramatically through puberty, thereby controlling gonadotropin release from the anterior pituitary. The P/Q-type Ca2⁺ current is shown in various neuronal centers with different degrees of expression (40, 41). Q-type channels are present on a subset of the neuropil region itself that releases vasopressin (20). Developmental change in the contribution of P/Q-type Ca2⁺ current is also demonstrated at several central synapses (38). Its contribution is greater on postnatal d 13–19 than on postnatal d 7–9.

Expression of T-type Ca2⁺ current also showed a clear change in development. The T-type current is classified as a low voltage-activated current. Some R-type currents are also activated in a similar voltage range (13, 16, 17). Therefore, in the present study the T-type current was identified by its sensitivity to Ni²⁺ and its insensitivity to SNX-482 (16) in addition to the low voltage activation. This type of current is demonstrated in mouse GnRH neurons in explant culture of the olfactory pit (7) and GT1 cells (10, 11). T-type Ca2⁺ current in GnRH neurons possibly activates small condu-

tance, Ca2⁺-activated K⁺ channels (SK channels), such as in midbrain dopaminergic neurons (42), thereby controlling action potential firing. According to the several reports concerning the firing pattern of mouse GnRH neurons, irregular spontaneous firing of single action potentials and irregular bursting of spikes are observed in these neurons (1–6, 43). As the SK channel is responsible for sustained tonic firing of single spikes (42), the T-type Ca2⁺ current may function as a regulator of SK channels in mouse and possibly rat GnRH neurons. The present results clearly demonstrate that the T-type current becomes active around the pubertal stage.

In conclusion, the present study revealed rat GnRH neurons functionally expressed L-, N-, and R-type Ca2⁺ channels both in neonates and around puberty and expressed the P/Q- and T-type Ca2⁺ channels around puberty. Cellular functions of these voltage-gated Ca2⁺ channels remain to be analyzed in future experiments.

Acknowledgments

We are grateful to Dr. Koichi Ishikawa for his suggestions on the method of dispersion of neurons. We also thank Drs. Hisashi Morii, Tsuyoshi Hamada, Keisuke Kaneishi, Tomohiro Hamada, and Masugi Nishihara for their help and valuable suggestions on the transgenic technique.

Received February 14, 2003. Accepted August 6, 2003.

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This work was supported in part by Grants-in-Aid for Scientific Research (C) 10670071 and 13680883 from the Japan Society for the Promotion of Science.

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