Gonadotropin-Releasing Hormone-1 Neuronal Activity Is Independent of Cyclic Nucleotide-Gated Channels

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Pulsatile release of GnRH-1 is essential for secretion of gonadotropin hormones. The frequency of GnRH-1 pulses is regulated during the reproductive cycle by numerous neurotransmitters. Cyclic nucleotide-gated (CNG) channels have been proposed as a mechanism to integrate the cAMP signal evoked by many neurotransmitters. This study reports the expression of the CNGA2 subunit in GnRH-1 neurons obtained from mouse nasal explants and shows the ability of GnRH-1 neurons to increase their activity in response to forskolin (activator of adenyl cyclases), or 3-isobutyl-1-methylxanthine (inhibitor of phosphodiesterases) even after removal of γ-aminobutyric acid (A)-ergic input. Next, the endogenous activity of adenyl cyclases was evaluated as a component of the oscillatory mechanism of GnRH-1 neurons. Inhibition of endogenous activity of adenyl cyclases did not alter GnRH-1 activity. The potential involvement of CNGA2 subunit in basal or induced activity was tested on GnRH-1 neurons obtained from CNGA2-deficient mice. Without up-regulation of CNGA1 or CNGA3, the absence of functional CNGA2 did not alter either the endogenous GnRH-1 neuronal activity or the response to forskolin, negating CNG channels from cAMP-sensitive mechanisms leading to changes in GnRH-1 neuronal activity. In addition, the potential role of CNGA2 subunit in the synchronization of calcium oscillations previously described was evaluated in GnRH-1 neurons from CNGA2-deficient explants. Synchronized calcium oscillations persisted in CNGA2-deficient GnRH-1 neurons. Taken together, these results indicate that CNGA2 channels are not necessary for either the response of GnRH-1 neurons to cAMP increases or the basal rhythmic activity of GnRH-1 neurons. (Endocrinology 149: 279–290, 2008)

GnRH-1 REGULATES REPRODUCTION, being an integral component of hypothalamic-pituitary-gonadal axis. The GnRH-1 neuronal population is comprised of relatively few cells (~800 in mouse) (1) that are not contained within a single anatomical nucleus. In rodents, the GnRH-1 neurons form bilateral continuums, on either side of midline, from the olfactory bulbs caudally to the hypothalamus. Independent of location, the majority of GnRH-1 axons target the pituitary portal blood where GnRH-1 is released in a pulsatile manner (2). The pulsatility of GnRH-1 secretion is essential for maintenance of anterior pituitary gonadotropin secretion (3), preventing desensitization of gonadotrope cells. The mechanisms involved in the pulsatile release of GnRH-1 remain unclear.

Reproduction is a multisensory response being modulated by physiological status as well as environmental conditions (2). As such, GnRH-1 neurons must integrate multiple signals (4, 5). Data from an immobilized GnRH-1 cell line, GT-1 cells, indicate that the secondary messenger cAMP is involved in many transduction pathways in these cells [nor-epinephrine, dopamine, acetylcholine, and γ-aminobutyric acid (GABA) (6); estradiol (7); and serotonin (8)]. Increasing cAMP levels with agonists (6), adenyl cyclase (AC) activators (6, 9, 10), or phosphodiesterase (PDE) inhibitors (11) increases GnRH-1 release, whereas decreasing cAMP levels with PDE overexpression (12, 13) decreases GnRH-1 release. Specific overexpression of PDE in GnRH-1 neurons in rats did not affect hypothalamic GnRH-1 levels but resulted in decreased amplitude of the preovulatory LH surge and impaired fertility in females and LH levels, and LH pulse frequency in ovariectomized rats were also attenuated (12), supporting the importance of cAMP-dependent modulation of GnRH-1 neuronal activity.

Transcripts for cyclic nucleotide-gated (CNG) channel subunits have been detected in GT-1 cells (6), and the involvement of CNG channels in the excitability of GT-1 cells has been evaluated. The functionality of CNG channels in GT-1 cells has been shown by cAMP-evoked microscopic as well as macroscopic currents (6). Moreover, forskolin (FSK) and cell-permeant cAMP (Sp-cAMPS) increase calcium oscillations, and pretreatment with l-cis-diltiazem (LCD), a common CNG channel blocker, inhibits Sp-cAMPS-induced oscillations (6). Because the CNGA2 subunit is essential for formation of functional CNG channels, a recent study specifically targeted this subunit in GT-1 cells with short interference RNA. A significant decrease in the interpulse interval for GnRH-1 secretion was found (14). A role of CNG channels in GnRH-1 neurons in vivo is supported by expression of CNGA2 subunit transcript in GnRH-1 neurons in rats and expression of the protein in GnRH-1 perikarya as well as in the external zone of the median eminence (15).

Native prenatal GnRH-1 neurons maintained in nasal ex-
plants have proven to be a useful model for evaluating GnRH-1 neuronal activity (16–19). In this in vitro model, GnRH-1 neurons, devoid of central nervous system cues, exhibit spontaneous electrical activity (20), are highly differentiated with respect to their electrophysiological properties, possess a wide variety of voltage- and ligand-gated ion channels (21, 22), and exhibit synchronous calcium oscillations (21, 22) and pulsatile GnRH-1 secretion (23–25). In two different species, the periodicity of synchronous calcium oscillations correlates with the periodicity of GnRH-1 secretion (21, 22), thereby linking synchronous calcium oscillations to secretory events. The goal of this study was to investigate whether cAMP modulated GnRH-1 neuronal activity and to evaluate the potential role of CNG channels under stimulation and basal conditions, using both wild-type (WT) mice and CNGA2 subunit-deficient mice (26). This work indicates that CNGA2 channels are not necessary for either the response of GnRH-1 neurons to cAMP increases or the basal rhythmic activity of GnRH-1 neurons.

Materials and Methods

In vitro nasal explants

Nasal regions were cultured as previously described (18) (Fig. 1A). Briefly, embryos were obtained from timed pregnant animals in accordance with NIH guidelines. Nasal pits of embryonic day 11.5 (E11.5)-stage NIH Swiss mice or CNGA2-deficient mice (26) were isolated under aseptic conditions and adhered onto coverslips by a plasma (Cocalico Biologicals, Reamstown, PA)/thrombin (Sigma Chemical Co., St. Louis, MO) clot. Nasal explants were maintained at 37°C in a humidified atmosphere with 5% CO2, in a defined serum-free medium (SFM) (18). In vitro nasal explants

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PCR on single GnRH-1 cells

cDNA libraries have been created from individual GnRH-1 cells maintained in nasal explants (27). Using these cDNA libraries, the presence of specific transcripts can be evaluated in individual GnRH-1 neurons and relative expression of that transcript within the GnRH-1 population determined (28–30). Briefly, GnRH-1-like neurons, identified in vitro by their bipolar morphology, association with outgrowing axons, and location within the explant, were removed with pulled glass capillaries. cDNA were produced, and PCR amplification was performed as previously described (27).

Based on the technique used to generate the cDNA pools, 3′-untranslated region biased primers are necessary. Primers were designed with the 5′ primer being less than 300 bases from the polyA site and the 3′ primer close to, but not into, the polyadenylation region. All primers were screened using BLAST to ensure specificity of binding. For each reaction, 30.5 μl H2O, 5 μl 10× PCR buffer (Applied Biosystems, Foster City, CA), 4 μl 25 mm MgCl2 (Applied Biosystems), 5 μl dNTP mix (25 μl of each 100 mm dNTP, 900 μl H2O), 2 μl 6.25 μm forward primer, 2 μl 6.25 μm reverse primer, and 0.5 μl AmpliTaq Gold (Applied Biosystems) were added to 1 μl template cDNA. PCR was performed at 94°C (10 min); 94°C (30 sec); 55, 60, or 65°C, depending on primers (30 sec); and 72°C (2 min) for 40 cycles with a postelongation at 72°C for 10 min. Amplified products were run on a 1.5% agarose gel. Specific bands of the predicted size were observed in the control total brain lane, whereas no bands were seen in water. All cDNA were initially screened by PCR for GnRH-1 (correct cell phenotype), III-tubulin, and L19 (two housekeeping genes, respectively microtubule and ribosomal; primer sequences given in Ref. 31). Used in previous studies (27, 32), only cells positive for all three transcripts were used in this study.

PCR were performed to determine the expression of CNGA1 (accession number NM_007723; forward primer 5′-TGC GAG AAA GAG TCG GTC TGG-3′, reverse primer 5′-TCT CCT TTT CAG GCC ACT TG-3′; product size, 248 bp; 65°C), CNGA2 (NM_007724; forward 5′-TCC CAA GGC ATG CAA GGT CT-3′, reverse 5′-CAG TAT CTC ATG CAG CAG TAG C-3′; 183 bp; 60°C), and CNGA3 (NM_009918; forward 5′-ATG GAT GTG TTA CGG GGC TG-3′, reverse 5′-TGG ACA TAT ATC CAC CGC CCC CAA G-3′; 156 bp; 65°C) pore-forming subunits of CNG channels in in vitro GnRH-1 cell cDNA 3 days in vitro (div n = 10–8; div (n = 15)). Two immortalized GnRH-1 cell lines, GT-1 (31) and NLT (34), were screened in parallel. A positive brain lane and a negative water lane validated the experimental run.

Calcium Green-1 calcium imaging was undertaken as reported previously (22). Briefly, the Calcium Green-1 AM (Molecular Probes, Eugene, OR) was diluted to 2.7 mM concentration in 20% pluronic F-127/DMSO solution (Molecular Probes). This solution was diluted 1:200 with SFM to a final Calcium Green-1 concentration of 13.5 μM. Nasal explants, maintained at 37°C in a 5% CO2 humidified incubator, were incubated with this loading solution for 20 min and then washed twice with fresh SFM (5 min wash each). Explants were mounted into a perfusion chamber and were continuously perfused with medium, at a rate of about 280 μl/min, using a gravity system (Warner Instruments, Hamden, CT) as inflow and a peristaltic pump as outflow. Drugs were applied by placing the inflow catheter as close as possible to the explant (~1–2 mm), thereby maximizing the onset of drug exposure (~5 sec). Calcium Green-1 was visualized using an inverted microscope (Nikon) through a x20 fluorescence objective and a digital CCD camera (Retiga, Qimaging, Burnaby, Canada) connected to a Macintosh computer. Experiments were piloted via imaging software (IPLab Spectrum, Scanalytics Inc., Rockville, MD) controlling the shutter (Uniblitz, Vincent Associates, Rochester, NY) and the acquisition. Excitation wavelengths were provided through a medium-width excitation bandpass filter at 465–495 nm, and emission was monitored through a 40-nm bandpass centered on 535 nm. Pictures were acquired each 20 sec for long-term recordings (100 min) or each second for short recordings (~20 min).

Calcium imaging recordings were performed from 6–10 div, and intracellular calcium fluctuations were analyzed a posteriori with IPLab software. Each cell, individually identified, was circled. Calcium Green-1 fluorescence intensity was plotted and analyzed with MATLAB (Mathworks, Natick, MA). When acquired at one picture/sec, a calcium fluctuation was first identified when a value was greater than the five previous and five subsequent points. Then, that calcium fluctuation had to be greater than the mean of the five previous and five next points plus a minimal value (which represented small fluctuations in baseline) to be
considered as a calcium oscillation (peak). The frequency of calcium oscillations was calculated as the number of detected calcium peaks per time unit (minute). When acquired at one picture/20 sec, neuronal activity was analyzed using a floating mean and a floating sd, both calculated on five points before and five points after the point of interest. A calcium peak was defined as a point value greater than the mean plus 2 sd (mean + 2 sd). The synchronization of calcium events was detected using a waveform analysis as previously described (22). In Results, n and N represent the number of cells and explants recorded, respectively. Statistical analysis was performed using paired t tests (to identify an effect of a drug on the peak frequency among a pool of cells), ANOVA (to compare the interpulse interval in WT mice and CNGA2-deficient mice), and a Wilcoxon-Mann-Whitney U test (to evaluate the shift in the cell population toward higher or lower frequencies in calcium oscillations). In all cases, a P value of 0.05 was chosen for significance.

Electrophysiological recordings

To ensure that calcium imaging could be used to evaluate GnRH-1 neuronal activity, simultaneous electrophysiological recordings were performed during calcium imaging under SFM control conditions. Under the same conditions previously described for calcium imaging, GnRH-1 neurons were recorded using the patch clamp technique in the der the same conditions previously described for calcium imaging, performed during calcium imaging under SFM control conditions. Un-

Fig. 2. Calcium imaging experimental paradigms. A, Two different paradigms were used. The first one (upper) was used to determine an appropriate drug concentration compatible with a reversible effect, stimulating period. The second one (lower) was used to induce a perturbation, conditioning period, before the stimulation. Both paradigms were initiated after a 5-min control period to ensure the quality of the cells and followed by a 5-min washout to examine the reversibility of the effect. A KCl stimulation at the end of the experiment was performed to verify the viability of the cells included in the analysis (B1, recorded field before the experiment; B2, loaded cells during the experiment; B3, after KCl).
differentiate and share many similarities with GnRH-1 cells in vivo (20, 28, 29, 31, 32).

Electrophysiological events correlate with calcium peaks

Calcium imaging of GnRH-1 cells in nasal explants allows one to monitor the activity of multiple GnRH-1 cells in a single experiment. This provides information on individual GnRH-1 cells as well as dynamics within the GnRH-1 neuronal population. However, to date, the relationship of electrical events and calcium peaks in GnRH-1 cells maintained in this model system has not been evaluated. Calcium imaging as a technique for evaluating GnRH-1 neuronal activity was validated by recording simultaneously the electrical activity from the neuronal membrane and the fluctuations in intracellular level of calcium from the cell soma. Here we show (Fig. 1B) that there is a direct temporal correlation (r = 0.99) between action potentials and calcium peaks in single GnRH-1 neurons (n = 5; N = 2).

cAMP-induced modulation of GnRH-1 neuronal activity

To ensure the stability of GnRH-1 neurons without exogenous perturbation, calcium imaging recordings were initially performed over the experimental paradigm periods in only SFM (Fig. 2A). No significant changes were detected: 1.35 ± 0.09 peaks/min during SFM period 1 vs. 1.46 ± 0.12 peaks/min during SFM period 2 vs. 1.54 ± 0.12 peaks/min during SFM period 3 (paired t test, P > 0.05 for all periods) (n = 64; N = 2; Fig. 3A).

Next, FSK, activator of AC, was tested at two different concentrations (1 and 10 \( \mu \)M). At 1 \( \mu \)M, a reversible significant increase in the frequency of calcium oscillations was observed in GnRH-1 cells [1.37 ± 0.23 peaks/min in SFM vs. 2.73 ± 0.27 peaks/min in FSK (paired t test, P < 0.01); 1.56 ± 0.22 peaks/min in washout SFM (P < 0.01); n = 32; N = 3; Fig. 3B; Fig. 3C1–2 shows significant shifts in the cell population (Wilcoxon-Mann-Whitney U test, P < 0.05)]. At 10 \( \mu \)M, this increase turned quickly into a sustained plateau of intracellular calcium and/or a complete arrest of calcium oscillations (n = 25; N = 1; data not shown). For additional experiments, only FSK at 1 \( \mu \)M was used. To determine whether endogenously synthesized cAMP could be an intrinsic mechanism for modulating GnRH-1 neuronal activity, IBMX (100 \( \mu \)M) was applied to inhibit PDE, enzymes that hydrolyze cAMP. Inhibiting degradation of cAMP induced an increase in the frequency of calcium oscillations [1.32 ± 0.15 peaks/min in SFM vs. 2.36 ± 0.17 peaks/min in IBMX (paired t test, P < 0.01); 1.30 ± 0.19 peaks/min in washout SFM (P < 0.01); n = 38; N = 2; Fig. 4A; Fig. 4B1–2 shows significant shifts in the cell population (Wilcoxon-Mann-Whitney U test, P < 0.05)], confirming the hypothesis of a cAMP sensitive mechanism for regulating GnRH-1 neuronal activity.

Endogenous GnRH-1 neuronal rhythmicity and cAMP production

To evaluate whether the endogenous activity of AC contributed to GnRH-1 neuronal activity, pharmacological blockade of AC was done using DDA (40 \( \mu \)M) (37). The blockade of cAMP production over a 6-min period did not alter the frequency in calcium oscillations in GnRH-1 neurons [1.42 ± 0.19 peaks/min in SFM vs. 1.45 ± 0.15 peaks/min in DDA (paired t test, P > 0.05); n = 48; N = 3]. These results indicate that cAMP modulates rather than triggers GnRH-1 neuronal rhythmicity.

FIG. 3. Effect of FSK on GnRH-1 neuronal activity. A, GnRH-1 neurons maintained in SFM did not show major spontaneous changes in neuronal activity over the total experimental time; B, FSK (1 \( \mu \)M) induced a reversible increase of calcium oscillations; C, histograms show the distribution of cells as a function of their frequency of calcium oscillations (peaks per minute: C1, a significant shift in the cell distribution (SFM, hatched bars) toward higher frequencies occurred upon exposure to FSK (black bars); Wilcoxon-Mann-Whitney U test, P < 0.05); C2, the shift induced by FSK was reversible with the cell distribution returning to lower frequencies during the washout period (gray bars; Wilcoxon-Mann-Whitney U test, P < 0.05).
neuronal activity in nasal explants. To ensure that the cAMP remaining after the 6-min treatment was not still promoting GnRH-1 neuronal activity, some recordings were performed for 16.5 min in the presence of DDA to reach the steady-state level of inhibition of AC blockade previously described in pituitary cells (38). Over 16.5 min, DDA application still failed to modify the activity of GnRH-1 neurons [1.47 ± 0.13 peaks/min for the first period (5.5 min), 1.48 ± 0.12 peaks/min for the next period (5.5 min), and 1.65 ± 0.12 for the last period (5.5 min) (paired t test, \( P > 0.05 \)); \( n = 34; N = 2 \)]. In addition to increasing intracellular cAMP level, FSK (1 µM) and IBMX (100 µM) are able to increase intracellular cGMP level in pituitary cells (38). To verify that the FSK-induced increases in calcium oscillations in GnRH-1 neurons were mediated by cAMP rather than cGMP, AC were blocked before application of FSK to prevent the FSK-induced synthesis of cAMP. The FSK-induced response was blocked, showing that the response was dependent on an increase in cAMP (1.73 ± 0.25 peaks/min in DDA vs. 2.00 ± 0.41 peaks/min in DDA+FSK (paired t test, \( P > 0.05 \)); \( n = 14; N = 1 \)].

**Direct or indirect action of cAMP on GnRH-1 neurons**

Extensive evidences indicate that glutamatergic and GABAergic neurons are important primary afferent inputs to GnRH-1 neurons *in vivo* (2). Although glutamatergic responses have been recorded in older explants (20), endogenous glutamatergic populations have not been shown. In addition, *in vivo*, tonic glutamatergic input is relatively low compared with GABAergic input (2). A subpopulation of GABAergic neurons, known to be present in nasal explants, regulates GnRH-1 neuronal activity via excitatory input (20, 39) and is important for synchronized activity (22). To confirm that FSK was not activating GABAergic neurons that would subsequently stimulate GnRH-1 neurons, a GABA\(_B\) receptor antagonist, BIC (20 µM), was used. Consistent with previous results, GnRH-1 neuronal activity decreased with blockade of GABA\(_B\) receptors \( \left( P < 0.01; \ n = 59; N = 2 \right); \) Fig. 5A). However, the FSK-induced increase in calcium oscillations in GnRH-1 cells still occurred after BIC pretreatment \[ 1.78 ± 0.14 peaks/min in BIC vs. 2.30 ± 0.16 peaks/min in BIC+FSK (paired t test, \( P < 0.01 \)); \] SFM control period \( (2.19 ± 0.15 \text{ peaks/min}) \) was significantly higher than BIC treatment; \( n = 59; N = 2 \); Fig. 5B; Fig. 5C1–2 shows significant shifts in the cell population (Wilcoxon-Mann-Whitney \( U \) test, \( P < 0.05 \)). These data suggest that cAMP acts directly on GnRH-1 neurons, increasing neuronal activity. To verify that GnRH-1 cells could respond to endogenous production of cAMP after GABAergic input removal, IBMX was applied in the presence of BIC. A significant increase in GnRH-1 calcium peaks was still observed \[ 1.35 ± 0.18 \text{ peaks/min in BIC vs. 1.67 ± 0.20 peaks/min in BIC+FSK (paired t test, \( P < 0.05 \)); } \] SFM control period \( (2.51 ± 0.85 \text{ peaks/min}) \) was significantly higher than BIC treatment; \( n = 46; N = 2 \). To further investigate the role of the endogenous AC activity in GnRH-1 neurons, deprived of GABAergic input, a 6- and 16.5-min blockade of AC with DDA was performed after BIC pretreatment. No significant changes in GnRH-1 neuronal activity were observed \[ 6 \text{-min blockade}: 0.80 ± 0.17 \text{ peaks/min in BIC vs. 1.02 ± 0.15 \text{ peaks/min in BIC+DDA (paired t test, } \( P > 0.05 \)); n = 33; N = 3; 16.5 \text{-min blockade}: 1.23 ± 0.23 \text{ peaks/min for the first 5.5 min, 1.43 ± 0.20 \text{ peaks/min for the next 5.5 min, and 1.46 ± 0.19 for the last 5.5 min (paired t test, } \( P > 0.05 \)); n = 22; N = 2 \].

**Involvement of CNGs in the cAMP-induced modulation of GnRH-1 neuronal activity**

PCR examination of cDNAs from single GnRH-1 cells from WT mice was performed for the CNG A1–3 subunits, the only subunits able to form a functional homomeric chan-
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tivity of GnRH-1 neurons (SFM, stimulation of calcium oscillations; C1, a significant shift in the ac-
per minute still occurred upon exposure of FSK ([1-peak/min bin after BIC exposure]); C2, even in presence of BIC, a
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ade of endogenous GABAergic input on GnRH-1 neurons by BIC (20
n

In contrast, CNGA2 transcript was found in GnRH-1 neurons
examined were negative for CNGA1 and CNGA3 subunits.
CNG Channels in GnRH-1 Neuronal Activity
nel when expressed heterologously (40). All GnRH-1 cells
examined were negative for CNGA1 and CNGA3 subunits.
In contrast, CNGA2 transcript was found in GnRH-1 neurons
(3 div, 60%; 6–8 div, 40%). CNGA2 channels are described to be more sensitive to cGMP than cAMP (41). Thus, a cGMP
analog that activates CNG channels but not cGMP-depen-
dent protein kinase (42), Rp-8-Br-cGMPs, was used to test the
involvement of CNGA2 channels in the regulation of
GnRH-1 neuronal activity. Application of Rp-8-Br-cGMPs
failed to stimulate GnRH-1 neurons [1.62 ± 0.19 peaks/min
in SFM vs. 1.47 ± 0.19 peaks/min in Rp-8-Br-cGMPs (paired
t test, P > 0.05); n = 26; N = 2]. Examination of the response of
all cells individually did not highlight a subpopulation response (22 of 26 cells did not respond to Rp-8-Br-cGMPs).
LCD (80 μm), a CNG channel blocker, was then applied on
nasal explants before the addition of FSK. Surprisingly, LCD
itself inhibited calcium oscillations in the majority of
GnRH-1 neurons (n = 32/42; N = 2 explants; Fig. 6A). LCD has been
classified as nonspecific to the CNGA2 subunit (41), and
modification of sodium current by LCD has been observed
in cardiomyocytes (43). Therefore, whole-cell recordings of
sodium current were performed on GnRH-1 neurons in the
absence of LCD, in the presence of LCD, and after washout
to investigate the availability of sodium channels (Fig. 6B).
Tetrodotoxin (1 μm), a sodium channel blocker, was initially
applied to confirm the nature of the recorded current and (as
predicted by Ref. 20), was found to abolish all the inward
current. The application of LCD induced a significant and
reversible shift toward lower potentials of the activation
curve [Vinact1/2 = −44.2 ± 0.1 mV in SFM (n = 2) vs. Vinact1/2 = −55.3 ± 0.3 mV in SFM + LCD (n = 2)] and of the steady-state
inactivation curve [Vinac1/2 = −39.0 ± 0.3 mV in SFM (n = 2) vs. Vinac1/2 = −56.4 ± 2.2 mV in SFM + LCD (n = 2) vs.
Vinac1/2 = −41 ± 5.3 mV in washout (n = 2); Fig. 6C],
indicating alteration of sodium channel properties. To our
knowledge, this is the first report of LCD modifying the
activation of sodium channels. Hashimoto et al. (43) also
report a decrease in the amplitude of the current recorded
in myocytes exposed to LCD (43). This was not found in
GnRH-1 neurons and may be the result of the differences
in resting potentials of these two cell types: myocytes −90
mV (44) vs. GnRH-1 neurons −50 mV (20) vs. the holding
potentials used (−90 mV). Hashimoto et al. (43) did find
LCD blockade to be highly dependent on the holding
potential, with higher potentials facilitating the blockade
of sodium current (100 μm LCD blocking 80 and 100% of
the current from holding potential −90 and −80 mV,
respectively. Consistent with Ref. 43, a pronounced effect on
the inactivation of sodium channels was found in GnRH-1
neurons exposed to LCD. For GnRH-1 neurons whose
resting potential is around −50 mV, this shift in the in-
activation curve represents a decrease of available sodium
channels of about 50% and might explain the loss of ex-
citability of GnRH-1 neurons observed in calcium
imaging.
GnRH-1 neuronal activity in CNGA2-deficient (KO) mice. Be-
cause LCD altered sodium current, current that directly modu-
lates the excitability of cells, nasal explants from CNGA2-
deficient mice were generated to test the involvement of
CNGs in cAMP-induced modulation of GnRH-1 neuronal
activity. Before generating nasal explants from CNGA2 KO
mice, the GnRH-1 system was examined in adult KOs. The

FIG. 5. Direct activation of GnRH-1 neuronal activity by FSK. Block-
ade of endogenous GABAergic input on GnRH-1 neurons by BIC (20
μm) decreased calcium oscillations. A, Example of a highly BIC-sen-
sitive cell [SFM = 1.71 ± 0.19 peaks/min; BIC = 1.21 ± 0.16 peaks/
min (paired t test, P < 0.01); washout SFM = 1.88 ± 0.16 peaks/min
(P < 0.01; n = 59; N = 2)]; B, BIC did not prevent the FSK-induced
stimulation of calcium oscillations; C1, a significant shift in the ac-
tivity of GnRH-1 neurons (SFM, hatched bars) toward fewer peaks per
minute occurred upon exposure to BIC (black bars; Wilcoxon-Mann-
Whitney U test, P < 0.05) (note example in A was a cell in the 0.5- to
1-peak/min bin after BIC exposure); C2, even in presence of BIC, a
significant shift in the activity of GnRH-1 neurons toward more peaks
per minute still occurred upon exposure of FSK (gray bars; Wilcoxon-
Mann-Whitney U test, P < 0.05).
FIG. 6. LCD effects on GnRH-1 neurons. A, LCD (80 μM) inhibited calcium oscillations in GnRH-1 neurons. Because the loss of excitability of GnRH-1 neurons was complete in the presence of LCD, sodium current recordings were performed to ensure the specificity of LCD blockade. B, The steady-state inactivation protocol used (inset) and the current family generated by conditioning prepulses (left) and testing pulses (right). The star indicates where the amplitude of the current was determined. The resulting steady-state inactivation curves (C) showed a reversible shift toward lower potentials in the presence of LCD (each point and vertical bar represents, respectively, mean and SEM of two experimental). The dotted line represents the resting potential described in GnRH-1 neurons (−50 mV) (20). Note that the percentage of available sodium channels as this membrane potential is lowered to about 40% with LCD in comparison with control (−90%).

The frequency of calcium oscillations in GnRH-1 neurons was not significantly different in CNGA2-deficient explants under normal conditions [1.49 ± 0.13 peaks/min in KO/SFM (n = 75; N = 5) vs. 1.59 ± 0.07 peaks/min in WT/SFM (n = 248; N = 13); ANOVA, P > 0.05]. Production of cAMP induced by FSK still resulted in an increase in the frequency of calcium oscillations in GnRH-1 neurons from CNGA2-deficient explants [1.10 ± 0.14 peaks/min in SFM vs. 1.92 ± 0.18 peaks/min in FSK; n = 36; N = 4 (paired t test, P < 0.01); Fig. 7A], suggesting another pathway leading to the increase in the frequency of calcium oscillations.

To evaluate whether GABAergic input was still present and excitatory in CNGA2-deficient explants, BIC was applied alone. In a manner similar to GnRH-1 cells in nasal explants from WT mice, the blockade of GABA<sub>A</sub> receptors led to an inhibition of the frequency of calcium oscillations [1.49 ± 0.13 peaks/min in KO/SFM vs. 1.18 ± 0.12 peaks/min in KO/BIC; n = 75; N = 5; (paired t test, P < 0.01)]. After removal of GABAergic inputs, the frequency of calcium oscillations in GnRH-1 neurons was still similar between CNGA2 KO and WT explants [1.89 ± 0.14 peaks/min in KO/BIC; n = 266; N = 9]; ANOVA, P > 0.05]. In CNGA2 KO explants, GnRH-1 neurons without GABAergic input still responded to FSK stimulation [1.18 ± 0.12 peaks/min vs. 1.89 ± 0.14 peaks/min (paired t test, P < 0.01); n = 75; N = 5]; Fig. 7B; Fig. 6C1–2 shows significant shifts in the cell population (Wilcoxon-Mann-Whitney U test, P < 0.05).
Synchronization of GnRH-1 neurons in CNGA2-deficient explants. The involvement of CNGA2 in the synchronization of GnRH-1 neurons was evaluated in long-term recordings (100 min, one picture/20 sec; Fig. 8A) by comparing inter-synchronized events in WT explants and CNGA2 KO explants. As previously described (22), GnRH-1 neurons from WT explants exhibited synchronized calcium oscillations with a periodicity of about 20 min (17.2 ± 1.8 min; N = 3). In CNGA2 KO explants, the synchronization among GnRH-1 neurons was persistent, showing the same periodicity (17.0 ± 2.0 min; N = 4; Fig. 8, B and C; ANOVA, P > 0.05).

Discussion

In addition to intrinsic regulatory mechanisms, a variety of neurotransmitters modulate GnRH-1 neurons in vivo (2). Hypothalamic explants have increased our understanding of the complex regulation of GnRH-1 release (2), but the fine tuning of cellular mechanisms leading to GnRH-1 secretion have been detailed in the immortalized cell line GT-1 (6, 8, 50, 51). cAMP is a ubiquitous second messenger evoked by the binding of many neurotransmitters to their receptors. CNG channels have been localized to GnRH-1 neurons in vivo (2).
vivo (15), and based on GT-1 cell data, have been proposed as an integrator of the cAMP signals that modulate GnRH-1 neuronal activity as well as a constitutive mechanism for basal GnRH-1 oscillatory activity (52). The goal of the present work was to evaluate the role of CNG channels in the neuronal activity of native embryonic GnRH-1 neurons obtained from mouse nasal explants. In contrast to data from GT-1 cells (6), cAMP signal and CNG channels are not essential for basal or stimulated neuronal activity of GnRH-1 neurons in this model.

To determine whether modifying the intracellular level of cAMP would alter GnRH-1 neuronal activity, the specific activator of AC, FSK, was applied. GnRH-1 calcium oscillations were stimulated. These data are consistent with observations on hypothalamic tissue from 28-d-old rats (53, 54) and correlate with data from GT-1 cells in which calcium oscillations (6), calcium level (9), and GnRH-1 release (6, 10) are stimulated by FSK. Interestingly, hypothalamic fragments from rats older than 30 d no longer respond to FSK (55), leading the authors to suggest that the responsiveness to FSK might be a characteristic of prepubertal animals. One neurotransmitter thought to be a potential switch to GnRH-1 neurons at puberty is GABA (56, 57). GABAergic neurons, present in nasal explants (39), are a major excitatory input to GnRH-1 neurons (20, 22). To determine whether GnRH-1 neurons were responding directly to FSK or responding to FSK-stimulated excitatory input, application of FSK was done after a conditioning treatment with bicuculline methyl ether (BIC), a GABA_A receptor antagonist. Under these conditions, GnRH-1 neurons still responded to FSK with an increase of calcium oscillations, suggesting their ability to integrate cAMP signals directly.

FSK pharmacologically activates AC, preventing evaluation of the endogenous activity of AC. Thus, experiments were performed with IBMX, a nonspecific inhibitor of PDE, enzymes that hydrolyze cAMP. Application of IBMX increased GnRH-1 calcium oscillations. This observation is consistent with data from GT-1 cells, showing stimulation of GnRH-1 release in the presence of IBMX correlated with an increase of intracellular cAMP (11). To verify that cAMP was endogenously synthesized by GnRH-1 neurons, rather than by GABAergic neurons, similar experiments were performed after removal of GABAergic input. An increase in GnRH-1 neuronal activity persisted. These data suggest that PDE are an essential component for regulating the basal and/or the induced activity of GnRH-1 neurons. This observation corroborates data obtained from GT-1 cells on basal (11) or stimulated (58) GnRH-1 release. To determine whether AC endogenous activity, as revealed by IBMX, was important for basal GnRH-1 neuronal activity, experiments were performed in the presence of AC inhibitor DDA. Interestingly, even if GnRH-1 neurons were modulated by FSK-induced increases in cAMP, the removal of basal cAMP production did not alter the basal GnRH-1 neuronal activity. This is in contrast to data from developing spinal neurons (37) and removes cAMP from the potential pacemaking mechanisms in GnRH-1 neurons.

A role for CNG channels in cAMP-induced responses in GnRH-1 neurons has been suggested (6). The expression of the CNG channels pore-forming subunits was examined in cDNA from single GnRH-1 neurons. Only the olfactory subunit CNGA2 was found, consistent with the detection of the CNGA2 transcript in GT-1 cells (6) and the detection of the CNGA2 protein in adult rat GnRH-1 neurons (15). At the time when GnRH-1 neurons in nasal explants are known to show synchronized calcium oscillations and GnRH-1 release, the CNGA2 subunit was detected in many but not all of the GnRH-1 neurons sampled (40%). Similar numbers of GnRH-1 cells expressing ERβ (60%) were found in a previous study (28) and produced dramatic changes in GnRH-1 neuronal activity (59).

When tested in this study, the commonly used CNG channel blocker LCD altered sodium current properties, consistent with previous data (43, 60). This, together with the fact that specific blockers for CNGA2 channels are unavailable (41), led us to examine GnRH-1 neuronal activity in nasal explants obtained from CNGA2-deficient mice (26). The basal activity of GnRH-1 neurons was not altered by the absence of functional CNGA2 channels. GnRH-1 neurons from CNGA2-deficient mice still exhibited calcium oscillations with a synchronization interval of about 20 min as in controls. The ability of GnRH-1 neurons from CNGA2-deficient mice to respond to FSK remained unchanged. To ensure that the lack of CNGA2 channels did not alter other cell types such as the GABAergic neurons, BIC was applied before the application of FSK. Both the inhibition of GABAergic input and the subsequent stimulation by FSK remained unchanged in CNGA2-deficient GnRH-1 neurons. Up-regulation of other CNGA pore-forming subunits was examined in CNGA2-deficient GnRH-1 neurons. Although an early developmental compensatory mechanism cannot be excluded, the absence of CNGA1 and CNGA3 subunits in CNGA2-deficient GnRH-1 neurons strongly suggests that CNG channels are not necessary for either basal or stimulated GnRH-1 neuronal activity. In addition, a specific stimulation of CNG channels in WT nasal explants by Rp-8-Br-cGMPs failed to stimulate the majority of GnRH-1 cells when individually examined post hoc.

Consistent with the idea that CNG channels are not the integrator of FSK-induced cAMP signal, data from GT-1 cells have shown that an intracellular calcium increase induced by FSK is dependent on both extracellular sodium and calcium (9). This observation is inconsistent with the pure calcium selectivity of olfactory CNG channels at physiological extracellular calcium concentration (61). Moreover, in contrast with GT-1 cells in which a variety of neurotransmitters can induce changes in cAMP correlated with changes in GnRH-1 release (6, 8), data from hypothalamic explants are not as consistent. The norepinephrine-induced GnRH-1 release, attributed to the activation of β1-adrenoreceptors in GT-1 (6), is inhibited by β1-adrenoreceptor antagonists and inhibitors of nitric oxide synthase in hypothalamic explants as well as the subsequent increase in prostaglandin E2 (2). Finally, the dopamine-induced GnRH-1 release is inhibited by α1-adrenoceptor antagonists (62), and only hypothalamic explants from supplemented-castrated females released GnRH-1 in response to cAMP (63), neuropeptide Y (2), or serotonin (64). Differences between data from GT-1 cells and hypothalamic explants could result from species differences, the steroidal environment, cell subpopulations, and/or developmental
differences. GT-1 cells are derived from mouse and might retain some embryonic properties because they were isolated from a tumor at the rostral boundary of the optic chiasm (33). In contrast, hypothalamic explants are usually dissected from rats, which are, moreover, either prepubertal or mature, intact or gonadectomized with or without supplement, males or females (53, 63, 65–69). In addition, due to the scattered distribution of GnRH-1 neurons in the brain, hypothalamic explants are made from the preoptic area (70), mediobasal hypothalamus (69), or median eminence (66) regions, and these sections might bias GnRH-1 subpopulations involved in GnRH-1 pulses or GnRH-1 surges (71). GT-1 cells and GnRH-1 cells in mouse nasal explants exhibit many similarities including electrophysiological properties and possession of a wide variety of voltage- and ligand-gated ion channels (20). Thus, both cells exhibit characteristics of well differentiated neurons. However, data presented in this study contrast data obtained from GT-1 cells (6). One explanation for these differences could be the maintenance of interneuronal networks in nasal explants, even though minimized, which could modulate the observed response: GABAergic (22), cholecystokininergic (31), purinergic (72), and catecholaminergic (73). To date, all of the identified modulators in mouse nasal explants are also present during development in vivo and thus may shape the final characteristics of GnRH-1 neurons postnatally.

In summary, the studies in this paper indicate that basal or stimulated GnRH-1 neuronal activity is not simply driven by CNG channels. However, the response of GnRH-1 neurons to FSK-induced cAMP increases demonstrates the ability of GnRH-1 neurons to integrate cAMP signals and thus to be a component for modulation by neurotransmitters. Two potential pathways could be activated in a rise in cAMP. One could be activation of protein kinase A by cAMP (74), and protein kinase A-dependent phosphorylations are known to modulate the properties of voltage-activated channels involved in membrane excitability such as sodium channels, potassium channels, and calcium channels, all found in GnRH-1 neurons (2, 20). A second pathway could be directly linked to the rise of cAMP such as hyperpolarization-activated CNG-modulated cation (HCN) channels recently shown to be in GnRH-1 neurons in vivo (75). HCN channels, sensitive to cyclic nucleotides (76) and to neurotransmitters (77), have been shown to be involved in rhythmic activity, control of the membrane potential, and neuronal responsiveness (78). Whether voltage-activated channels or HCN channels, together with cAMP signals, are involved in basal neuronal activity and/or oscillatory activity of GnRH-1 neurons remains to be determined.

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