Decreased Expression of A-Kinase Anchoring Protein 150 in GT1 Neurons Decreases Neuron Excitability and Frequency of Intrinsic Gonadotropin-Releasing Hormone Pulses

Qiumei Chen, Richard I. Weiner, and Brigitte E. Blackman

Center for Reproductive Sciences, Department of Obstetrics, Gynecology, and Reproductive Sciences, University of California, San Francisco, School of Medicine, San Francisco, California 94143

The frequency of intrinsic pulsatile GnRH secretion from endogenous GnRH neurons and GT1 GnRH cell lines is stimulated by increased intracellular cAMP levels. The downstream molecules comprising the cAMP signaling pathway are organized in microdomains by a family of scaffolding proteins, A-kinase anchoring proteins (AKAPs). These molecules tether protein kinase A, cAMP-specific phosphodiesterases, phosphatases to known substrates. In neurons AKAP150 organizes many of the signaling molecules known to regulate the excitability and intrinsic pulsatile activity of GnRH neurons. AKAP150 was expressed in both the GT1-1 and GT1-7 cells. We determined the role of AKAP150 in coordinating GT1-1 cell excitability and intrinsic GnRH pulsatile secretion by lowering AKAP150 levels with a small interfering RNA (siRNA) adenovirus construct to AKAP150 (Ad-AKAP150-siRNA). Infection with Ad-AKAP150-siRNA specifically decreased AKAP150 mRNA levels by 74% and protein levels by 53% relative to uninfected cells or cells infected with a luciferase control adenovirus siRNA vector. In GT1 cells, spontaneous Ca\textsuperscript{2+} oscillations, an index of neuron excitability, are stimulated by increased levels of intracellular cAMP and lowered by decreased levels. The frequency of spontaneous Ca\textsuperscript{2+} oscillations in Ad-AKAP150-siRNA-treated GT1-1 cells decreased by 47.2% relative to controls. A dramatic decrease in the number of spontaneous GnRH pulses was also observed after infection with Ad-AKAP150-siRNA. The interpulse interval increased to 143 ± 20.25 min in Ad-AKAP150-siRNA infected cells from 32.2 ± 7.3 min in luciferase control adenovirus siRNA vector-infected cells. These data demonstrate an important role of AKAP150 in coordinating signaling events regulating the frequency of intrinsic pulsatile GnRH secretion. (Endocrinology 151: 281–290, 2010)
cAMP through the activation of AC (9). Pharmacologically increasing cAMP concentrations by treatment with forskolin or 8-Bromo-cAMP mimicked the stimulation of GnRH release by norepinephrine or dopamine.

GnRH neurons are spontaneously excitable as shown by the appearance of spontaneous Ca\textsuperscript{2+} oscillations seen in fura-2-loaded cells (10). Because each Ca\textsuperscript{2+} oscillation was preceded by an action potential, the frequency of Ca\textsuperscript{2+} oscillations is an index of neuron excitability. Increases in intracellular cAMP levels resulted in an increase in Ca\textsuperscript{2+} oscillations and neuron excitability (10). Genetically decreasing the intracellular cAMP concentration by the expression of a constitutively active cAMP-dependent phosphodiesterase (PDE4D1) lowered both the frequency of spontaneous Ca\textsuperscript{2+} oscillations and the frequency of intrinsic pulsatile GnRH release (11). The frequency of pulsatile GnRH release was also decreased in castrated GPR4 transgenic rats in which PDE4D1 expression was genetically targeted to GnRH neurons (12). cAMP gated Ca\textsuperscript{2+} channels (CNG) are a downstream component of the cAMP signaling pathway, regulating GT1 cell excitability and intrinsic GnRH pulse frequency. GT1 cells and rat GnRH neurons express all three subunits of the CNG channel, and GT1 cells have electrophysiologically characteristic CNG channels (13, 14). Decreasing CNG channel expression in GT1 cells by infection with an adenovirus expressing a specific CNG channel small interfering RNA (siRNA) resulted in a similar decrease in cell excitability and intrinsic GnRH pulse frequency as seen with overexpressing PDE4D1.

From a variety of studies, it is clear that the cAMP signaling events do not occur globally throughout cells. Rather signaling molecules are organized into microdomains within neurons by molecules that tether signaling molecules to relevant substrates. A large family of more than 50 A-kinase anchoring proteins (AKAPs) was identified. Multivalent AKAPs bring protein kinase A (PKA) along with other regulatory molecules in close molecular proximity to relevant substrates (for reviews see Refs. 15–18). The restriction of PKA to specific domains in the cell also permits the enzyme to be activated by locally fluctuating pools of cAMP. AKAP79/150 (79 human/150 rodent) is expressed predominantly in the brain and central nervous system. It tethers PKA to AC 5 and AC 6 (19), β2-adrenergic receptor (20–22), protein kinase C (PKC), α-amino-3-hydroxyl-5-methyl-4-isoxazole propionic acid receptor (23), KCNQ K\textsuperscript{+} channels (24), L-type Ca\textsuperscript{2+} channel (Cav1.2) (25, 26), and the calcium/calmodulin-dependent phosphatase (PP2B)/calcineurin (27, 28). In AKAP150 knockout mice, the localization of PKA holoenzyme was excluded from dendritic spines in hippocampal neurons (29). This was associated with a loss in bidirectional regulation of 2-amino-3-hydroxy-5-methyl-4-isoxazol propionic acid receptors in the hippocampus. Alterations in complex behaviors including memory retention, motor function and anxiety were observed in knockout mice consistent with AKAP150 playing an important role in the spatial and temporal organization of signaling molecules throughout the brain.

AC5 and AC6, CNG channels, and L-type Ca\textsuperscript{2+} channels were shown to be involved in cAMP signaling regulating GnRH secretion from GT1 cells. Therefore, AKAP150 was an excellent candidate for the spatial and temporal organization of the cAMP signaling pathways involved in regulating intrinsic GnRH pulsatility. We first show that AKAP150 is expressed in GT1 cells and that decreasing its expression with a specific siRNA resulted in decreased cell excitability and the frequency of spontaneous GnRH pulses.

### Materials and Methods

#### Cell culture

GT1-1 cells (passages 14–23) were cultured on 10-cm culture plates in DMEM (Invitrogen Corp., Carlsbad, CA) supplemented with 5% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT), 5% horse serum (HyClone Laboratories), 100 U/ml penicillin, and 100 µg/ml streptomycin.

#### Adenoviral siRNA constructs

Two pairs of oligos (AKAP150 no. 1 and AKAP150 no. 2) were synthesized from sequences corresponding to AKAP150 cDNA sequence (NP_001094941) (Table 1). These oligos were annealed and ligated into linearized pSIREN-Shuttle (CLONTECH, Palo Alto, CA). The negative control luciferase (Luc) siRNA supplied with the vector was also cloned into pSIREN-Shuttle. Both the AKAP150 siRNA (Ad-AKAP150-siRNA) and the Luc siRNA (Ad-Luc-siRNA) were transferred from the shuttle vector into pAdenoX (CLONTECH) using the recommended protocol. The recombinant adenoviral DNA was transfected into HEK 293

### Table 1. Sequences of the siRNA oligonucleotides designed to the mouse AKAP150 mRNA

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKAP150 no. 1F</td>
<td>gatccGGCAAAAGAGAAATGTGCATTTTCAAGAGAATGACGACTTCTCTGGCCTTTTTTTACGCCTGtg</td>
</tr>
<tr>
<td>AKAP150 no. 1R</td>
<td>aatgccACCGGTAATAAAAAGGGAAAGAGAAATGTGCATTTTCAAGAGAATGACGACTTTCTGGAATTAGACGACTCTCTTTCGcg</td>
</tr>
<tr>
<td>AKAP150 no. 2F</td>
<td>gatccGGCAAAAGAGAAATGTGCATTTTCAAGAGAATGACGACTTCTCTGGCCTTTTTTTACGCCTGtg</td>
</tr>
<tr>
<td>AKAP150 no. 2R</td>
<td>aatgccACCGGTAATAAAAAGGGAAAGAGAAATGTGCATTTTCAAGAGAATGACGACTTTCTGGAATTAGACGACTCTCTTTCGcg</td>
</tr>
</tbody>
</table>

Sequences corresponding to the AKAP150 mRNA are shown in bold.
cells to produce viral particles. Cell lysates were then used to reinfect HEK 293 cells for large-scale viral production. The virus was purified on two consecutive cesium chloride gradients, dila-
yzed in formulation buffer [2.5% glycerol, 25 mM NaCl, and
20 mM Tris-HCl (pH 8.0)] and titered using the adenoviral rapid
titer kit (CLONTECH). Viral titers were 2.8 × 10⁹ pfu/ml for
Ad-AKAP150-siRNA and 2.3 × 10⁹ pfu/ml for Ad-Luc-siRNA.

RT-PCR

GT1-1 and GT1-7 cells were grown on 10-cm dishes until
70% confluence. Total RNA was isolated using 1 ml Trizol (In-
vitrogen). Total RNA was isolated from mouse cortex. cDNA
was synthesized using 2 μg total RNA. Primer sequences were
as follows: forward, 5'-ATGGAGACCCAGTTTCTGA-3' and
reverse primer, 5'-GTGGTTTTTCTCTCTTG-3'. The expected
product size was 97 bp.

Real-time quantitative RT-PCR analysis

GT1-1 cells plated in six-well plates were infected with either
Ad-AKAP150-siRNA or Ad-Luc-siRNA at 5 multiplicity of
infection (MOI; the average number of viral particles per cell) were
washed with PBS and lysed with 0.5 ml Trizol (Invitrogen).
Total RNA was prepared according to the recommended pro-
tocol. cDNA was synthesized from 1 μg of total RNA using the
iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time
quantitative PCR was performed using SYBR Advantage
PCR mix (CLONTECH) with an iCycler thermal cycler (Bio-
Rad). We used the following primers: AKAP150 forward, 5'-
AGGATGGGGCTTCTCTAAAG-3' and reverse, 5'-GGGTC-
TGCGTTTATCTCC-3'; β-actin forward, 5'-GTCCACAC-
CCGCCACACTTT-3' and reverse, 5'-GACCCATTTCCA-
CCATCAC-3'. The data were collected and analyzed using
the comparative threshold cycle method using β-actin ex-
pression as the reference gene. Data were collected in triplicate
from three separate experiments analyzed using GraphPad Prism
(GraphPad, San Diego, CA). Statistically significant differences
were determined by t test.

Western blotting

COS-7 cells were plated into six-well plates at a density of 3 ×
10⁵ cells/well. Cells were transfected with 1 μg of AKAP150
cDNA into a mammalian expression vector using Lipofectamine
(Invitrogen). After 24 h the cells were transfected with 1 μg
pSiren-AKAP150 no. 1/no. 2 cDNA. Cells were incubated for
24 h and then washed twice in cold PBS and total cell lysate was
collected using membrane protein extraction kit containing a
complete protease inhibitor cocktail (Roche, Indianapolis, IN).
For Western blots on GT1-1 cells, cells were cultured on 10-cm
dishes and infected with 5 MOI Ad-AKAP150-siRNA or Ad-
Luc-siRNA. Protein was extracted 48 h after infection using the
membrane protein extraction kit containing a
Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN).

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Perfusion studies

GT1-1 cells (300,000 cells/well) were plated on Matrigel (BD
Biosciences, Discovery Labware, Franklin Lakes, NJ)-coated,
25-mm plastic coverslips (Thermo Fisher Scientific, Rochester, NY) in six-well plates. When the cells reached 50–60% confluency, they were infected with 5 MOI of Ad-AKAP150-siRNA or Ad-Luc-siRNA. Cells were incubated for 24 h after infection. The media were then replaced with Opti-
MEM (Invitrogen), and incubated for an additional 24 h before
sampling. The coverslips were placed in a modified Sykes-Moore
chamber (Bellco Glass, Vineland NJ), and the cells were
perfused with oxygenated Locke’s medium (154 mM NaCl, 5.6
mM KCl, 2.2 mM CaCl₂, 1 mM MgCl₂, 6 mM NaHCO₃, 10
mM glucose, 2 mM HEPES) supplemented with 0.1% BSA and 20 µM
bacitracin at a flow rate of 70–100 µl/min. Chambers were
washed for 60 min and then samples were collected every 2 min
for 3 h. Each sample was boiled for 3 min cooled on ice and then
stored at −80 C for RIA.

GnRH RIA

Levels of GnRH in the media from the perfusion studies were
determined by a RIA using rabbit antibody R1245 (obtained from T.
Nett, College of Veterinary Medicine and Biomedical
Sciences, Colorado State University, Fort Collins, CO). This
antiserum was specific for intact GnRH. Synthetic human GnRH
(Sigma, St. Louis, MO) was used for the standard and 125I-
[β-Tryp]GnRH was purchased from PerkinElmer (Norwalk, CT).
All samples from an experiment were analyzed in the same assay.
The limit of detection of the assay was 0.25–25 pg/tube, and
the intraassay coefficient of variation was 7.2%. The limit of detec-
tion of the assay was defined as 90% of maximal binding. Analy-
ysis of the GnRH pulse data was performed using the hormone
pulse analysis software Cluster 8 (Michael L. Johnson,
University of Virginia, Charlottesville, VA). Cluster analysis was
performed on measurements done in singlicate. The coefficient of
variation was determined from intraassay controls. Cluster size
or nadir was defined by two points that significantly increased or
decreased with a t statistic of 4.

Ca²⁺ assays

Intracellular Ca²⁺ concentration was measured using fluores-
cence ratio imaging with MetaFluor imaging software (Universal
Imaging Corp., Westchester, PA) as previously described (30).
Briefly, GT1-1 cells cultured on Matrigel-coated, 25-mm glass
coverslips were loaded with fura-2 (Molecular Probes, Eugene,
OR) by incubation in 5 µM fura-2-AM for 30 min at 37 C in
oxygenated Locke’s medium supplemented with 0.1% BSA.
The cells were then washed in fresh Locke’s medium for 15 min.
Coverslips were placed in a temperature-controlled modified
Sykes-Moore chamber mounted on a TE2000 inverted fluores-
cence microscope (Nikon, Tokyo, Japan). Cells were per-
fused with Locke’s medium or Locke’s medium containing 10
µM forskolin at a flow rate of 50 µl/min. Fura-2 fluorescence
at 510 nm was measured at five second intervals for 18 min
and a further 2 min after the addition of forskolin at excita-
tion settings of 340 and 380 nm. Approximately 40–60 cells/cov-
erslip were imaged and four coverslips were studied for the
control GT1-1 cells as well as the Ad-AKAP150-siRNA- or
Ad-Luc-siRNA-infected cells.
Quantification of Ca\textsuperscript{2+} concentration and Ca\textsuperscript{2+} oscillations

To distinguish living cells from dead cells, only cells that showed a 20% increase in Ca\textsuperscript{2+} concentration after treatment with forskolin for 5 min were used for analysis. The intracellular Ca\textsuperscript{2+} concentration was estimated from the ratio of the fluorescence intensities and comparison with fura-2 standards using the fura-2 Ca\textsuperscript{2+} calibration kit (Invitrogen) (31). These values were plotted in Raster plots using Transform (Fortner Software, Sterling, VA). Ca\textsuperscript{2+} oscillations were quantified using JPULSAR\textsuperscript{1} (written by Dr. Xiaoyu Pan, Department of Molecular and Cellular Biology, University of California, Davis, CA), a program modified from PULSAR (32), to detect peaks in intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) oscillations. The SD was determined by using the mean of the SDs identified by time series analysis using a 5-sec window. The coefficient of variation for calcium imaging was described by the formula: $Y = 4.1827X + 0.4826$. The peak detection parameters for JPULSAR, G(1)–G(5), were 1.77, 1.05, 0.73, 0.53, and 0.39. A smoothing window of 25 sec was used to determine segmented baseline values.

Results

Expression of AKAP150 in GT1-1 and GT1-7 GnRH neurons

AKAP150 expression was shown to be high in the forebrain regions including the cerebral cortex (33). We determined whether AKAP150 was expressed in GnRH neurons using RT-PCR. Specific primers designed to the AKAP150 cDNA sequences yielded a single predicted band of 97 bp with mRNA from both GT1-1 and GT1-7 cells (Fig. 1A). An identical size band was observed with mRNA extracted from mouse cerebral cortex. A strong fluorescence signal associated with the plasma membrane was observed in GT1 cells transfected with AKAP150-red fluorescent protein (RFP) (Fig. 1C).

Development of AKAP150 siRNA

We designed two siRNAs specific to the mouse AKAP150 cDNA (AKAP150 no. 1; AKAP150 no. 2; Table 1). Before cloning these oligonucleotides into the Adenovirus expression vector, we tested their ability to knockdown expression of AKAP150-RFP cDNA. We performed these experiments in COS cells transiently expressing the AKAP150 cDNA. The oligonucleotides were cloned into pSIREN-Shuttle and expressed in COS cells transiently cotransfected with a AKAP150 expression construct. Western blot analysis showed that both AKAP150 no. 1 and AKAP150 no. 2 were equally effective in decreasing the expression of the AKAP150 cDNA (Fig. 1B). Because only a small portion of GT1 cells are transiently transfected by available techniques, for subsequent experiments in GT1 cells, we developed an adenovirus vector to direct expression of the AKAP150 no. 1 siRNA. The AKAP150 no. 1 siRNA (Ad-AKAP150-siRNA) as well as the control luciferase siRNA (Ad-Luc-siRNA) were cloned into an adenovirus vector for all further studies.

Inhibition of AKAP150 mRNA and protein by Ad-AKAP150-siRNA treatment

We measured AKAP150 mRNA levels in GT1-1 cells 48 h after infection by real-time quantitative RT-PCR. At 5 MOI, Ad-AKAP150-siRNA caused a 74% average decrease in mRNA levels of AKAP150 relative to uninfected cells (Fig. 2).

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FIG. 1. Specific primers were designed to the sequence of AKAP150 and used in RT-PCR to determine expression (A). PCR on cDNA from GT1-1, GT1-7 cells, and mouse cortex show expression of AKAP150. B, Whole-cell extracts from untransfected COS 7 cells or cells transiently transfected with AKAP150-RFP cDNA alone or with the control luciferase (Luc) siRNA, AKAP150 siRNA no. 1, or AKAP150 siRNA no. 2, respectively, were immunoblotted with anti-AKAP150 antibody. Both AKAP-siRNA no. 1 and AKAP150-siRNA no. 2 but not Luc-siRNA inhibited the expression of AKAP150 in transiently transfected COS 7 cells. WB, Western blot; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. C, AKAP150-RFP transfected into GT1-1 cells is expressed at the plasma membrane.

FIG. 2. Quantitative RT-PCR showed that Ad-AKAP150-siRNA decreased AKAP150 mRNA expression by approximately 74%. GT1-1 cells were infected with Ad-AKAP150-siRNA (5 MOI) or Ad-Luc-siRNA (5 MOI) and RNA was isolated 48 h after infection. The data were analyzed using the comparative threshold cycle (Ct) method with $\beta$-actin as the reference gene. The data, expressed as a ratio of the control AKAP150 mRNA in uninfected cells, represent the mean ± SEM from three separate experiments.
Infection with 5 MOI of Ad-Luc-siRNA. The Ad-AKAP150-siRNA appeared specific because no affect was observed on the mRNA levels of \( \beta \)-actin or GnRH (data not shown). All subsequent experiments were done with 5 MOI of the siRNA vectors because infection of GT1 cells with a MOI above 5 was shown to have a nonspecific effect on spontaneous GnRH pulses and Ca\(^{2+} \) oscillations (11). Furthermore, infection with 5 MOI of the same adenovirus vector expressing green fluorescent protein (GFP) resulted in GFP expression in 90–95% of GT1 cells (data not shown).

A specific band of approximately 150 kDa, corresponding to the AKAP150 protein, was observed in immunoblots of membrane protein extracted from GT1-1 cells (Fig. 3A). The blots were quantified and band intensity was normalized for loading using an antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In agreement with findings in transfected COS cells, a 54% decrease in the intensity of the AKAP150 band relative to the uninfected GT1-1 cells was observed after infection with 5 MOI of the Ad-AKAP150-siRNA relative to uninfected controls (Fig. 3B). Infection with 5 MOI of Ad-Luc-siRNA had no effect on AKAP protein levels.

**Effect of Ad-AKAP150-siRNA infection on frequency of Ca\(^{2+} \) oscillations**

We then asked whether lowering the levels of AKAP150 expression in GT1-1 cells would affect the occurrence of spontaneous Ca\(^{2+} \) oscillations. Calcium was measured using fura-2 imaging in living GT1-1 cells by fluorescence microscopy (Fig. 4). In untreated, fura-2-labeled cells spontaneous Ca\(^{2+} \) oscillations were observed with a similar frequency to those previously reported (10). Quantitation of the Ca\(^{2+} \) oscillations showed a significant 47.2% decrease (from 4.4 to 2.3 oscillations per 18 min experiment, \( P < 0.0001 \)) in GT1-1 cells infected with the Ad-AKAP150-siRNA relative to the uninfected cells (Fig. 5A). No difference was observed in the Ad-Luc-siRNA infected cells (Fig. 5A). Consistent with these findings, the interpulse interval increased more than 2-fold in Ad-AKAP150-siRNA-treated cells relative to the uninfected or Ad-Luc-siRNA infected cells (Fig. 5B).
or cells infected with the Ad-AKAP150-siRNA or Ad-Luc-siRNA were perifused and samples obtained every 2 min for 3 h for measurement of GnRH by RIA. A significant decrease in the number of spontaneous GnRH pulses per collection period was observed after infection with Ad-AKAP150-siRNA, whereas no difference was seen between uninfected cells and the Ad-Luc-siRNA infected cells (Table 2). The interpulse interval was significantly increased ($P < 0.003$) by infection with the Ad-AKAP150-siRNA ($143.20 \pm 20.25$ min) vs. uninfected ($32.2 \pm 7.3$ min) or Ad-Luc-siRNA infected cells ($32.7 \pm 5.2$ min). Interestingly, no significant change in the pulse amplitude was observed (Fig. 6 and Table 2).

**Discussion**

Substantial data exist that the level of cAMP in GT1 cells regulates spontaneous cell excitability and the frequency of intrinsic GnRH pulses (11, 14). GT1 GnRH neurons express several different isoforms of adenylyl cyclase including predominantly the Ca$^{2+}$ insensitive forms of ACs 2, 4, and 7, the Ca$^{2+}$ sensitive forms, ACs 3, 5, 6, and 9 (negatively regulated by Ca$^{2+}$), and AC 1 an isoform activated by Ca$^{2+}$ (9, 34, 35). Synthesis of cAMP in response to both intracellular Ca$^{2+}$ concentrations (34) and feedback via PKA inhibition of AC 5/6 (9, 36) suggest fine tuning of cAMP concentrations as a focal point for the regulation of GnRH secretion. Work by several groups has shown that small local changes in cAMP concentrations or even possibly concentration gradients of cAMP may account for the molecular specificity of cAMP signaling, emphasizing the importance of subcellular organization (for review, see Ref. 37). Increases in cAMP can initiate downstream signaling events by directly binding to target proteins, e.g. the PKA regulatory subunit, CNG channels (38) and exchange proteins directly activated by cAMP (39) or indirectly by alterations in the activity of signaling molecules after phosphorylation by activated PKA, e.g. Cav1.2 channel. The downstream signaling events mediating the effects of cAMP on cell excitability and the initiation and

**TABLE 2.** Summary of GnRH pulse parameters determined by cluster analysis

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Ad-Luc-siRNA</th>
<th>Ad-AKAP150-siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of pulses/experiment</td>
<td>$3.2 \pm 0.5$ (n = 7)</td>
<td>$3.3 \pm 0.5$ (n = 7)</td>
<td>$1 \pm 0.3$ $a,b$ (n = 7)</td>
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<tr>
<td>Interpulse interval (min)</td>
<td>$32.2 \pm 7.3$ (n = 22)</td>
<td>$32.7 \pm 5.2$ (n = 23)</td>
<td>$143 \pm 20.25$ $c,d$ (n = 7)</td>
</tr>
<tr>
<td>Pulse amplitude (percent increase)</td>
<td>$184.6 \pm 26.58$ (n = 22)</td>
<td>$194.3 \pm 37.08$ (n = 23)</td>
<td>$142.5 \pm 6.6$ (n = 7)</td>
</tr>
</tbody>
</table>

The values are mean ± SEM.

$^a P = 0.0034$ relative to wild type (WT); $^b P = 0.0068$ relative to Ad-Luc-siRNA; $^c P = 0.0037$ relative to WT; $^d P = 0.0017$ relative to Ad-Luc-siRNA; $^e P = ns$ relative to WT and Ad-Luc-siRNA.
Ad-AKAP150-siRNA decreased spontaneous Ca\(_{\text{m}}\) mRNA by 74% and protein by 53% by infection with the plasma membrane. The lowering of the levels of AKAP150 cells, dense fluorescence was observed associated with the cells. When RFP-labeled AKAP150 was expressed in GT1 timing the frequency of GnRH pulses. AKAP binding provides potential feedback mechanisms for the AKAP/cAMP/PKA and PP2B in close proximity to the Cav1.2 channel, effects of increased cAMP levels and phosphorylation of target proteins by PKA are regulated by PDE activity and protein phosphatases, respectively.

The purpose of the current study was to better understand how cAMP signaling events are spatially and temporally organized to result in spontaneous GnRH pulses with a frequency of 24 min. In muscle and the heart, it is clear that the cAMP signaling pathways are highly organized into microdomains by the sequestration of signaling molecules. The best understood of the tethering molecules maintaining the subcellular distribution and temporal organization of signaling molecules are the AKAP proteins. We hypothesized that the AKAP79/150 (79 human/150 rodent) scaffolding protein, localized to the plasma membrane via a polybasic region (40) with a high expression in neurons (41, 42) could provide a mechanism for organizing these molecules in relevant microdomains within GT1 cells. AKAP79/150 binds protein PP2B/calcineurin (28) and PKC (43) in addition to PKA. By organizing molecules that bivalently regulate signaling events, e.g. bringing both PKA and PP2B in close proximity to the Cav1.2 channel, AKAP binding provides potential feedback mechanisms timing the frequency of GnRH pulses.

AKAP150 mRNA and protein were present in GT1 cells. When RFP-labeled AKAP150 was expressed in GT1 cells, dense fluorescence was observed associated with the plasma membrane. The lowering of the levels of AKAP150 mRNA by 74% and protein by 53% by infection with the Ad-AKAP150-siRNA decreased spontaneous Ca\(^{2+}\) oscillations by 71% and dramatically decreased the number of spontaneous GnRH pulses (increased the interpulse frequency of GnRH pulses). No effect was observed on the amplitude of spontaneous GnRH pulses. We hypothesized that the decrease in neuron excitability and spontaneous GnRH pulsatility was a result of the disruption of the organization of signaling molecules known to be tethered to AKAP150 possibly including PKA, PKC, and PP2B.

Performing these experiments depended on the ability of expressing specific siRNAs that would sufficiently lower AKAP150 expression to alter cell function. We demonstrated that the siRNA used in the experiments dramatically lowered the level of AKAP mRNA and protein levels in transfected COS cells and in GT1 cells. No nonspecific effects were seen on nonrelated gene expression, i.e. β-actin or GnRH.

A second key requirement for success of using siRNA expression in GT1 cells was the ability to direct expression to the majority of GT1 cells in the cultures. We had previously used adenovirus vectors to direct expression of PDE4D1 to GT1 cells (11). In these studies as well as the current studies, as long as the viral load was not raised above 5 MOI, little if any nonspecific effect of viral infection was observed. As a further control, the same viral vector-expressing luciferase was used to infect cells at 5 MOI. In this way we controlled for both viral infection and effects of expressing a siRNA. No effects on the frequency of Ca\(^{2+}\) oscillations or GnRH pulses were observed after infection with 5 MOI of Ad-Luc-siRNA relative to uninfected cells. Infection of GT1 cells with 5 MOI of an adenovirus vector expressing GFP resulted in observable green fluorescence in greater than 90% of the cells.

The decrease in cell excitability and frequency of spontaneous GnRH pulses in GT1 cells resulting from the siRNA-induced decrease in AKAP150 expression was similar to that seen by lowering cAMP levels by expression of PDE4D1 or lowering CNG channel expression using a siRNA (11, 44). Also, the lack of effect on GnRH pulse amplitude of lowering AKAP150 expression was observed after the over expression of PDE4D1 and lowering CNG channel expression. These findings are consistent with the hypothesis that all three treatments involved decreased cAMP signaling at the plasma membrane resulting in decreased cell excitability. Potential mechanisms that could be involved include the integral membrane proteins CNG channels, AC 5/6, Cav1.2 channels, the inwardly rectifying potassium channel (Kir2.1/KCNJ2), β1-adrenergic receptor and PKA, PKC, and PP2B tethered to these molecules by AKAP150.

Binding of cAMP to the CNG channel subunits opens the channel allowing cations that increase the depolarization drive to enter the neurons (14). The magnitude of the cAMP-regulated conductance through the CNG channels is not sufficient to trigger an action potential. Instead the

**FIG. 6.** Spontaneous GnRH release in uninfected GT1-1 neurons (A), neurons infected for 48 h with 5 MOI of Ad-Luc-siRNA (B), or 5 MOI of Ad-AKAP150-siRNA (C). Samples were obtained every 2 min for 180 min from perfused cells. Data are shown for three of seven experiments for each treatment. GnRH levels were measured by RIA and data were analyzed for pulsatile secretion using cluster analysis (*, pulse). IPI, Interpulse interval.
increase in the resting potential presumably leads to the activation of inwardly rectified K⁺ channels that are responsible for triggering the action potentials in GT1 cells (45, 46). By decreasing the number of CNG channels by infection with the Ad-CNG-siRNA, the cAMP levels in the neuron presumably took longer to increase the resting potential sufficiently to activate the inwardly rectified K⁺ channels. This resulted in fewer Ca²⁺ oscillations, i.e., action potentials. This decrease in spontaneous neuron excitability resulted in a decrease in the frequency of spontaneous GnRH pulses (44). The expression of PDE4D1 lowers intracellular cAMP levels and decreases cell excitability consistent with the hypothesis that the changes are mediated in part through decreases in cation conductance through CNG channels. How AKAP150 would directly affect CNG channel activity is not clear because, however, the indirect effect of decreased localized concentrations of cAMP by disruption of AKAP/AC complexes for example could account for the decrease in neuron excitability.

Oscillatory increases in intracellular Ca²⁺ are generated in GT1 cells by the influx of Ca²⁺ through voltage-gated Ca²⁺ channels (4, 10). The enhancement of Cav1.2 channel activity was bidirectionally regulated in cultured hippocampal neurons by PKA and PP2B tethered to the channel by AKAP150 (26). Both the activation by PKA phosphorylation and inactivation by dephosphorylation by PP2B required expression of AKAP150. Interestingly, anchored PP2B dominantly suppressed enhancement by PKA.

AKAP150 mediated feedback and potentially bidirectional regulation is the inactivation/activation of AC 5 and AC 6 activity by AKAP150-tethered PKA (19). When cAMP levels are locally increased by increased AC 5/6 activity, tethered PKA is activated resulting in phosphorylation and decreased cAMP formation. Phosphorylation of AC 5/6 by tethered PKA would be reversed by the phosphatase PP2B. Additionally, PDEs constitute another potential bidirectional feedback mechanism for regulating cAMP levels in discrete compartments. Both the Ca²⁺/calmodulin-sensitive PDE1B and the PKA-regulated PDE4D (specifically PDE4D3) and PDE4B subtypes are expressed in GnRH neurons (47). Phosphorylation of PDE4D3 by PKA activates the enzymes ability to hydrolyze cAMP. To date PDE subtypes have not been shown to bind to AKAP79/150; however, PDE4D3 has been shown to form a complex with Yotiao (AKAP9) (48) a membrane-associated AKAP expressed in brain (49, 50); the presence of this complex in GnRH neurons has not yet been investigated. In GT1 cells treatment with the PKA inhibitor H89 increased intracellular cAMP concentrations, possibly via the removal of PDE4D3 activation, and the loss of AC 5/6 down-regulation ultimately led to an increased GnRH secretion (9). However, because H89 is known to have several nonspecific targets, these results do not conclusively prove that PKA is involved only in GnRH secretion via the regulation of negative feedback.

AKAP79/150 has also been shown to interact with the carboxy terminus of β1-adrenergic receptor (21, 51), promoting receptor resensitization and recycling. The β1-adrenergic receptor is expressed in GnRH neurons and plays a role in GnRH secretion (7). Another potential target is the inwardly rectifying potassium channel Kir2.1 (KCNJ2), which is shown to have an enhanced response to elevated intracellular cAMP when complexed to AKAP79 (52).

In GT1 cells these feedback mechanisms could result in the waxing and waning of cAMP levels in the vicinity of the plasma membrane constituting a timing mechanism for cell excitability and GnRH pulsatility. The cycling of cAMP levels is a known timing mechanism for reproduction in the slime mold (53) and was proposed as a mechanism in timing the rate of contraction of the heart (54). To date direct measurements of cAMP levels in GT1 GnRH neurons have not been achieved. However, global fluctuations of cAMP concentrations in GT1 cells may not reflect the precision of localized concentrations of cAMP mediated through the assembly of macromolecular complexes involving AKAP150. Currently available cAMP sensors facilitating the measurement of cAMP concentrations in specific microdomains may be more informative (for review see Ref. 55).

No changes in the magnitude of spontaneous GnRH pulses was consistently observed in GT1 cells after decreased AKAP150 and CNG channel expression and increased PDE4D1 expression. Clearly large global pharmacologically induced increases in cAMP levels induced by forskolin or 8-bromoadenosine-cAMP caused dramatic increases in GnRH release from GT1 cells (56). The physiological relevance of pharmacological induced changes have frequently been questioned, but the emergence of data on the importance of the organization of signaling complexes in discrete domains further challenges the relevance of these findings. Spontaneous GnRH pulses are likely to be regulated by highly organized signaling complexes within discrete intracellular domains, a hypothesis consistent with the findings of this paper. The magnitude of pulses may be restricted by the participation of only small populations of signaling molecules organized within these signaling complexes.

We conclude that AKAP150 plays a significant role in organizing cAMP signaling complexes involved in regulating GT1 cell excitability and the frequency of intrinsic pulsatile GnRH secretion. Additional experiments addressing the dynamics of bidirectional regulation of cAMP...
signaling molecules are necessary to draw further conclusions. These results also raise numerous questions on the organization of additional signaling pathways involved. Understanding the spatial and temporal organization of signaling complexes will vastly increase our understanding of the regulation of intrinsic pulsatile secretion.

Acknowledgments

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References

42. Sarkar D, Erlichman J, Rubin CS 1984 Identification of a calmodulin-binding protein that co-purifies with the regulatory subunit of brain protein kinase II. J Biol Chem 259:9840–9846
46. Costantin JL, Charles AC 2001 Modulation of Ca(2+) signaling by K(+) channels in a hypothalamic neuronal cell line (GT1-1). J Neurophysiol 85:295–304
54. Sculptoreanu A, Rotman E, Takahashi M, Scheuer T, Catterall WA 1993 Voltage-dependent potentiation of the activity of cardiac L-type calcium channel α1L subunits due to phosphorylation by cAMP-dependent protein kinase. Proc Natl Acad Sci USA 90:10135–10139