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Recruitment of Individually (All-or-None) Responding Cells, Rather than Amplitude Enhancement, Is the Single-Cell Mechanism Subserving the Dose–Responsive Activation of Intracellular Calcium Second Messenger Signaling by the Human Luteinizing-Hormone Receptor

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We have investigated at the single-cell level how the human LH receptor mediates a dose–responsive increase in intracellular free calcium-ion concentrations ($[Ca^{2+}]_i$). In human embryonic kidney cells (293 cells) stably transfected with the full-length human LH receptor cDNA. Intact dimeric LH, but not LH β- or α-subunits, evoked specific $[Ca^{2+}]_i$ signals. High-resolution fluorescence (fura-2) video-microscopy demonstrated cell-to-cell variability in $[Ca^{2+}]_i$ signaling responses in individual cells, viz., an all-or-none spike (9%), spike-and-plateau (25%), or plateau (52%) types of temporal signal. Oscillatory $[Ca^{2+}]_i$ responses were observed in 12–14% of LH-stimulated cells unrelated to LH concentration. The LH dose–response originated by higher concentrations of LH recruiting more individually responding cells (rather than altering $[Ca^{2+}]_i$ signal amplitude), and eliciting a $[Ca^{2+}]_i$ rise more rapidly, i.e., at reduced latency. Cobalt did not abolish the LH-stimulated $[Ca^{2+}]_i$ spike-and-plateau response, but decreased the percentage of cells with a plateau pattern. Quench experiments demonstrated influx of Mn^{2+} following the $[Ca^{2+}]_i$ spike, thus directly documenting divalent cation inflow during the plateau phase. Adenylyl-cyclase activation with forskolin or treatment with a cAMP analog failed to elicit the biphasic $[Ca^{2+}]_i$ response, and pertussis toxin (PTX) did not alter LH-stimulated $[Ca^{2+}]_i$ signaling. However, overnight preincubation with LH reduced the percentage of $[Ca^{2+}]_i$-responding cells following re-exposure to LH to 5.7% (vs 72% in control), suggesting LH-induced desensitization of the LH-receptor directed $[Ca^{2+}]_i$ signal.

In summary, the present studies of human LH receptor signal transduction at the single-cell level show that increasing concentrations of LH achieve a dose-dependent intracellular Ca^{2+} signaling response by recruiting an increasing number of $[Ca^{2+}]_i$-responding cells, while concomitantly decreasing the temporal latency of the biphasic $[Ca^{2+}]_i$ signal without altering the amplitude of its spike phase. Prolonged exposure to LH appears to desensitize the LH receptor-driven $[Ca^{2+}]_i$ signal.

Key Words: Gonadotrophin; receptor; LH; signaling; calcium.

Introduction

The LH receptor belongs to a family of G protein-coupled receptors with seven putative transmembrane-spanning domains (reviewed in *1*). Activation of the LH receptor induces dual second messenger signals that include the cAMP–protein kinase A (PKA) pathway and an effector system presumptively linked to stimulation of phospholipase C *(1–3)*. The LH receptor in the ovary is expressed in both theca and well-differentiated granulosa-luteal cells, and participates at least via the well-recognized cAMP–PKA pathway in ovulation and luteinization of the maturing Graafian follicle with a resultant induction of specific steroidogenic genes.

Since ovarian granulosa and luteal cells show diverse size, morphology, and cytodifferentiation, heterogeneous $[Ca^{2+}]_i$ signals among different individual gonadal cells may be owing to variable cell types rather than pleiotropic transduction by the LH receptor. In addition, availability of human gonadal tissue is limited for experimental use, thus making difficult detailed studies of signaling by the human LH receptor. Accordingly, both to provide a more homogeneous cell population of LH-responsive cells and to allow a more tractable experimental model of LH-induced $[Ca^{2+}]_i$

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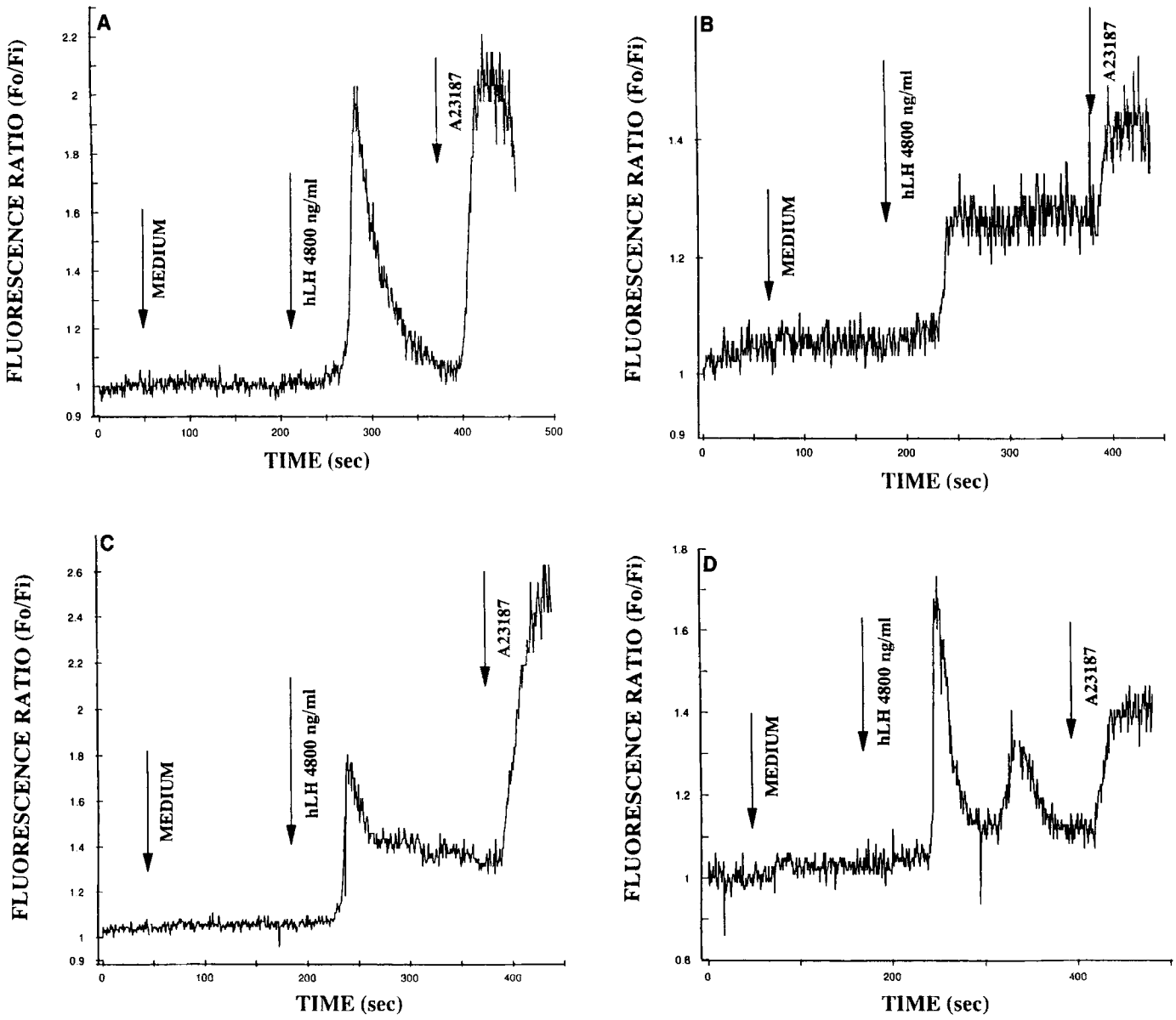

Fig. 1. Serial fura-2 emitted fluorescence intensity ratios (F_o/F_i) are depicted for illustrative single 293 cells stably transfected with the full-length human LH receptor cDNA. Data are successive (100–200 ms) intracellular calcium ion concentration ($[Ca^{2+}]_i$) changes in individual 293 cells stimulated with hLH (4800 ng/mL): **(A)** solitary spike pattern, as observed in 9% of cells studied; **(B)** plateau pattern, as identified in 52% of cells; **(C)** spike-and-plateau pattern, as recognized in 25% of cells; and **(D)** oscillatory pattern, as seen in 12% of cells. At the end of each experiment, the calcium ionophore A23187 was administered to test cell responsiveness.

signaling, in the present investigation we took advantage of clonal human embryonic kidney (293) cells stably transfected with the full-length human LH receptor cDNA *(4)*. In addition, we applied the technology of single-cell $[Ca^{2+}]_i$ signal monitoring in order to examine the mechanisms of the LH dose–response on this second messenger signaling pathway at the single-cell level without the confounding influences of cell population responses *(5,6)*. These studies in single clonal human LH receptor-bearing cells show for the first time that higher concentrations of LH achieve a dose–response in $[Ca^{2+}]_i$ signaling by recruiting an increased number of individually responding cells generating an all-or-none biphasic $[Ca^{2+}]_i$ signal, which occurs at decreased latency.

Results

LH Dose–Response Studies

In studies including 35 experiments, 20 batches of cells, and over 350 monitored cells, purified human LH at a concentration of 4800 ng/mL evoked intracellular calcium ($[Ca^{2+}]_i$) responses in approx 82% of cells. The $[Ca^{2+}]_i$ responses included a spike (9.1%), spike-and-plateau (25%), and plateau pattern (52%). LH at 480 ng/mL elicited spike (24%), spike-and-plateau (24%), or plateau patterns (40%), each of which lasted about 5–10 min. Oscillatory responses were observed in some cells (12–14%), and could not be related consistently to the LH concentration (Fig. 1). A slightly increased number of oscillating cells was found

Table 1
Effects of Different Concentrations of hLH on LH-Induced $[Ca^{2+}]_i$ Responses in 293 Cells Transfected with hLH Receptor cDNA

	LH (4800 ng/mL)	LH (480 ng/mL)	*P* value
Total number of cells studied	61	63	NA
Number of responding cells	44 (72.1%)	25 (39.7%)	$P < 0.0005$
Number of nonresponding cells	17 (27.9%)	38 (60.3%)	$P = 0.0005$
Maximal $[Ca^{2+}]_i$ increase from basal (fold)	1.48 ± 0.04	1.48 ± 0.04	NS
Time required to achieve half-max. value (sec)	45 ± 2.5	99 ± 8.2	$P = 0.0001$

Data are expressed as mean ± SEM numbers of cells (percentages) and collected from four individual experiments. NS = not significant, NA = not applicable. *P* values derive from chi-square and Wilcoxon tests to evaluate the null hypothesis of equal numbers and times of responding cells, respectively.

Table 2
Effects of Cobalt Chloride (4 m*M*) on hLH-Induced $[Ca^{2+}]_i$ Responses in 293 Cells Transfected with hLH Receptor cDNA

	LH	LH with Co	*P* value
Total number of cells studied	53	57	NA
Number of responding cells	40 (75.5%)	42 (73.7%)	NS
Number of nonresponding cells	13 (24.5%)	15 (26.3%)	NS
Maximal $[Ca^{2+}]_i$ increase from basal (fold)	1.56 ± 0.04	1.88 ± 0.08	$P = 0.0005$
Time required to achieve half-max. value (sec)	45 ± 2.8	64 ± 5.4	$P = 0.0033$
Spike	4 (10%)	17 (41%)	$P < 0.05$
Spike-plateau	10 (25%)	7 (17%)	NS
Plateau	21 (53%)	9 (21%)	$P < 0.05$
Oscillation	5 (13%)	9 (21%)	NS

Data are expressed as mean ± SEM and collected from three individual experiments. NS = not significant, NA = not applicable. Comparisons were performed statistically as defined in the legend of Table 1. The LH concentration used here was 4800 ng/mL.

after calcium influx was blocked with cobalt chloride. The lowest LH concentration of 48 ng/mL was unable to evoke $[Ca^{2+}]_i$ responses in single cells ($N = 32$).

The magnitude of the $[Ca^{2+}]_i$ peak did not depend on the LH concentration (Table 1). The concentration of LH of 4800 ng/mL elicited the same maximal $[Ca^{2+}]_i$ rise over baseline (mean 1.48-fold) as 480 ng/mL. However, higher LH concentrations recruited more responding cells. The higher LH concentration also elicited a $[Ca^{2+}]_i$ rise more rapidly than the lower concentration. In paired experiments, the time required to achieve a half-maximal value of $[Ca^{2+}]_i$ after the higher dose of LH was 44 ± 2.5 s compared to 99 ± 8.2 s after the lower dose ($p < 0.005$).

Dependence on Extracellular Calcium of LH-Induced Intracellular Calcium Rise

To test if the LH-induced $[Ca^{2+}]_i$ rise depends on extracellular calcium, cobalt chloride was added with LH (Table 2). To this end, the cells were first loaded in Cunningham chambers with medium. After the basal level of $[Ca^{2+}]_i$ was imaged, medium with cobalt chloride (4 m*M*) was infused into the cell chamber, and further imaging was performed 10 min later. Thereafter, hLH (4800 ng/mL) with cobalt chloride (4 m*M*) in medium was delivered. Cobalt chloride, which blocks divalent cation uptake nonselectively, induced a slightly higher (1.88 ± 0.8 vs 1.56 ± 0.04-fold, $P < 0.005$) and temporally delayed (64 ± 5.4 vs 45 ± 2.9 s, $p < 0.005$) $[Ca^{2+}]_i$ peak, and significantly increased the percentage of spike patterns alone (from 10–41%, $P < 0.005$) (Fig. 2). Cobalt decreased the plateau response from 53% to 21% ($P < 0.05$).

Effects of Pertussis Toxin Pretreatment on the LH-Induced Intracellular Calcium Rise

To test if the LH-induced $[Ca^{2+}]_i$ rise is mediated via a pertussis-toxin- (PTX) sensitive G-protein(s), 293 cells were pretreated with PTX (500 ng/mL) for 12 h. This dose has been shown to result in ADP-ribosylation of a 41-kDa protein in ovarian cells *(7)*. On the day of the experiment, the cells were kept in medium with PTX (500 ng/mL). Cell viability was no different, as assessed by vital-dye exclusion in both control and PTX-treated cells. Compared to control cells, PTX-exposed cells showed an LH-induced $[Ca^{2+}]_i$ rise that was no different with respect to either the $[Ca^{2+}]_i$ peak value or latency to peak (Table 3).

Effects of cAMP Effectors on $[Ca^{2+}]_i$

To test if the $[Ca^{2+}]_i$ rise associated with activation of the LH receptor is mediated via the cAMP pathway, the cell-permeable cAMP analog, 8-bromo-cAMP (1 m*M* for $N = 20$ cells and 2 m*M* for $N = 16$ cells), or the adenylyl-cyclase activator, forskolin (10 μ*M*, $N = 4$ and 100 μM, $N = 21$ cells)

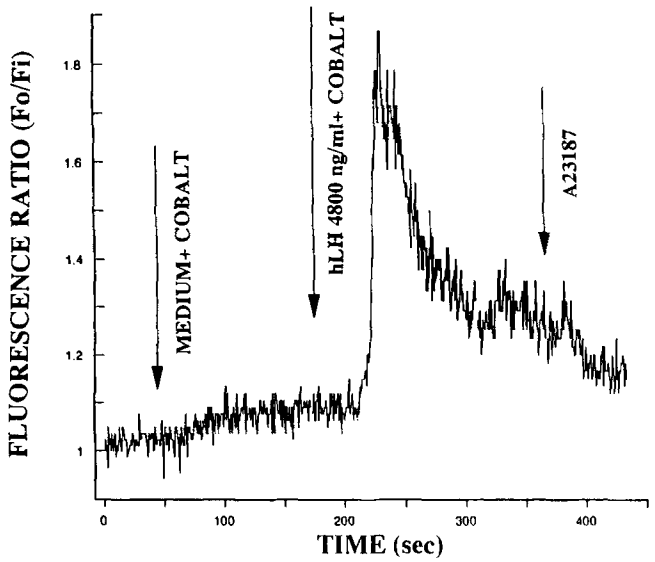

Fig. 2. Serial $[Ca^{2+}]_i$ responses plotted as fura-2-emitted fluorescence intensity (F_o/F_i) ratios stimulated by hLH in the presence of cobalt chloride in an individual 293 cell stably transfected with the hLH receptor cDNA. Cells were loaded into a Cunningham chamber with medium. After the basal level of $[Ca^{2+}]_i$ was imaged, medium with cobalt chloride (4 m*M*) was infused into the chamber, and imaging was continued. Ten minutes later, hLH (4800 ng/mL) was administered with cobalt chloride (4 m*M*) in medium, with a resultant increased percentage of cells responding with a predominant spike pattern rather than spike and plateau. A23187 was administered at the end of the experiment.

Table 3
Summary of PTX Effects on hLH-Induced $[Ca^{2+}]_i$ Responses in 293 Cells Transfected with hLH Receptor cDNA

	Cells not exposed to PT	Cells treated with PT (500 ng/mL)	*P* value
Total number of cells studied	32	38	NA
Number of responding cells	27 (84.4%)	27 (71.1%)	NS
Number of nonresponding cells	5 (15.6%)	11 (28.9%)	NS
Cell viability with trypan blue	70%, 79%	78%, 72%	
Maximal $[Ca^{2+}]_i$ increase from basal (fold)	1.79 ± 0.08	1.86 ± 0.09	NS
Time required to achieve half-max. value (sec)	52 ± 5.1	53 ± 4.8	NS

Data are expressed as mean ± SEM numbers of cells (percentage) and collected from two individual experiments. NS = not significant, NA = not applicable.

was administered. Neither agonist elicited a $[Ca^{2+}]_i$ rise. However, forskolin stimulated a cAMP increase 100 times baseline in this cell line.

LH-Induced Desensitization of $[Ca^{2+}]_i$ Rise

Incubation with hLH (4800 ng/mL) compared to control solvent overnight yielded a much lower percentage of cells responding to hLH (4800 ng/mL) with a biphasic $[Ca^{2+}]_i$ signal (5.7%, $N = 35$ cells, in four experiments vs 72% for control cells incubated overnight without LH). Loss of biphasic signal consisted primarily of loss of the spike phase.

Specificity of LH Effect

Specificity of the LH response was studied using human LH β-subunit (4.8 μg/mL, $N = 20$ cells and 48 μg/mL, $N = 20$ cells), α-subunit (48 μg/mL, $N = 20$ cells), ovine FSH (1000 ng/mL, $N = 15$ cells), and bovine TSH (0.1 mU/mL, $N = 12$ cells), all of which failed to evoke a $[Ca^{2+}]_i$ signal.

Specificity of 293 Cell Line Stably Transfected with Human LH Receptor

Another 293 cell line transfected with an α-inhibin CRE luciferase reporter construct, but without the LH receptor *(4)* was used as a negative control in order to test if the LH-stimulated $[Ca^{2+}]_i$ response is specific to 293 cells transfected with the human LH receptor. After stimulation with human LH 4800 ng/mL, 293 cells transfected with the α-inhibin CRE luciferase reporter construct alone did not have any $[Ca^{2+}]_i$ responses ($N = 22$ cells). No spontaneous oscillations occurred.

Manganese Quench Experiments

In our Mn^{2+} quench experiments, almost every cell (total $N = 8$) that responded to hLH (4800 ng/mL) exhibited Mn^{2+} influx following the $[Ca^{2+}]_i$ increase (Fig. 3). The majority of Mn^{2+} influx occurred only following the initial LH-stimulated $[Ca^{2+}]_i$ spike, resulting in quenching of the fura-II fluo-

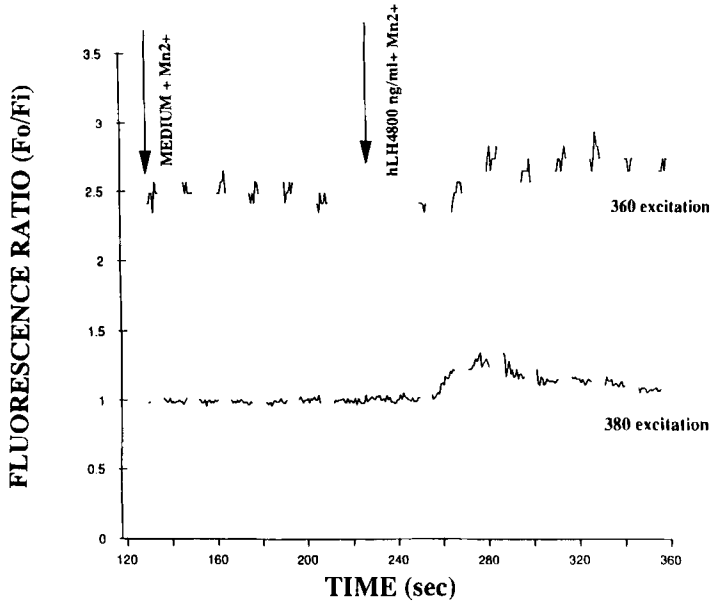

Fig. 3. Manganese quench technique showing extracellular cation influx after LH stimulation of a single 293 cell transfected with human LH receptor cDNA. Fura-2 fluorescence intensity was measured at two excitation wavelengths, for which the two records are given; namely, 360 nm (Ca^{2+}-independent) and 380 nm (Ca^{2+}-dependent) to allow sequential monitoring of changes in $[Ca^{2+}]_i$ (380 nm) and Mn^{2+} influx (360 nm, independent of changes in $[Ca^{2+}]_i$) in the same cell. After recording baseline fluorescence intensity, Mn^{2+}-containing medium was infused and the basal rate of Mn^{2+} influx recorded. Then, the effects of hLH (4800 ng/mL) in Mn^{2+}-containing medium were assessed. In other experiments, six cells responding to hLH exhibited Mn^{2+} influx following the spike-like $[Ca^{2+}]_i$ increase (two experiments, total eight cells).

rescence signal excited by illumination at 360 nm. Thus, calcium signaling following the spike reflects uptake.

Spatial Distribution of Intracellular Calcium

The spatial distribution of $[Ca^{2+}]_i$ in single 293 cells after stimulation by hLH 4800 ng/mL is displayed in Fig. 4. Each image represents a distribution of $[Ca^{2+}]_i$ in the same cell separated by an interval of 200 ms. The $[Ca^{2+}]_i$ increases in these cells were characterized spatially by rises initiated at the margins of the cells, which spread sequentially to other parts of the cell.

Discussion

The present single-cell studies of clonal human embryonic kidney cells (293 cells) stably transfected with the full-length human LH receptor cDNA demonstrate that increased concentrations of LH achieve an LH receptor-mediated $[Ca^{2+}]_i$ dose–response by recruiting a higher percentage of responding target cells, while concomitantly decreasing the response latency. In contrast, the amplitude of the $[Ca^{2+}]_i$ rise is not LH dose-dependent. In particular, the spike component of the $[Ca^{2+}]_i$ signal tends to be all-or-none, with no agonist-dependent amplitude variation. In addition, we discovered a spectrum of individual intracellular calcium ($[Ca^{2+}]_i$) second-messenger responses following human LH receptor activation. We found that the range of intracellular $[Ca^{2+}]$ signals driven by LH includes oscillatory (12–14%), spike (9%), spike-and-plateau (25%), and plateau (52%) patterns. When cellular calcium uptake was blocked by cobalt chloride *(8)*, LH still induced spike-like $[Ca^{2+}]_i$ rises, but with a significantly greater latency and significantly fewer cells showing a plateau pattern. Furthermore, Mn^{2+} quench experiments demonstrated directly that human LH receptor activation triggers divalent cation entry into responsive cells immediately following the spike phase of the $[Ca^{2+}]_i$ signal.

In other studies of 293 cells transfected with rat LH receptor cDNA *(9)*, the amplitude of the $[Ca^{2+}]_i$ response showed some LH dose dependence, which may well reflect species differences in LH receptor behavior or technical differences in study conditions. Importantly, our finding that different LH concentrations can evoke apparently identical $[Ca^{2+}]_i$ responses in clonal cells suggests the possible existence of threshold-dependent mechanisms within individual target cells of LH activation. The exact nature of these mechanisms has not yet been defined. In addition, using the human LH receptor, we found that about 12–14% of LH-stimulated $[Ca^{2+}]_i$ responses were oscillatory, which included both baseline-level rhythmic spikes and sinusoidal oscillations that remained above baseline. The occurrence of oscillatory behavior was not related to the concentration of LH. In other receptor and cell systems, $[Ca^{2+}]_i$ oscillations are believed to represent a cycle of discharge and reuptake of calcium by intracellular stores *(8–11)*. The $[Ca^{2+}]_i$ oscillations observed here after human LH receptor activation may be contingent on specific biochemical properties of the host cell as well as the gonadotrophin receptor subtype, since oscillations are not so readily observed following LH or FSH-induced $[Ca^{2+}]_i$ rises in swine granulosa

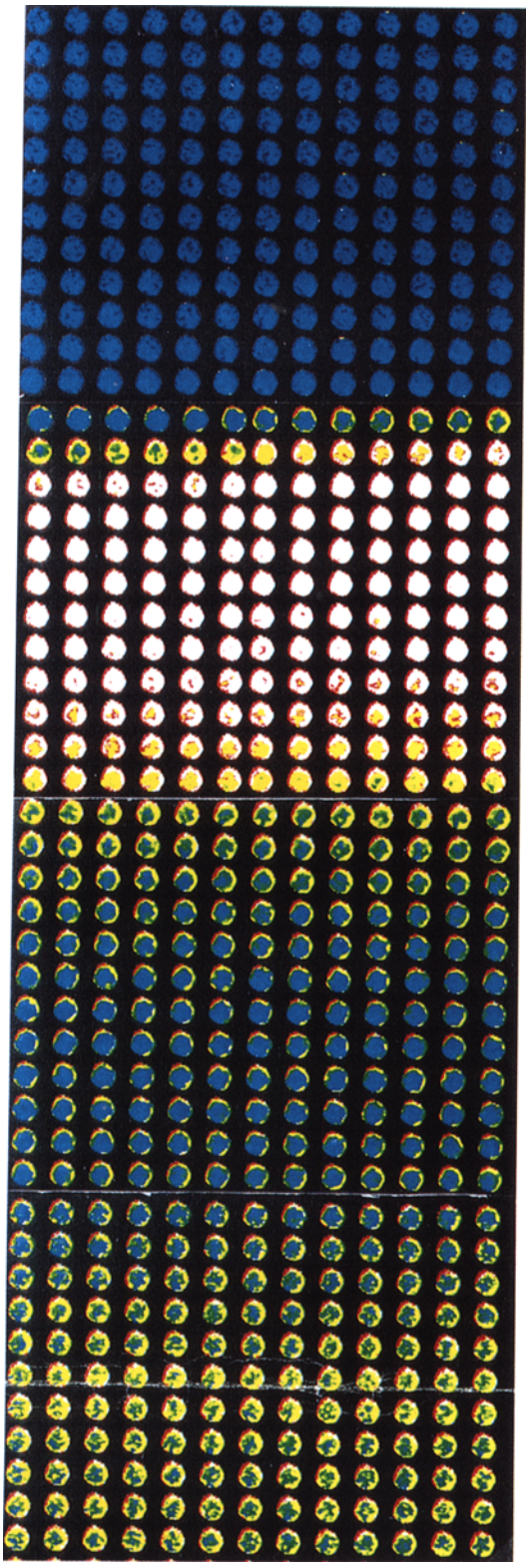

Fig. 4. Spatial distribution of $[Ca^{2+}]_i$ over time following hLH (4800 ng/mL) stimulation in one individual 293 cell stably transfected with hLH receptor cDNA. The sequence of images reads from left-to-right across individual rows beginning at the top. Successive images represent the spatial distribution of $[Ca^{2+}]_i$ portrayed in pseudocolor in the same single cell separated by an interval of 200 ms. White > red > yellow > blue denotes increased $[Ca^{2+}]_i$. This figure was taken from an oscillatory pattern of $[Ca^{2+}]_i$ rise, with only the first cycle of changes shown.

cells, but are seen in response to LH-action on porcine theca cells (*5*, and unpublished).

A marked loss of expression of the LH-stimulated spike-and-plateau $[Ca^{2+}]_i$ signal was demonstrable following overnight exposure of LH receptor-bearing cells to the homologous agonist. This attenuation of responsiveness seen in vitro might also occur in granulosa-luteal cells in vivo immediately following the midcycle LH surge, when LH concentrations rise 3- to 30-fold. Loss of responsiveness to repetitive gonadotrophin (agonist) exposure can include so-called receptor uncoupling *(1)*. The terminal cytoplasmic tail of the LH receptor appears to be necessary for uncoupling of receptor-activated adenylyl cyclase. The present stable human LH receptor transfection model showing markedly attenuated $[Ca^{2+}]_i$ signaling following homologous agonist pretreatment should allow a more systematic investigation of biochemical mechanisms that mediate loss of $[Ca^{2+}]_i$ responsiveness to sequential gonadotropic stimuli, since the mechanisms subserving loss of $[Ca^{2+}]_i$ signaling may or may not mimic those already mapped for the cAMP second messenger system *(1)*.

In the present studies, the cell-permeable cAMP analog, 8-bromo-cAMP, and the direct adenylyl-cyclase activator, forskolin, failed to elicit a $[Ca^{2+}]_i$ rise, which is consistent with other inferences that LH receptor-related $[Ca^{2+}]_i$ signal transduction is not mediated via the cAMP pathway *(1,3)*. Rather, activation of the phospholipase C pathway appears to be linked to LH-driven $[Ca^{2+}]_i$ signaling, which is akin to inferences using the cloned dog TSH receptor that also mediates the formation of both cAMP and inositol phosphates *(12)*. Of note, our LH dose–response studies and others in luteal or 293 or L-cells bearing bovine, murine, or rat LH receptors, as well as human LH receptor-transfected *Xenopus oocytes*, all show that the concentration of hCG or LH required to elicit half-maximal stimulation of inositol phosphate production is about 20- to 30-fold higher than that yielding 50% stimulation of adenylyl cyclase *(1,2,9)*. The exact molecular mechanisms that mediate this disparity are not yet fully understood *(2)*.

The spatial distribution of $[Ca^{2+}]_i$ increases evoked by human LH receptor activation consisted of initiation ("kindling") sites at the periphery of the individually responsive cells with further progressive propagation over the remainder of the cell. This is similar to observations in 293 cells transfected with rat LH receptor cDNA *(9)*. A discrete point of origin also seems to characterize the spatial distribution of $[Ca^{2+}]_i$ following stimulation with angiotensin II in porcine granulosa calls *(6)*, but in this circumstance $[Ca^{2+}]_i$ rises propagate as radically extending waves. In this context, we noted that angiotensin II, but not LH-induced $[Ca^{2+}]_i$ increases in granulosa cells are mediated through PT-sensitive G-protein(s). Thus, different receptors, host cell environments, the cellular milieu, and G-proteins mediating $[Ca^{2+}]_i$ signals may contribute to the different spatial distributions of intracellular calcium following stimulation by distinct agonists.

Materials and Methods

Materials

Human embryonic kidney cells (293 cells) stably transfected with the full-length human LH receptor cDNA and α-inhibin CRE luciferase reporter construct were donated by A. J. W. Hsueh (Department of Gynecology/Obstetrics, Stanford University School of Medicine, Palo Alto, CA) *(4)*. The clones are identified as FMEP2GEN. We first corroborated the specificity of LH action in this clonal model by treating the cells with different pituitary hormones and growth factors. Treatment with hLH, or hCG, consistently resulted in a dose-dependent stimulation of cyclic AMP accumulation and luciferase activity at a minimally effective dose of 0.3–1.0 ng/mL. In contrast, treatment with recombinant human FSH or TSH was ineffective. Human LH (I–3, SA 5100 IU/mg) was obtained from the Hormone Distribution Office, National Pituitary Agency (NPA), NIAMDD, NIH (Bethesda, MD). Human pituitary α-subunit (for radioiodination, NIDDK-hLHalpha-I-SIAFP-1, AFP-3491A, NIDDK-NIH, Bethesda, MD) and LH β-subunit (for radioiodination, NIDDK-hLHbeta-I-SIAFP-1, AFP-3477A, NIDDK-NIH, Bethesda, MD) were also provided by NPA. Bovine TSH was obtained from Ann Dunn (Department of Internal Medicine, University of Virginia Health Sciences Center, Charlottesville, Virginia, VA). Ovine FSH (NIADDK-oFSH-17, AFP-6446C) was provided by NIADDK-NIH, Bethesda, MD. Purified PTX was a gift of Erik Hewlett (Department of Pharmacology, University of Virginia Health Sciences Center, Charlottesville, VA). Geneticin, DMEM, and fetal bovine serum were obtained from Gibco Co. (Grand Island, NY). Fura-2 and calcium ionophore 4-Br-A23187 were purchased from Calbiochem (San Diego, CA), and forskolin, 8-Bromo-cAMP, HEPES, BSA (Fraction V), and poly-L-lysine from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

Human embryonic kidney cells (293 cells) stably transfected with the full-length human LH receptor cDNA (above) were maintained in DMEM with 5% FBS, Geneticin 100 μg/mL, and penicillin/streptomycin as monolayer cultures at 37°C in a humidified 95% air/5% CO_2 environment. All cells used in these experiments were used after four to seven passages. The experiments were then performed 2 d after repassaging. One day before the experiment, the medium was changed to serum-free DMEM with 0.1% BSA, 10 m*M* HEPES, without Geneticin.

Measurement of Cytosolic Ca^{2+} in Single Target Cells

The cells were collected and washed in defined medium containing NaCl 127 m*M*, KCl 5 m*M*, MgCl 2 m*M*, KH_2PO_4 0.5 m*M*, $NaHCO_3$ 5 m*M*, HEPES 10 m*M*, glucose 10 m*M*, BSA 0.1%, $CaCl_2$ 1.8 m*M*, and penicillin /streptomycin *(5)*. The cell suspension was transfused into Cunningham chamber slides treated with poly-L-lysine (the slides were precoated with 0.1% poly-L-lysine in water for 10 min and then rinsed with distilled water), as described for granulosa cells *(5,6)*. The slides were put in a humidified dish to prevent drying between loading and washes. After 1 h of incubation to allow the cells to attach to the slides, the cells were loaded with fura-2 acetoxymethylester (5 μ*M*) at 37°C for 20 min, and then washed with medium and left at room temperature for 20 min to allow cytoplasmic deesterification of the fura-2/AM dye. Thereafter, the slides with Cunningham chambers were placed on the stage of a Zeiss Axioplan microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with epifluorescence illumination. A field containing cells was imaged using an Axioplan Nikon microscope equipped with a fluor x20. The excitation wavelength was selected by narrow-bandpass filter (e.g., 360 and 380 nm, half-bandwidth, 2 nm, Corian, Holiston, MA). Video images were obtained over 7 min for each slide using a silicon-intensified target camera (SIT-68 DAGE, MTI) and stored on 3/4-in. broadcast-quality videotape (Video Products, VA). At the end of each experiment, the calcium ionophore, 4-Br-A23187, was delivered to the cells to verify cellular $[Ca^{2+}]_i$ responses.

Manganese Quench Technique

Fura-2 fluorescence intensity was measured at two excitation wavelengths, 360 nm (Ca^{2+}-independent) and 380 nm (Ca^{2+}-dependent), to allow sequential monitoring of changes in $[Ca^{2+}]_i$ (380 nm) and Mn^{2+} influx (360 nm independent of changes in $[Ca^{2+}]_i$). After a baseline was recorded in Ca^{2+}-containing medium, Mn^{2+}-containing medium (Mn^{2+} 0.9 m*M* and Ca^{2+} 0.9 m*M*) was infused into the Cunningham chamber and the basal rate of Mn^{2+} influx was observed at two excitation wavelengths. Then, the effects of hLH (delivered in Mn^{2+}-containing medium) were assessed by similar dual-wavelength monitoring.

Image Analysis

Serial images from individual cells were captured on a Quantex QT-7 digital analyzer using the RADTIME program *(5,6)*. This program creates a radiance file, which can be imported into a spreadsheet where the 380-nm fluorescence values are converted to the final graphical form F_o/F_i (F_o is the initial 380 nm fluorescence emission intensity value and F_i is the 380 nm fluorescence intensity at time i).

Study of Spatial Distribution of Intracellular Calcium

A real-time disk was used to map the $[Ca^{2+}]_i$ spatial distributions at standard video rates (33 frames/s). A Gould IP8500 image processing system equipped with a time-based corrector and a real-time disk was used to create a new video record of each original fluorescence video. This new Gould video differed from its original, in that each frame was captured and repeated 15 times. Data were digitized and processed with a quantex QX7 image analyzer to capture every 0.2 s without frame averaging. Thus, a frame-

by-frame movie of the original record was created, which allowed a "real-time" measurement of changing cellular $[Ca^{2+}]_i$. A fluorescence image obtained under basal conditions (F_o) was divided by each subsequent image (F_i) after LH delivery. The spatial distribution of changing $[Ca^{2+}]_i$ was then produced from the ratio images (F_o/F_i) and represented in pseudocolor *(6)*.

Statistics

Data were evaluated by analysis of variance with post hoc testing via Duncan's new multiple-range test, the binomial distribution, chi-squared testing, or the nonparametric Wilcoxon rank sum test, as noted. All experiments were replicated on at least three independent occasions.

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