Vol. 154, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1988 Pages 398-403

BIPHASIC ACTIVATION OF CYTOSOLIC FREE CALCIUM AND LH RESPONSES BY GONADOTROPIN-RELEASING HORMONE

Keiichi Tasaka, Stanko S. Stojilkovic, Shun-Ichiro Izumi, and Kevin J. Catt

Endocrinology and Reproduction Research Branch,
National Institute of Child Health and Human Development,
National Institutes of Health, Bethesda, MD 20892

Received May 12, 1988

Summary: Gonadotropin-releasing hormone (GnRH) stimulates rapid peak increases in $[Ca^{2+}]i$ and LH release, followed by lower but sustained elevations of both $[Ca^{2+}]i$ and hormone secretion. Omission of extracellular Ca^{2+} only slightly decreased the peak of $[Ca^{2+}]i$, but reduced the peak LH response by 40% and prevented the prolonged increases in $[Ca^{2+}]i$ and LH release. Dihydropyridine calcium antagonists did not affect the peak $[Ca^{2+}]i$ and LH responses, but reduced the sustained increases by up to 50%. Whereas GnRH-induced mobilization of intracellular calcium initiates the LH peak, and Ca^{2+} entry through dihydropyridine-insensitive channels contributes to the peak and plateau phases of LH release, dihydropyridine-sensitive L-type Ca^{2+} channels participate only in the sustained phase of gonadotropin secretion.

Gonadotropin-releasing hormone (GnRH) binds to plasma membrane receptors in pituitary gonadotrophs and stimulates the secretion of LH and FSH. The mechanism by which GnRH elicits its biological actions includes rapid changes in polyphosphoinositide hydrolysis that are characteristic of several Ca^{2+} mobilizing hormones (1,2). Degradation of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C results in the formation of inositol 1,4,5-triphosphate ($InsP_3$) and 1,2 diacylglycerol (DG), which respectively mobilize Ca^{2+} from intracellular stores (3) and activate protein kinase C (4). In rat pituitary gonadotrophs, GnRH induces minor increases of cytosolic free calcium concentration ($[Ca^{2+}]i$) as measured by the Quin-2 method (5-7). However, Quin-2 signals have not been adequate to permit the dynamic analysis of changes in $[Ca^{2+}]i$ during stimulation by GnRH. Dyes with lower affinity and higher fluorescence intensity, such as Fura-2 acetoxymethylester (Fura-2 AM), possess advantages over Quin-2 for measurement of basal and elevated levels of $[Ca^{2+}]i$ (8). The present analysis of the effects of GnRH in Fura-2 loaded pituitary cells has revealed biphasic changes in $[Ca^{2+}]i$ during gonadotroph activation. The dependence of these changes on extracellular calcium ($[Ca^{2+}]e$) was analyzed and compared with the dynamic profile of LH secretion during column perifusion of GnRH-stimulated pituitary cells.

[1]The abbreviations used are: GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; BSA, bovine serum albumin; EGTA, [ethylene bis(oxyethyl-enenitrilo)] tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid.

0006-291X/88 $1.50

EXPERIMENTAL PROCEDURES

Pituitary glands were removed from adult female Sprague-Dawley rats (200 - 250 g) two weeks after ovariectomy and subjected to controlled trypsinization as previously described (9). The enzyme-dispersed cells were resuspended in Medium 199 (M199) with 10% horse serum for 3 hr prior to use for $[Ca^{2+}]i$ studies. Measurements of $[Ca^{2+}]i$ was performed according to methods described by Grynkievich et al (8). Briefly, twenty million cells were resuspended in 20 ml M199 containing 25 mM HEPES and 0.3% bovine serum albumin (BSA) and incubated with 2 μM Fura-2 AM (Calbiochem Inc.), added from stock solution dissolved in dimethylsulfoxide, for 30 min at 37° C. Cells were then washed twice and resuspended in Hank's balanced salt solution with 25 mM HEPES and 0.01% BSA. 3-5 million cells were used for $[Ca^{2+}]i$ measurements by fluorescence analysis (excitation wavelength 340 nM and emission wavelength 500 nM) in a 3 ml cuvette in a Perkin Elmer spectrophotometer fitted with a magnetic stirrer and thermostatted cuvette holder. Following each experiment, F_{max} and F_{min} were determined after lysing the cells by addition of Triton X-100 (Calbiochem) in the presence of $[Ca^{2+}]e$, and by adding 5 mM EGTA (Sigma) with 50 mM Tris (Sigma) after cell lysis. The fluorescence of extracellular Fura-2 was estimated by adding 50 μM $MnCl_2$ and 200 μM Ca^{2+} DPTPA. $[Ca^{2+}]i$ was calibrated after correction of fluorescence for extracellular Fura-2 leakage as previously described (10).

Column perifusions were performed on 3-day cultured cells with exposure to 10 nM GnRH for 15 min, under previously reported conditions (11). Briefly, 10 x 10^6 cells were incubated on preswollen Cytodex-1 beads (Pharmacia) for 3 days, then collected and resuspended in M199 with 25 mM HEPES and 0.01% BSA and loaded into 500 μl perifusion chambers (Endotronics, Minneapolis, MN). Cells were perifused with the same medium for 90 min at a flow rate of 0.6 ml/min, and then with control medium (containing 500 μM Ca^{2+}); control medium + 1 μM nifedipine or 1 μM nicardipine (Sigma, St. Louis, MO.) or Ca^{2+}-free medium (Ca^{2+}-deficient medium + 100 μM EGTA). GnRH (10 nM) dissolved in conditioned medium was added for 15 min as indicated; each set of experiments was performed with the same batch of cells. Fractions were collected every 30 sec and stored at -20° C prior to radioimmunoassay using the RP-2 rat LH standard provided by the National Pituitary Agency, Baltimore, MD. Significances for differences between means were derived by Student's t test.

RESULTS AND DISCUSSION

GnRH-induced changes in $[Ca^{2+}]i$ of pituitary cells incubated in control medium are characterized by a rapid peak increase within 20 seconds, followed by a decline to a lower level that is sustained for at least 10 min. (Fig. 1A). The rapid first phase was little affected by the omission of extracellular calcium and addition of 100 μM EGTA ($[Ca^{2+}]e$ < 20 nM) (Fig. 1B) and was not inhibited by addition of the voltage sensitive Ca^{2+} channel blockers (12), nifedipine and nicardipine (Fig. 1C). In contrast, the second phase of $[Ca^{2+}]i$ increase during GnRH action was completely abolished in calcium-free medium (Fig. 1B), and was partially inhibited by addition of nifedipine (Fig. 1C) or nicardipine (not shown).

The mean resting level of $[Ca^{2+}]i$ was 148 ± 25 nM, and the $[Ca^{2+}]i$ changes during GnRH stimulation under each incubation condition are summarized in Fig. 2. The magnitude of the first phase of the calcium response was determined as the height of the peak above resting levels, and that of the second phase was determined as the elevation of $[Ca^{2+}]i$ above the basal level 5 min after addition of GnRH. The first phase of the calcium response showed little change under any of the incubation conditions employed (Ca^{2+}-free medium plus EGTA, or control medium plus nifedipine or nicardipine), and was largely independent of extracellular calcium. However, the second, prolonged phase of the calcium response was abolished by omission of $[Ca^{2+}]e$ and was reduced by addition of 1 μM nifedipine (to 53 ± 4%) or nicardipine (to 72 ± 5%). Thus, the agonist-induced increase in $[Ca^{2+}]i$ differed both quantitatively and

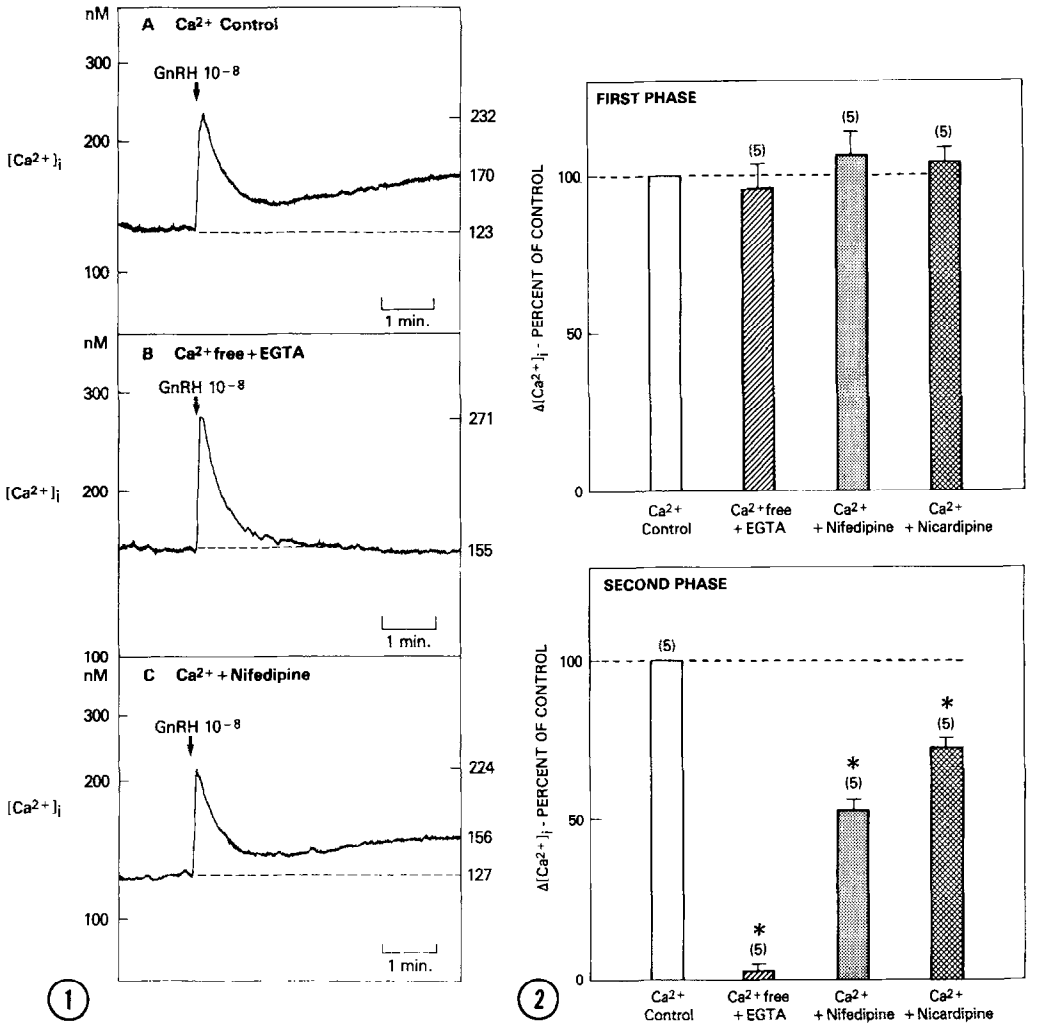

Fig. 1. $[Ca^{2+}]i$ changes in pituitary cells stimulated by 10 nM GnRH. A, medium containing 500 μM $CaCl_2$; B, calcium-deficient medium containing 100 μM EGTA; C, medium containing 500 μM $CaCl_2$ + 1 μM nifedipine; D, medium containing 500 μM $CaCl_2$ + 1 μM nicardipine. Similar changes were observed in 5 independent experiments.

Fig. 2. Relative changes in the first (peak) and second (plateau) components of the $[Ca^{2+}]i$ response to GnRH. Increases during the first phase represent the maximum rise above the resting level, and those in the second phase were measured 5 min after addition of GnRH. Increases in each phase are expressed as percentages of the control values (*, $p < 0.01$, t-test).

qualitatively with time after the addition of GnRH, and in particular in its sensitivity to external calcium and blockers of L-type calcium channels.

The kinetics of the LH response to GnRH were analyzed during cell column perifusion to permit comparison with the rapid changes in $[Ca^{2+}]i$ described above. In perifused pituitary cells, LH release was detectable within 6 seconds of exposure to GnRH. The rapid initial peak of LH release reached a maximum in 1.5 min, and was followed by a lower and sustained phase of hormone secretion (Fig. 3). Omission of calcium from the perifusion medium reduced the magnitude of the first phase, and completely inhibited the second phase of LH secretion.

Addition of nifedipine or nicardipine had little effect on the first phase, but consistently reduced the magnitude of the second phase of LH secretion. The results of 5 similar column perifusion studies are summarized in Fig. 4. Peak phase secretion was determined as the integrated LH release during the first 3 min, and second phase secretion was determined as the subsequent integrated release of LH, each being expressed as a percentage of the corresponding responses observed in normal $[Ca^{2+}]e$ medium. In the combined data, the initial peak of LH release was reduced to 63 ± 6% by omission of $[Ca^{2+}]e$ but was unaffected by addition of nifedipine or nicardipine, whereas the second, sustained phase of LH secretion was completely blocked by the omission of $[Ca^{2+}]e$ and was reduced by nifedipine (to 61 ± 10%) or nicardipine (to 72 ± 7%).

The results of this study have shown that GnRH stimulates two distinct phases of elevated $[Ca^{2+}]i$ and LH secretion in cultured pituitary cells. The rapid peak elevation of

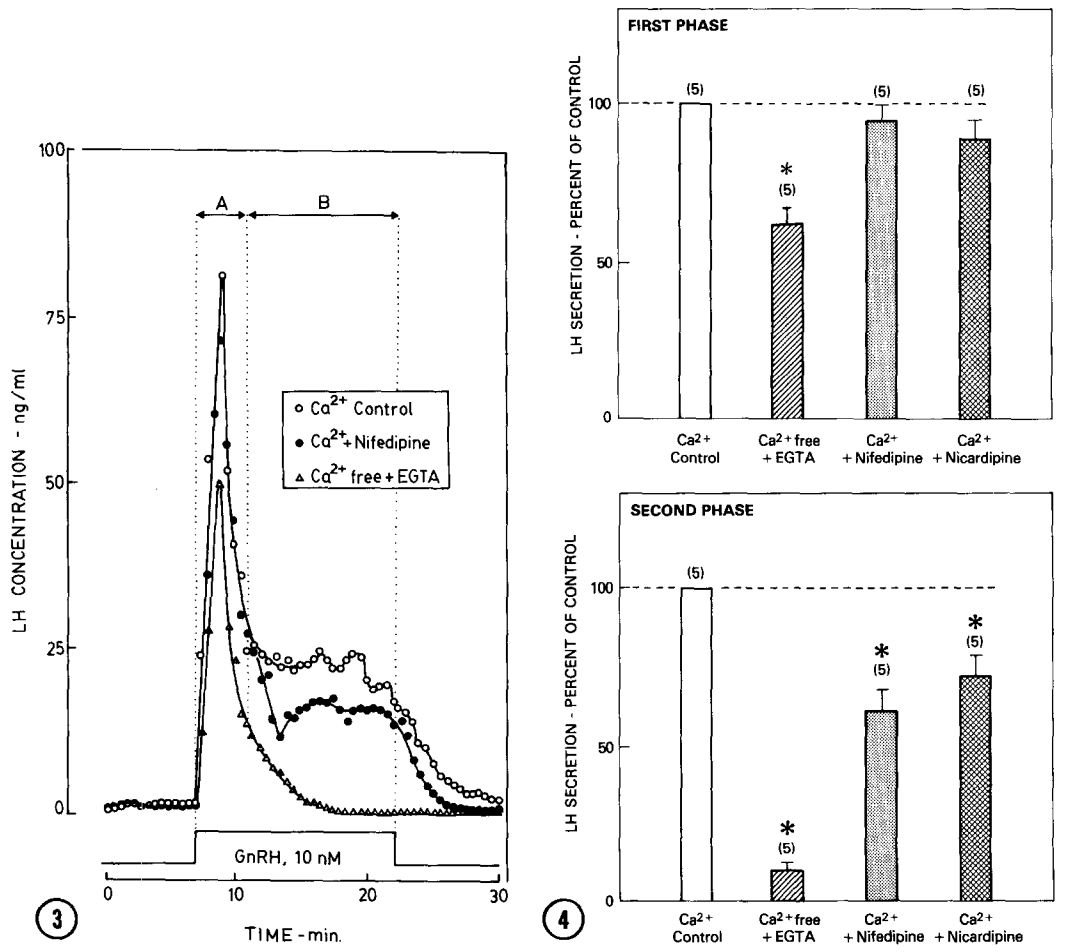

Fig. 3. Profile of LH secretion stimulated by 10 nM GnRH, showing the initial peak (A) and the sustained plateau phase (B). Results are representative of data from 5 similar experiments, each performed under the conditions described in Fig. 1.

Fig. 4. LH release measured during the peak and plateau phases of secretion stimulated by 10 nM GnRH (see Fig. 3). Increases are expressed as percentages of the corresponding control values in each experiment (*, $p < 0.01$).

$[Ca^{2+}]i$ is largely attributable to mobilization of Ca^{2+} from intracellular stores as a consequence of $InsP_3$ production, and shows little dependence on entry of extracellular calcium. However, the initial peak of LH release is reduced in magnitude in the absence of extracellular calcium, but is completely unaffected by dihydropyridine channel antagonists and thus does not involve calcium entry through L-type VSCC (13,14). In contrast to the peak responses, the sustained increases in $[Ca^{2+}]i$ and LH release are highly dependent on extracellular calcium and are partially mediated by entry through dihydropyridine-sensitive L-type VSCC. The calcium requirement for the prolonged component of LH secretion is consistent with the major dependence of GnRH action in static cell cultures on calcium entry from extracellular sources (15), following the initial mobilization of intracellular calcium (16).

The reduction in magnitude of the LH peak response in the absence of extracellular calcium, with little change in the $[Ca^{2+}]i$ peak, reflects the greater rapidity of the calcium response to GnRH. Since the LH peak outlasts the calcium spike and is in part supported by the sustained phase of the GnRH-induced rise in cytosolic calcium, it is significantly impaired by omission of extracellular calcium. The participation of VSCC in the overall secretory response to GnRH has been previously indicated by studies with nitrendipine and the calcium channel agonist, Bay K 8644 (6,16). The present results have shown that calcium entry through VSCC participates in the maintenance of the second, sustained phases of elevated $[Ca^{2+}]i$ and LH secretion. However, the inability of dihydropyridine agonists, including nitrendipine (6) and nifedipine or nicardipine, to inhibit completely the second phase of LH secretion suggests that calcium channels other than VSCC are also involved in the maintenance of the LH response during GnRH action.

Many agonist-stimulated cells show an initial rapid increase in cytosolic free calcium during the first minute of activation, derived primarily by calcium mobilization from intracellular stores such as the endoplasmic reticulum (17). This is frequently followed by a less prominent but prolonged second phase or plateau of elevated cytosolic calcium that is largely or completely dependent on calcium influx across the plasma membrane (18,19). Such influx may be secondary to the initial action of Ins-1,4,5-P_3 on calcium mobilization from pools located near the plasma membrane (20,21), and/or to the potential actions of Ins-1,3,4,5-P_4 (22) or other second messengers responsible for calcium entry and maintenance of the cell response (23).

We have previously observed that LH responses to short (2 min) pulses of GnRH are potentiated by Bay K 8644 and attenuated by nitrendipine, consistent with the participation of voltage-dependent calcium channels as well as mobilization of internal stores in GnRH action (6). That the rise in $[Ca^{2+}]i$ in GnRH-stimulated gonadotrophs originates partly from intracellular Ca^{2+} pools and partly from influx was further demonstrated by studies with Quin-2 in enriched gonadotrophs from normal female rats (7). The independence of the initial phase of GnRH-stimulated LH secretion from calcium entry through voltage-dependent calcium channels was recently indicated by kinetic studies on the effects of nifedipine and D600 on LH release in rat and chicken pituitary cells (24). The present findings have clearly defined the primary and secondary phases of calcium mobilization and influx during GnRH action, and have

demonstrated the dependence of the prolonged phase of cytosolic calcium elevation and sustained LH secretion upon the influx of calcium through both L-type VSCC and dihydropyridine-insensitive channels in the plasma membrane.

REFERENCES

1. Michel, R.H., Kirk, C.J., Jones, L.M., Downes, C.P., and Creba, J.A. (1981) Philos. Trans. R. Soc. Lond. B. Biol. Sci. 296, 123-138.
2. Downes, P., and Michel, R.H. (1982) Cell Calcium 3, 467-502.
3. Berridge, M.J., and Irvine, R.F. (1984) Nature, 312, 315-321.
4. Nishizuka, Y. (1984) Nature 308, 693-698.
5. Clapper, D.L., and Conn, P.M. (1985) Biol Reprod. 32, 269-278.
6. Chang, J.P., McCoy, E.E., Graeter, J., Tasaka, K., and Catt, K.J. (1986) J. Biol. Chem. 261, 9105-9108.
7. Limor, R., Ayalon, D., Capponi, A.M., and Naor, Z. (1987) Endocrinology 120, 497-503.
8. Grynkiewicz, G., Poenie, M., and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440-3450.
9. Hyde, C.L., Childs, G., Wahl, L.M., Naor, Z., and Catt, K.J. (1982) Endocrinology 111, 1421-1423.
10. Rink, T.J., and Pozzan, T. (1985) Cell Calcium 6, 134-144.
11. Chang, J.P., Graeter, J., and Catt, K.J. (1987) Endocrinology 120, 1837-1845.
12. Glossmann, H., Ferry, D.R., Striessnig, J., Goll, A. and Moosburger, K. (1987) TIPS 8, 95-100.
13. Nowycky, M.C., Fox, A.P., and Tsien, R.W. (1985) Nature 316, 440-443.
14. Kokubun, S. and Reuter, H. (1984) Proc. Natl. Acad. Sci. USA 81, 4824-4827.
15. Bates, M.D., and Conn, P.M. (1984) Endocrinology 115, 1380-1385.
16. Chang, J.P., Stojilkovic, S.S., Graeter, J.S. and Catt, K.J. (1988) Endocrinology, in press.
17. Tan, K.N., and Tashjian, A.H. (1984) J. Biol. Chem., 427-434.
18. Albert, P.R., and Tashjian, A.H. (1984) J. Biol. Chem. 259, 15350-15359.
19. Gershengorn, M.C., and Thaw, C. (1985) J. Biol. Chem. 116, 591-596.
20. Putney, J.W. (1976) Cell Calcium 7, 1-12.
21. Guillemette, G., Balla, T., Baukal, A.J., and Catt, K.J. (1987) J. Biol. Chem. 262, 1010-1015.
22. Irvine, R.F., and Moor, R.M. (1986) Biochem. J. 240, 917-920.
23. Albert, P.R., Wolfson, G., and Tashjian, A.H. (1987) J. Biol. Chem. 262, 6577-6581.
24. Smith, C.E., Wakefield, I., King, J.A., Naor, Z., Millar, R.P., and Davidson, J.S. (1987) FEBS Letters 12, 247-250.