Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 6566–6570, September 1988
Physiological Sciences

Gonadotropin-releasing hormone-induced Ca^{2+} transients in single identified gonadotropes require both intracellular Ca^{2+} mobilization and Ca^{2+} influx

(fura-2/signal transduction/inositol trisphosphate/diacylglycerol/peptide hormone secretion)

GARY A. SHANGOLD*, SHAWN N. MURPHY†, AND RICHARD J. MILLER†‡

Departments of †Pharmacological and Physiological Sciences and of *Obstetrics and Gynecology, University of Chicago, Chicago, IL 60637

Communicated by Hewson Swift, June 2, 1988 (received for review April 18, 1988)

ABSTRACT **We examined the effects of gonadotropin-releasing hormone (GnRH) on the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in single rat anterior pituitary gonadotropes identified by a reverse hemolytic plaque assay. Concentrations of GnRH >10 pM elicited increases in $[Ca^{2+}]_i$ in identified cells but not in others. In contrast, depolarization induced by 50 mM K^+ increased $[Ca^{2+}]_i$ in all cells. Ca^{2+} transients induced by GnRH exhibited a complex time course. After an initial rapid rise, the $[Ca^{2+}]_i$ fell to near basal levels only to be followed by a secondary extended rise and fall. Analysis of the Ca^{2+} transients on a rapid time base revealed that responses frequently consisted of several rapid oscillations in $[Ca^{2+}]_i$. Removal of extracellular Ca^{2+} or addition of the dihydropyridine Ca^{2+}-channel blocker nitrendipine completely blocked the secondary rise in $[Ca^{2+}]_i$ but had no effect whatsoever on the initial spike. Nitrendipine also blocked 50 mM K^+-induced increases in $[Ca^{2+}]_i$ in identified gonadotropes. The secondary rise induced by GnRH could be enhanced by a phorbol ester in a nitrendipine-sensitive fashion. Multiple spike responses to GnRH stimulation of the same cell could only be obtained if subsequent Ca^{2+} influx was permitted either by allowing a secondary rise to occur or by producing a Ca^{2+} transient by depolarizing the cells with 50 mM K^+. It therefore appears that the response to GnRH consists of an initial phase of Ca^{2+} mobilization, probably mediated by inositol trisphosphate, and a subsequent phase of Ca^{2+} influx through nitrendipine-sensitive Ca^{2+} channels that may be activated by protein kinase C. The relative roles of these phases in the control of gonadotropin secretion are discussed.**

Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) plays a pivotal role in triggering the secretion of neurotransmitters and hormones (1). For example, Ca^{2+} appears to trigger the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from pituitary gonadotropes as well as hormone secretion from other cells of the anterior pituitary (2). Normally the resting $[Ca^{2+}]_i$ in most cells is about 0.1 μM (3). Stimulus-induced increases in $[Ca^{2+}]_i$ that initiate events such as hormone secretion can potentially occur by two major mechanisms: (*i*) a transient increase in the permeability of the plasma membrane allowing the influx of Ca^{2+} into the cell down its electrochemical gradient (4) or (*ii*) the mobilization of Ca^{2+} from intracellular bound stores (3, 5). To completely understand the role of Ca^{2+} in the control of gonadotropin secretion precise determinations of gonadotropin-releasing hormone (GnRH)-induced changes in gonadotrope $[Ca^{2+}]_i$ are required. Such studies have been problematic, however, as gonadotropes represent only 5–15% of anterior pituitary cells (6). Thus, although it has been demonstrated that GnRH does produce some increase in $[Ca^{2+}]_i$ in anterior pituitary cell populations enriched in gonadotropes (7, 8), no precise data concerning the characteristics of this response are available. Indeed, to obtain such data it is essential to measure $[Ca^{2+}]_i$ either in completely pure populations of gonadotropes or, preferably, in single identified cells. It is possible to achieve these ends by the use of the reverse hemolytic plaque procedure (9) and instrumentation allowing the precise determination of $[Ca^{2+}]_i$ fluctuations in single cells (10). We now report the use of these techniques to show that GnRH produces Ca^{2+} transients in identified gonadotropes with a very characteristic and complex time course. The role of these transients in the control of gonadotropin release is discussed.

MATERIALS AND METHODS

Cell Culture. Female 35-day-old Sprague–Dawley rats were decapitated, their pituitaries were removed, and adenohypophyses were dissected from neurohypophyses. Anterior pituitaries were then enzymatically dispersed for 20 min with 0.25% trypsin (bovine, type XII-S, Sigma) in a metabolic shaker at 37°C. Cells were then filtered through organza cloth (Tetko, Elmsford, NY), diluted to 50 ml in medium 199 with Hanks' salts and L-glutamine (GIBCO), and centrifuged at 350 × *g* for 10 min. The supernatant was removed, and cells were resuspended by trituration through a Pasteur pipette in 5 ml of medium and then were plated on sterile, poly(L-lysine)-coated (10 μg/ml, Sigma) etched-grid coverslips (Bellco). The cell density was ≈2.5 × 10^5 cells per coverslip. The cell yield was ≈4 × 10^5 cells per rat. Cells were incubated for 2 days in 60-mm Falcon culture dishes, with medium 199 containing 10% (vol/vol) heat-inactivated horse serum (GIBCO) and 2.5% (vol/vol) fetal bovine serum (GIBCO) and supplemented with penicillin (5 units/ml) and streptomycin (50 μg/ml), in a 5% CO_2/95% air atmosphere in a water-jacketed incubator at 37°C.

Identification of Isolated Gonadotropes. Sheep erythrocytes (Colorado Serum, Denver) were washed four times in 0.9% NaCl and conjugated to protein A (0.5 mg/ml, Sigma) with $CrCl_2$ (0.2 mg/ml). After further washing, cells were then mixed with LH antiserum (1:5), guinea pig complement (1:10, GIBCO), and GnRH (100 nM, Peninsula Laboratories, San Carlos, CA). Final dilutions of antiserum, complement, and GnRH were 1:25, 1:67, and 20 nM, respectively. LH antiserum was kindly provided by Nira Ben-Jonathan (Department of Physiology, University of Indiana) (11). Cunningham chambers were created on each coverslip by fixing smaller plain coverslips in place with double-stick tape, and ≈50 μl of the hemolysis suspension was then injected into each

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; $InsP_3$, inositol trisphosphate.

‡To whom reprint requests should be addressed.

chamber. After 45–60 min of incubation at 37°C, chambers were inspected under an inverted-head phase-contrast microscope. Location of hemolytic plaques was noted on grid-marked paper. The hemolysis suspension was then flushed out of the chambers, and the roofs of the chambers were lifted off; coverslips containing pituitary cells were then placed into medium 199 and incubated overnight.

Measurement of $[Ca^{2+}]_i$. All measurements were made on single cells. $[Ca^{2+}]_i$ was determined by using a microspectrofluorimeter, which has been described (10, 12), to monitor the Ca^{2+}-sensitive fluorescent chelator fura-2 (Molecular Probes, Plano, TX). Gonadotropes were loaded with the dye by incubation in 2 μM fura-2 pentakis(acetoxymethyl ester), which is membrane-permeant, for 1 hr at 37°C in serum-free culture medium. After this incubation, the cells were washed twice in Hepes-buffered Hanks' solution (see below) and incubated for an additional 30 min. The coverslips containing the loaded and washed cells were mounted in a flow-through chamber for viewing where the contents of the bath were changed every 10 sec. The gonadotropes were located relative to the etched grid. Experiments were performed at 25°C. The cells were usually maintained in a bath of Hepes-buffered Hanks' balanced salts solution (137 mM NaCl/5.4 mM KCl/0.41 mM $MgSO_4$/0.49 mM $MgCl_2$/1.26 mM $CaCl_2$/0.44 mM KH_2PO_4/0.64 mM Na_2HPO_4/3 mM $NaHCO_3$/5.5 mM glucose/20 mM Hepes at pH 7.45). Ca^{2+}-free experiments were performed with Ca^{2+} removed and 20 μM EGTA added to the medium. Each cell was excited alternately with 340-nm and 380-nm light at 60 Hz. Emissions from each wavelength were collected and averaged, and the 340 nm/380 nm ratio was calculated every second. $[Ca^{2+}]_i$ was calculated, after background correction, from the equation $[Ca^{2+}]_i = K(R - R_{min})/(R_{max} - R)$. The constants of this equation were determined by measuring fura-2 fluorescence from cells with $[Ca^{2+}]_i \geq 30$ μM and cells with $[Ca^{2+}]_i$ approaching 0 nM (13). The maximum ratio (R_{max}) was determined when $[Ca^{2+}]_i \geq 30$ μM, and this condition was attained in the cytosol by adding ionomycin (50 μM) and the metabolic inhibitors 2-deoxy-D-glucose (10 mM) and antimycin A (25 μM). The minimum ratio (R_{min}) was reached asymptotically after the addition of 20 mM EGTA to the same cells. The maximum fluorescence with 380-nm excitation obtained at $[Ca^{2+}]_i \approx 0$ was divided by the minimum fluorescence at this wavelength obtained at $[Ca^{2+}]_i \geq 30$ μM and this ratio was multiplied by the dissociation constant for fura-2 (224 nM, see ref. 14) to obtain the constant K. This procedure was carried out with seven cells, and R_{max}, R_{min}, and K were determined to be (mean ± SEM) 8.13 ± 0.53, 0.399 ± 0.011, and 2063 ± 226.

RESULTS

General Characteristics of the GnRH Response. Experiments were performed on 51 cells identified by use of the reverse hemolytic plaque assay (Fig. 1). Basal $[Ca^{2+}]_i$ in these cells was 88 ± 10 nM; however, this varied over quite a wide range (42–170 nM). When an identified gonadotrope was perfused with medium containing GnRH, increases in $[Ca^{2+}]_i$ were observed that had several typical characteristics. The threshold concentration of GnRH required to elicit a response varied from cell to cell. However, responses to concentrations as low as 10 pM were frequently observed. In some cells the threshold was higher than this, although all cells responded to GnRH at ≥100 pM. The peak magnitude and duration of GnRH-induced Ca^{2+} transients also varied over quite a wide range (Figs. 1–6). However, the qualitative characteristics of the responses observed were remarkably similar in each case. Ca^{2+} transients were never observed when GnRH was added to a randomly selected cell not identified by the plaque assay (n = 11). A typical response to

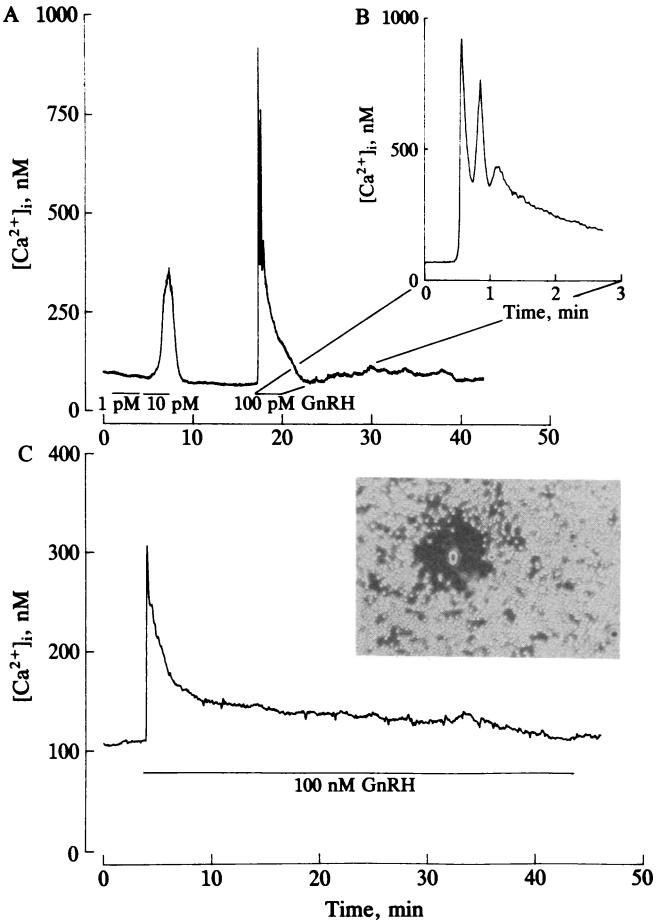

FIG. 1. (*A*) Effect of increasing concentrations of GnRH on $[Ca^{2+}]_i$ in a single gonadotrope. Note the appearance of an initial spike at 100 pM GnRH and relatively noisy secondary rise. (*B*) Spike portion of response to 100 pM GnRH at higher time resolution. Note the oscillations in $[Ca^{2+}]_i$. (*C*) $[Ca^{2+}]_i$ transient induced in a gonadotrope by a high concentration of GnRH (100 nM). (*Inset*) Gonadotrope identified in reverse hemolytic plaque assay.

GnRH stimulation can be seen in Fig. 1*A*. At 1 pM no response was observed. However, at 10 pM a $[Ca^{2+}]_i$ transient was clearly evident. At 100 pM a larger initial "spike" was seen that consisted of several large fluctuations (Fig. 1*B*). This was followed by a secondary rise in $[Ca^{2+}]_i$ during which the trace became relatively noisy (see also Figs. 2–6). The length of the secondary rise was variable but it often lasted >40 min. The secondary phase was observed even if the GnRH had been washed out. The secondary rise was not always discernible at the lowest concentrations of GnRH utilized (10 pM), although even at this concentration it was clearly evident in some instances (e.g., Fig. 5*B*). Fig. 1*C* illustrates a $[Ca^{2+}]_i$ transient evoked by a very high concentration of GnRH (100 nM). Such concentrations are frequently used in studies of gonadotropin secretion (2). In this case the spike and secondary rise merged to produce a "spike/plateau" waveform such as has been observed in studies of the mechanism of action of thyrotropin-releasing hormone (15). In the face of such a high GnRH concentration, $[Ca^{2+}]_i$ actually remained elevated above basal levels for a very extended period of time. GnRH at 10 nM gave a peak rise in $[Ca^{2+}]_i$ of 424.8 ± 73 nM above basal levels (see above, n = 7).

It is also evident that the overall response to GnRH could often be resolved into multiple oscillations in $[Ca^{2+}]_i$. The response illustrated in Fig. 2 is a good example of this (but also see Figs. 1*B*, 4*B*, and 5*A*). Fig. 2 shows frequent

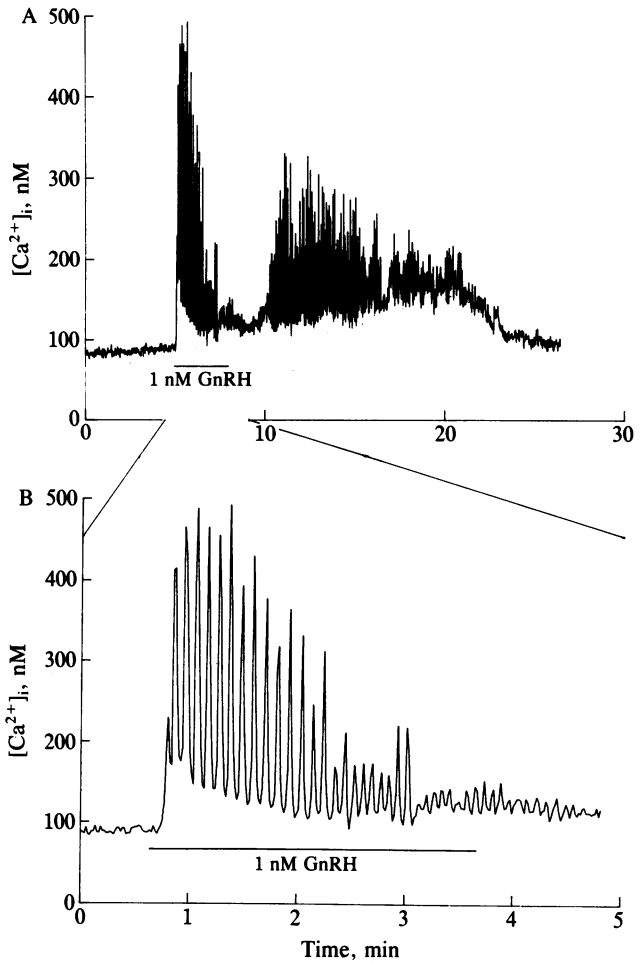

FIG. 2. (*A*) Typical spike and secondary rise induced in a gonadotrope by GnRH (1 nM). (*B*) Spike portion of the response at higher time resolution. Note that the response is composed of multiple oscillations in $[Ca^{2+}]_i$.

oscillations in $[Ca^{2+}]_i$ during both the initial peak and the secondary rise of the response. Similar rapid fluctuations in $[Ca^{2+}]_i$ have been shown to occur during agonist-induced responses in other cell types (16).

Relative Roles of Intra- and Extracellular Ca^{2+} in the Response to GnRH. The different phases of the Ca^{2+} transient appeared to reflect the involvement of Ca^{2+} from different sources. Fig. 3 illustrates the production of multiple responses to GnRH on a single cell. Initially a spike and secondary rise were observed. If we applied GnRH again during the period of the secondary rise, the subsequent $[Ca^{2+}]_i$ spike was attenuated or absent. However, if we waited for the secondary rise to subside, then another full response could be achieved. This process could be repeated many times. Thus it appears that the secondary rise is required in order to produce the spike response more than once. The situation is further clarified in Fig. 4*A*. It can be seen that $[Ca^{2+}]_i$ decreased upon removal of extracellular Ca^{2+}. Once a new resting $[Ca^{2+}]_i$ was established, GnRH was applied. A single spike response was now obtained, but no secondary rise. Application of GnRH a second time proved to be completely ineffective. We then applied a Ca^{2+} load to the cell by depolarizing it (50 mM K^+) in Ca^{2+}-containing medium. This allows Ca^{2+} to enter the cell through voltage-sensitive Ca^{2+} channels and to more rapidly

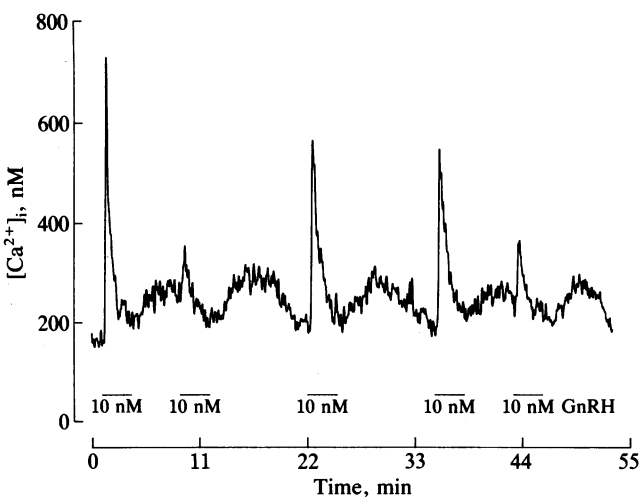

FIG. 3. Effect of multiple additions of GnRH (10 nM) on $[Ca^{2+}]_i$ in a single gonadotrope. The first addition of GnRH produced the typical spike and secondary rise in $[Ca^{2+}]_i$. Subsequent additions of GnRH produced a complete response only if added after the secondary rise had subsided. If GnRH was added during the secondary rise, the spike portion of the response was absent or attenuated.

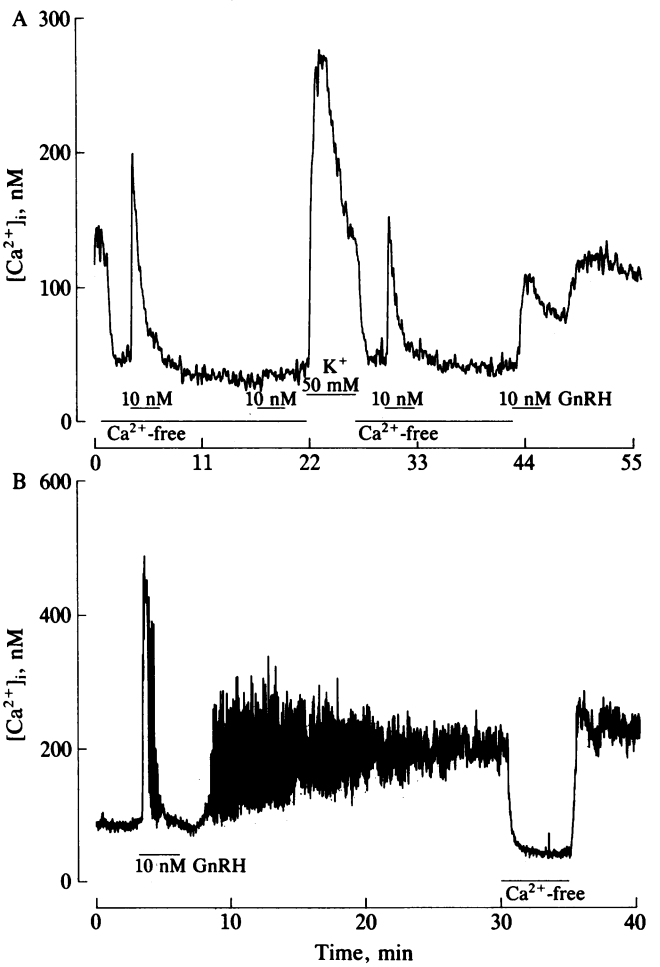

FIG. 4. The secondary rise is due to Ca^{2+} influx. (*A*) With this cell, removal of extracellular Ca^{2+} caused $[Ca^{2+}]_i$ to fall. Addition of GnRH (10 nM) elicited a spike but no secondary rise. Subsequent addition of GnRH elicited no response. The cell was then depolarized in Ca^{2+}-containing medium to promote Ca^{2+} influx. On removal of extracellular Ca^{2+} a GnRH spike response could again be produced. (*B*) Addition of GnRH to this cell produced a spike and a long-lasting secondary rise. Extracellular Ca^{2+} was removed during the secondary rise, and $[Ca^{2+}]_i$ fell only to increase again on readmission of extracellular Ca^{2+}.

refill the intracellular stores. Such responses were observed in all anterior pituitary cells. After a switch back to Ca^{2+}-free medium, GnRH again produced a spike response but no secondary rise. Finally, in Ca^{2+}-containing medium, a GnRH-induced spike and secondary rise could be produced. These results indicate that the initial spike of the Ca^{2+} transient is independent of extracellular Ca^{2+} and presumably results from mobilization of intracellular Ca^{2+} stores. The secondary rise in $[Ca^{2+}]_i$ represents Ca^{2+} influx. This influx is required to reload the Ca^{2+} stores that are mobilized and apparently depleted during the initial, spike portion of the response. Reloading of the stores mobilized by GnRH can also be achieved artificially by causing Ca^{2+} influx into the cell as a result of depolarization. The dependence of the secondary rise on extracellular Ca^{2+} can also be clearly seen in Fig. 4*B*. In this case a spike and secondary rise was produced. Removal of extracellular Ca^{2+} during the period of the secondary rise caused $[Ca^{2+}]_i$ to decline immediately. However, when extracellular Ca^{2+} was reintroduced, $[Ca^{2+}]_i$ immediately rose again to stimulated plateau levels.

Nature of the Pathway for Ca^{2+} Influx. It is likely that the initial spike of the Ca^{2+} transient is produced by inositol trisphosphate ($InsP_3$) synthesized in response to GnRH (2, 3, 5). However, the nature of the pathway responsible for the subsequent influx of Ca^{2+} is not immediately obvious. Several possibilities might be envisaged (5). However, the experiments illustrated in Fig. 5 provided a clear answer. The Ca^{2+} influx during the secondary phase could always be completely blocked by the dihydropyridine Ca^{2+}-channel blocker nitrendipine (see also Fig. 6). Indeed, in the presence of nitrendipine the initial Ca^{2+} spike could be produced but it was never followed by a secondary rise, nor could multiple spike responses be produced (e.g., Fig. 5*A*). Thus the addition of nitrendipine precisely mimicked the effects of removing extracellular Ca^{2+}. These data indicate that GnRH produces the secondary rise in $[Ca^{2+}]_i$ by promoting Ca^{2+} influx through dihydropyridine-sensitive, L-type Ca^{2+} channels (17).

GnRH has been shown to stimulate the production of both $InsP_3$ and diacylglycerol. As $InsP_3$ is presumably responsible for the initial Ca^{2+} spike, we speculated that diacylglycerol might be involved in triggering the subsequent activation of L-type channels. In support of such a proposal, we observed that addition of phorbol 12-myristate 13-acetate produced a slow increase in $[Ca^{2+}]_i$ and enhanced the size of the secondary rise produced by GnRH (Fig. 6). The entire secondary response could be immediately reversed by the addition of nitrendipine.

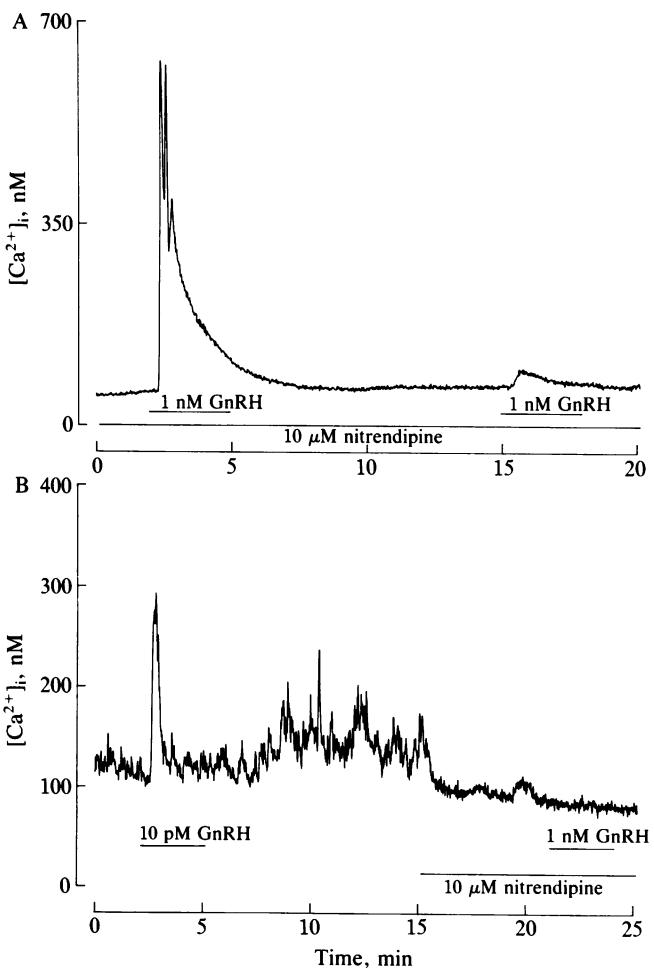

FIG. 5. GnRH-induced Ca^{2+} influx utilizes L-type Ca^{2+} channels. (*A*) In the presence of nitrendipine (10 μM) only the spike portion of the GnRH response could be produced and no subsequent responses could be produced. (*B*) After the production of a spike response to GnRH (10 pM), the secondary rise was inhibited by nitrendipine (10 μM).

DISCUSSION

Several studies have demonstrated the importance of Ca^{2+} in triggering the release of gonadotropin (2). However, the precise source of the Ca^{2+} involved in this response is somewhat controversial (2, 18, 19). In the experiments reported here, we demonstrated that the Ca^{2+} transient elicited by GnRH derives from two sources: intracellular stores and the extracellular milieu. Similar dual effects have been observed in the action of other releasing factors (15, 20). In the case of GnRH the pathway for Ca^{2+} influx appears to be related to the L-type Ca^{2+} channel previously observed in several cell types, as it is effectively blocked by the dihydropyridine nitrendipine (17). It might be imagined that GnRH could depolarize gonadotropes, resulting in the opening of L channels. However, electrophysiological studies have shown that GnRH does not depolarize these cells but leads to the appearance of voltage fluctuations resulting from the opening of a Ca^{2+}-permeable channel in the plasma membrane (21, 22). This channel can be blocked by nifedipine (21, 22). Activation of these Ca^{2+}-permeable channels requires the intervention of a diffusible second messenger (22). The studies reported here, based on the effects of phorbol

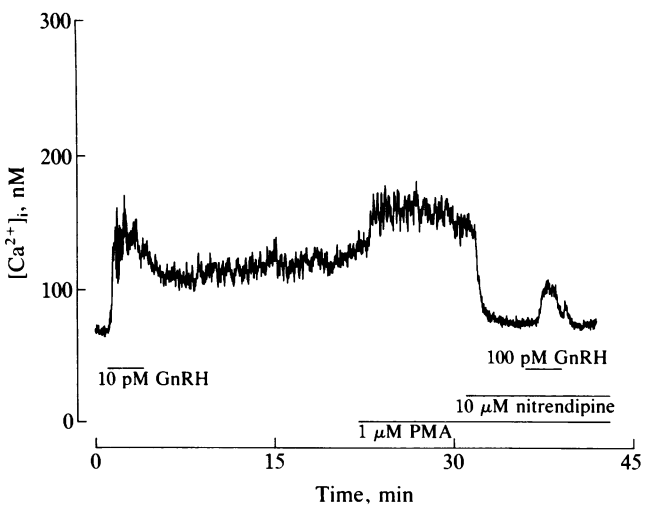

FIG. 6. Secondary rise of GnRH-induced $[Ca^{2+}]_i$ transient can be enhanced by phorbol ester. Phorbol 12-myristate 13-acetate (PMA, 1 μM) enhanced the secondary rise as in this experiment using 10 pM GnRH. The entire secondary response could be blocked by nitrendipine (10 μM).

ester, suggest the possibility that the diffusible second messenger could be diacylglycerol. Indeed, GnRH stimulates the breakdown of phosphatidylinositol bisphosphate in gonadotropes, producing diacylglycerol and InsP_3 (2, 23, 24). As GnRH does not depolarize gonadotropes, the mechanism by which it causes L-channel opening is unclear. One possibility is that the channels become modified in such a way that their voltage dependence of activation is shifted in the hyperpolarizing direction, allowing them to open at resting membrane potentials. Such effects are produced, for example, by dihydropyridine Ca^{2+}-channel agonists such as BAY K 8644 (25). Perhaps protein kinase C-mediated phosphorylation of the channel in this case produces a conformational change that results in gating behavior similar to that observed with this drug. Considering the effects of GnRH on breakdown of phosphatidylinositol bisphosphate, it seems reasonable to speculate that the initial spike of the GnRH-induced Ca^{2+} transient is the result of InsP_3-induced Ca^{2+} mobilization. The significance of the multiple oscillations in $[Ca^{2+}]_i$ that we observed is difficult to assess. It has been proposed, however, that the frequency in addition to the amplitude of such transients may be important in Ca^{2+} signaling (16). In some cells the interaction of InsP_3 with its intracellular receptor can be inhibited by Ca^{2+} (26). This negative-feedback system may provide a mechanism by which such transients can be produced.

It is of interest to consider the relationship between the various phases of the GnRH-induced Ca^{2+} transients we observed and the GnRH-induced release of pituitary hormones. There is no consensus as to the role of GnRH-induced inositolphospholipid breakdown in this process. Thus, evidence for (19) and against (2, 27) the importance of intracellular Ca^{2+} mobilization has been presented. Space does not allow a thorough discussion of the literature relating to this important point. Furthermore, as both LH and FSH can be released in response to GnRH, it is possible that the $[Ca^{2+}]_i$ requirement may differ in these two cases. However, the following should be noted. LH release has been shown to be partially or completely dependent on Ca^{2+} influx (2, 19). This Ca^{2+} influx apparently involves L-type channels (19, 28), although there is also some disagreement on this point (2, 29). However, recent studies, which monitored LH release on a relatively rapid time base, indicated that L-channel blockers did not block an initial spike of LH release but did block subsequent release over an extended time course (19). The nitrendipine-sensitive and -insensitive portions of LH release reported in that study exhibit a close temporal correlation with the spike and secondary rise phases of the Ca^{2+} transients reported here. Thus it may be that both phases of the Ca^{2+} response participate in the regulation of LH release. The initial spike and reloading phases of the Ca^{2+} transient may be important in the normal response of gonadotropes to pulsatile stimulation by GnRH *in situ* (30).

The results presented here are important for understanding the precise role of Ca^{2+} in the release of gonadotropins. However, many questions remain to be completely addressed. As discussed above it is of particular interest to consider the relative role of $[Ca^{2+}]_i$ in the release of FSH and LH. As these two hormones are frequently found in the same cells (6, 31) it would be interesting to know whether or not their release was regulated conjointly. Some evidence for separate mechanisms of control has been presented (31, 32). An answer to this question will require the simultaneous assay of hormone release and $[Ca^{2+}]_i$ at the single-cell level. Current techniques make such experiments feasible (33).

We are indebted to Prof. Nira Ben-Jonathan (Department of Physiology, University of Indiana) for providing the LH antiserum used in this study and to her and Dr. Tom Croxton for instructing us in the reverse hemolytic plaque assay. The research was supported by Public Health Service Grants DA02121, DA02575, and MH40165 and by grants from Miles Laboratories and Marion Laboratories. G.A.S. was also supported by Public Health Service Grant HD00708 and the Mother's Aid Research Fund, and S.N.M. by Public Health Service Grant GM07151-11.

1. Baker, P. F. (1987) in *Structure and Physiology of the Slow Inward Calcium Channel,* eds. Venter, J. C. & Triggle, D. (Liss, New York), pp. 247–265.
2. Conn, P. M., Huckle, W. R., Andrews, W. V. & McArdle, C. A. (1987) *Recent Prog. Horm. Res.* **43,** 29–68.
3. Berridge, M. J. (1984) *Biochem. J.* **220,** 345–360.
4. Tsien, R. W. (1983) *Annu. Rev. Physiol.* **45,** 341–358.
5. Putney, J. W. (1986) *Cell Calcium.* **7,** 1–12.
6. Childs, G. V. (1984) *Endocrinology* **114,** 499–502.
7. Limor, R., Ayalon, D., Capponi, A. M., Childs, G. V. & Naor, Z. (1987) *Endocrinology* **120,** 497–503.
8. Clapper, D. L. & Conn, P. M. (1985) *Biol. Reprod.* **32,** 269–278.
9. Neill, J. D. & Frawley, S. C. (1983) *Endocrinology* **112,** 1135–1145.
10. Thayer, S. A., Murphy, S. N. & Miller, R. J. (1986) *Mol. Pharmacol.* **30,** 305–309.
11. Croxton, T. L., Armstrong, W. M.-D. & Ben-Jonathan, N. (1988) *Methods Enzymol.*, in press.
12. Thayer, S. A. & Miller, R. J. (1988) *Pflügers Arch.*, in press.
13. Scanlon, M., Williams, D. A. & Fay, F. S. (1987) *J. Biol. Chem.* **262,** 6308–6312.
14. Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985) *J. Biol. Chem.* **260,** 3440–3450.
15. Winger, B. P., Wuarin, F., Zahnd, G. R., Wollheim, C. B. & Schlegl, W. (1987) *Endocrinology* **121,** 2222–2228.
16. Woods, N. M., Cuthbertson, K. S. R. & Cobbold, P.H. (1986) *Nature (London)* **319,** 600–602.
17. Miller, R. J. (1987) *Science* **235,** 46–52.
18. Bates, M. D. & Conn, P. M. (1984) *Endocrinology* **115,** 1380–1385.
19. Smith, C. E., Wakefield, I., King, J. A., Naor, Z., Miller, R. P. & Davidson, J. S. (1987) *FEBS Lett.* **225,** 247–250.
20. Albert, P. A. & Tashjian, A. H. (1984) *J. Biol. Chem.* **259,** 5827–5832.
21. Mason, W. T. & Waring, D. W. (1985) *Neuroendocrinology* **41,** 258–268.
22. Mason, W. T. & Waring, D. W. (1986) *Neuroendocrinology* **43,** 205–219.
23. Kiesel, L. & Catt, K. J. (1984) *Arch. Biochem. Biophys.* **231,** 202–210.
24. Andrews. W. V. & Conn, P. M. (1986) *Endocrinology* **118,** 1148–1158.
25. Sanguinetti, M. C., Krafte, D. S. & Kass, S. C. (1986) *J. Gen. Physiol.* **88,** 369–392.
26. Worley, P. F., Barabau, J. M., Supattapone, S., Wilson, U. S. & Snyder, S. H. (1987) *J. Biol. Chem.* **262,** 12132–12136.
27. Naor, Z., Katikineni, M., Loumaye, E., Vela, A. G., Dufau, M. L. & Catt, K. J. (1982) *Mol. Cell. Endocrinol.* **27,** 213–220.
28. Chang, J. P., McCoy, E. E., Graeter, J., Tasaka, K. & Catt, K. J. (1986) *J. Biol. Chem.* **261,** 9105–9108.
29. Conn, P. M., Rogers, D. C. & Seay, S. G. (1983) *Endocrinology* **113,** 1592–1595.
30. Veldhuis, J. D., Evans, W. S., Rogol, A. D., Drake, C. R., Thorner, M. O., Merriam, G. R. & Johnson, M. L. (1984) *J. Clin. Endocrinol. Metab.* **59,** 96–102.
31. Lloyd, J. M. & Childs, G. V. (1988) *Endocrinology* **122,** 1282–1290.
32. Denef, C., Hautekeete, E., Deevals, R. & De Wolf, A. (1980) *Endocrinology* **106,** 724–729.
33. Neher, E. (1988) *J. Physiol. (London)* **395,** 193–214.