tions without probe were first incubated for 20 min on ice. Sequences of the octamer-heptamer (octhep), Prl 1P, and CRH sites were as described [J. M. Mathis, D. Simmons, X. He, L. W. Swanson, M. G. Rosenfeld, *EMBO J.* **11**, 2551 (1992)]. The DE2 site is 5′-GAGTGGAGATCCAACAGCATCCTTAATTAAGTTCCT-3′.
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23. Approximately 100,000 cpm of ^{35}S-labeled protein were added at room temperature to 20 μl of binding buffer [10 mM Hepes (pH 7.8), 50 mM KCl, and 5% glycerol]. After 15 min at room temperature, 0.5 μl of DSS in dimethyl sulfoxide (DMSO; 5 mg/ml) or DMSO alone was added to the reactions. After 5 min, we stopped the reaction by adding SDS sample buffer (*22*). The sample was boiled before analyses by SDS-PAGE. To quantitate cross-linking efficiency, we excised monomers and dimers from the gel and measured them by scintillation counting.
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31. Rat-1 cells were plated at low density the day before transfection. Transfections were performed by the calcium phosphate method as described (*22*). Cells were harvested after 48 hours, and luciferase activity measured. HeLa cells were transfected with the lipofectant DOTAP (Boehringer Mannheim) according to the manufacturer's instructions. Cells were harvested after 36 to 48 hours.
32. We used RNA from rat neonatal skin with random hexamers and avian myeloblastosis virus RT to generate cDNA that was cloned into λZAP II (Stratagene). This library was screened with a ^{32}P-labeled probe corresponding to the entire NH_2-terminus and the POU domain of Skn-1i. Three independent clones were isolated that together corresponded to the entire common region between Skn-1i and Skn-1a. One of the clones, Skn-1a, contained a 5′-sequence that predicted a different NH_2-terminus as a result of alternative splicing. The expression of both forms in skin was demonstrated with the PCR with primers specific for each form and with RNA hybridization studies (*18*).
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Rhythmic Exocytosis Stimulated by GnRH-Induced Calcium Oscillations in Rat Gonadotropes

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In pituitary gonadotropes, gonadotropin-releasing hormone (GnRH) induces the rhythmic release of Ca^{2+} from an inositol 1,4,5-trisphosphate (IP_3)–sensitive store. Simultaneous measurement of the concentration of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) and exocytosis in single identified gonadotropes showed that each elevation of $[Ca^{2+}]_i$ induced a burst of exocytosis. These phenomena were largely suppressed by buffering of $[Ca^{2+}]_i$ but persisted in the absence of extracellular Ca^{2+}. Activation of voltage-gated Ca^{2+} channels by brief depolarizations seldom supplied enough Ca^{2+} for exocytosis, but $[Ca^{2+}]_i$ elevations induced by photolysis of caged IP_3 did trigger exocytosis, confirming that GnRH-stimulated gonadotropic hormone secretion is closely coupled to intracellular Ca^{2+} release. Agonist-induced oscillations of $[Ca^{2+}]_i$ in secretory cells may be a mechanism to optimize the secretory output while avoiding the toxic effects of sustained elevation of $[Ca^{2+}]_i$.

Although many cells display oscillations in $[Ca^{2+}]_i$ in response to agonists, physiological roles for these oscillations are unclear (*1*). Pituitary gonadotropes exhibit oscillations in $[Ca^{2+}]_i$ in response to their natural stimulating hormone, GnRH (*2*). Each cycle of increase in $[Ca^{2+}]_i$ hyperpolarizes the cell by opening apamin-sensitive Ca^{2+}-activated K^+ channels (*3–5*). This allows voltage-gated Na^+ and Ca^{2+} channels to recover from inactivation and then to fire action potentials when $[Ca^{2+}]_i$ declines again and the cell depolarizes (*5*). Thus, GnRH stimulates both the entry of extracellular Ca^{2+} through voltage-gated Ca^{2+} channels and the release of intracellular Ca^{2+} in gonadotropes. Understanding the relative contribution of these mechanisms to GnRH-induced secretion of gonadotropic hormones (*6*), and particularly whether each cycle of increase in $[Ca^{2+}]_i$ can trigger secretion, has been difficult because of the low temporal resolution of traditional assays for hormone secretion. We used high temporal resolution capacitance measurements (*7*), which monitor changes in cell membrane capacitance (ΔC_m) resulting from exocytosis of secretory vesicles, to measure simultaneously exocytosis and $[Ca^{2+}]_i$ in identified gonadotropes of adult male rats (*8*).

GnRH-induced $[Ca^{2+}]_i$ oscillations are readily seen with fluorescent indicators, even in gonadotropes voltage-clamped to potentials (−90 mV) at which voltage-gated Ca^{2+} channels are closed. A 10-s application of GnRH caused $[Ca^{2+}]_i$ to oscillate (Fig. 1A). In 40 cells, the resting $[Ca^{2+}]_i$ was 109 ± 16 nM, and the maximal $[Ca^{2+}]_i$ induced by a brief application of GnRH (40 or 50 nM) was 3.3 ± 0.2 μM. The rising phase of each cycle of $[Ca^{2+}]_i$ elevation was accompanied by an increase in C_m (Fig. 1B) and by an increase in the rate of exocytosis ($\Delta C_m/\Delta t$) (Fig. 1C). In later cycles of the $[Ca^{2+}]_i$ oscillation (for

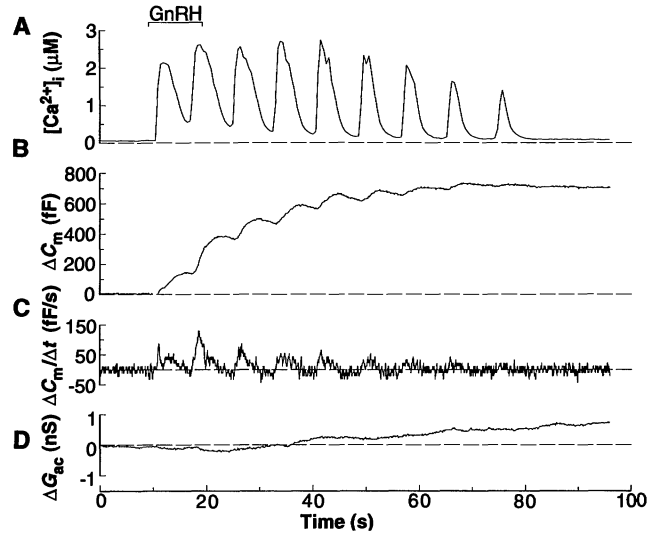

Fig. 1. Time course of GnRH-induced oscillations in $[Ca^{2+}]_i$ and accompanying bursts of exocytosis. (**A**) $[Ca^{2+}]_i$. (**B**) ΔC_m. (**C**) $\Delta C_m/\Delta t$. (**D**) ΔG_{ac}. GnRH (40 nM) was applied during the 10-s period marked with a bar. The initial membrane capacitance was 6.8 pF. To reduce contamination of capacitance signal by conductance changes, we voltage-clamped the cell at a holding potential of −90 mV to shut off most voltage-gated ionic channels, and the GnRH-induced rhythmic increase in Ca^{2+}-activated K^+ conductance was blocked by extracellular apamin and TEA (*3*). Ionic conductance changes were indeed minimal under these conditions (ΔG_{ac} trace).

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example, after the third cycle), increases of C_m and the maximum rate of exocytosis became smaller. A similar sequence of $[Ca^{2+}]_i$ elevations and exocytosis could be repeated six to ten times by brief application of GnRH every 2 min. After each $[Ca^{2+}]_i$ elevation, when $[Ca^{2+}]_i$ fell C_m decreased slightly, presumably reflecting endocytosis. As in chromaffin cells (9) and melanotropes (*10*), endocytosis could be gradual and small or abrupt and large. Rapid endocytosis usually followed a burst of fast exocytosis. Such variability in endocytosis has also been observed in pancreatic β cells recorded with the perforated-patch technique (*11*), which minimizes loss of intracellular components during whole-cell recording.

When the $[Ca^{2+}]_i$ oscillation stopped, the cumulative ΔC_m was ~700 fF, which corresponds to an increase of >10% of the membrane surface area (Fig. 1B). On the basis of published electron microscopy (*12*), we estimate that there are ~10,000 small secretory vesicles in a male rat gonadotrope and that between 600 to 1000 vesicles are located within one vesicle diameter (average diameter ~200 nm) of the membrane. Each vesicle should contribute a capacitance of 1.3 fF. Hence, more than 540 vesicles were released after a single 10-s application of GnRH (Fig. 1).

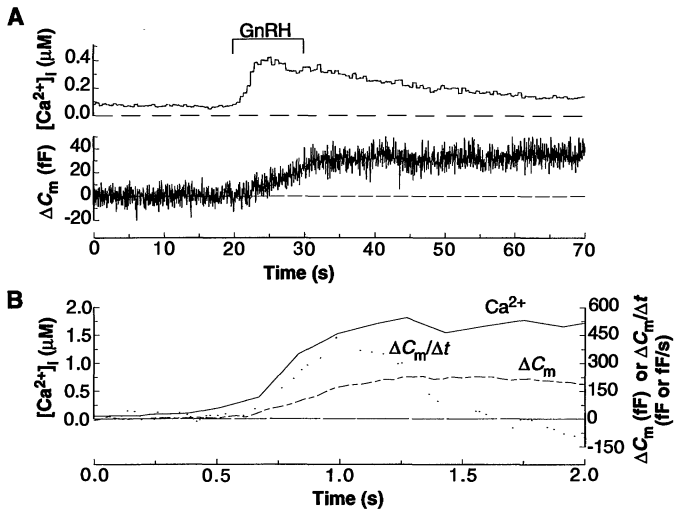

Fig. 2. Dependence of exocytosis on an increase in $[Ca^{2+}]_i$. (**A**) Damping of exocytosis by buffering of intracellular Ca^{2+}. The pipette solution contained 6 mM $CaCl_2$ and 10 mM EGTA (67 nM free Ca^{2+}), and the cell was held at −70 mV. Initially, $[Ca^{2+}]_i$ was 75 nM, and the membrane capacitance was 5.4 pF. After application of GnRH, membrane capacitance increased by less than 30 fF. (**B**) Temporal relation of $[Ca^{2+}]_i$, ΔC_m, and $\Delta C_m/\Delta t$ during the first GnRH-induced elevation of $[Ca^{2+}]_i$ in a cell without Ca^{2+} chelator. The value of $\Delta C_m/\Delta t$ reached a maximum when $[Ca^{2+}]_i$ was 1.48 μM even though $[Ca^{2+}]_i$ stayed above this concentration for more than 1 s. The $[Ca^{2+}]_i$ was sampled every 100 ms; the pipette solution was as in Fig. 1. The holding potential was −70 mV and the initial membrane capacitance was 6.2 pF.

To investigate whether GnRH-induced exocytosis requires an increase in $[Ca^{2+}]_i$, we examined the response to GnRH of cells loaded with an intracellular solution containing a Ca^{2+} chelator (Fig. 2A). Application of GnRH to these cells resulted in one small increase in $[Ca^{2+}]_i$ (to 0.51 ± 0.07 μM; n = 10) and a very small cumulative increase in C_m (8.7 ± 3.1 fF), which was 37 times smaller than that in cells in which intracellular Ca^{2+} was not chelated (321 ± 37 fF; n = 54). Thus, GnRH induced little exocytosis when the increase in $[Ca^{2+}]_i$ was suppressed. We examined the dependence of exocytosis on $[Ca^{2+}]_i$ during the first elevation of $[Ca^{2+}]_i$ in cells without intracellular Ca^{2+} chelator (Fig. 2B). The onset of exocytosis could be reliably detected ($\Delta C_m/\Delta t$ > 50 fF/s) at $[Ca^{2+}]_i$ >272 ± 26 nM (n = 20). In most cells, $\Delta C_m/\Delta t$ increased initially with the increase in $[Ca^{2+}]_i$ but began to fall before $[Ca^{2+}]_i$ peaked (Fig. 2B) (33 of 36 experiments; in the remainder, maximum $\Delta C_m/\Delta t$ coincided with maximum $[Ca^{2+}]_i$). In 40 experiments, the maximum $\Delta C_m/\Delta t$ (292 ± 35 fF/s, corresponding to 224 ± 27 vesicles per second; range, <20 to 853 fF/s) occurred when $[Ca^{2+}]_i$ was 2.2 ± 0.1 μM. The decline in $\Delta C_m/\Delta t$ while $[Ca^{2+}]_i$ was still rising in the first elevation probably reflects the depletion of a finite pool of readily releasable vesicles (9, *13*) and also suggests that a sustained elevation of $[Ca^{2+}]_i$ would not result in a faster secretion rate (see also Fig. 3A). During oscillations in $[Ca^{2+}]_i$ (Fig. 1), $\Delta C_m/\Delta t$ rose and fell with each elevation; thus, additional vesicles must be mobilized to the releasable pool between each cycle. Consistent with this observation, transient elevation of $[Ca^{2+}]_i$ produces a much faster rate of secretion than prolonged elevation of $[Ca^{2+}]_i$ in chromaffin cells (9).

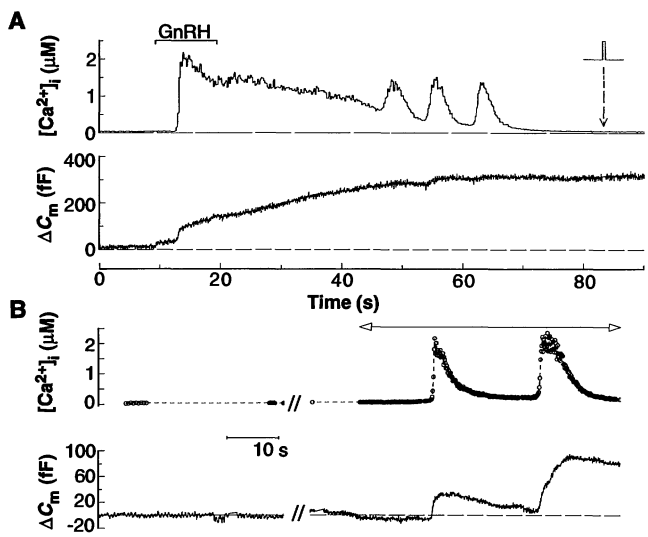

Fig. 3. Exocytosis triggered by release of intracellular Ca^{2+}. (**A**) Ca^{2+} oscillations and exocytosis in a cell bathed in a Ca^{2+}-free solution containing 3 mM Mg^{2+} and 1 mM EGTA for 2 min before application of GnRH. The holding potential was −70 mV, and initial membrane capacitance was 6.0 pF. At 82 s, a 0.5-s depolarizing voltage step to 0 mV failed to induce any change in $[Ca^{2+}]_i$ because extracellular Ca^{2+} was absent. (**B**) Increases of $[Ca^{2+}]_i$ and exocytosis after slow photolysis of intracellular caged IP_3. Pipette solution was as in Fig. 1 with caged IP_3 (10 μM; Calbiochem) (*20*). For measurement of the initial resting $[Ca^{2+}]_i$ without photolysis of caged IP_3, the shutter was opened infrequently (as indicated by the circles) for 14 ms, and the fluorescence was integrated for 5 ms. The initial low resting $[Ca^{2+}]_i$ and the flat ΔC_m trace (C_m was 5.2 pF) indicate that contamination of free IP_3 in the caged IP_3 was below the threshold for triggering intracellular Ca^{2+} release. When exposure to ultraviolet (UV) light was increased by opening of the shutter for 55 ms every 100 ms (indicated by horizontal arrow), sufficient IP_3 was continuously generated to induce two elevations of $[Ca^{2+}]_i$, each accompanied by increases in C_m. Because the UV illumination was focused through an objective, only the caged IP_3 in the cell and in the tip of the pipette was photolysed.

Two lines of evidence argue that release of intracellular Ca^{2+} from IP_3-sensitive stores underlies GnRH-induced exocytosis. First, neither elevations in $[Ca^{2+}]_i$ nor exocytosis requires extracellular Ca^{2+} (Fig. 3A) (*14*). The cumulative GnRH-induced increase in C_m in the absence of extracellular Ca^{2+} was 284 ± 65 fF (n = 5), as compared with 321 fF in the presence of 2 or 5 mM extracellular Ca^{2+}. Second, gradual photolysis of caged IP_3 triggered agonist-independent $[Ca^{2+}]_i$ elevations and exocytosis (Fig. 3B) (n = 9). These results contrast with those from mast cells, in

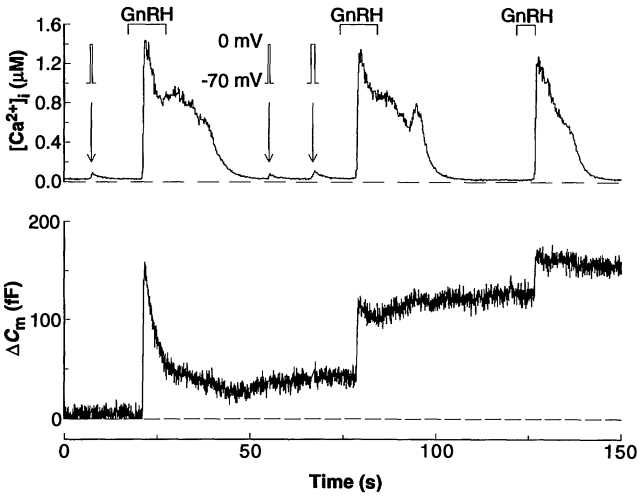

Fig. 4. Lack of effect of depolarizing voltage steps on exocytosis. A 0.5-s voltage step to 0 mV (first arrow) resulted in a small transient rise of $[Ca^{2+}]_i$ and no detected increase in C_m. In contrast, applications of GnRH resulted in increases in $[Ca^{2+}]_i$ as well as increases in C_m. Two further depolarizations (of 0.5 and 1 s) still failed to supply enough Ca^{2+} to trigger exocytosis. Note that the first large exocytosis is followed by rapid endocytosis. The cell was voltage-clamped at −70 mV, and the initial membrane capacitance was 5.0 pF.

which intracellular Ca^{2+} release is not tightly coupled to secretion (*15*).

We also examined the contribution to exocytosis of voltage-gated entry of extracellular Ca^{2+} (2 mM). As in lactotrophs (*16*), 0.5- or 1-s depolarizations in gonadotropes usually resulted in a transient increase in $[Ca^{2+}]_i$ that was too small (<100 nM) to trigger exocytosis (Fig. 4) (n = 11). In contrast, exocytosis could be triggered in chromaffin cells when the average $[Ca^{2+}]_i$ was raised to 100 nM by depolarization, but not by intracellular dialysis of solutions containing ≤200 nM Ca^{2+} (*9*). This depolarization-induced exocytosis was attributed to local high $[Ca^{2+}]_i$ (underestimated during mean Ca^{2+} measurements) near the vesicles when secretory sites are in close proximity to the voltage-gated Ca^{2+} channels. Thus, in gonadotropes, most vesicles may be further away from the Ca^{2+} channels.

Hormone-stimulated exocytosis from a pituitary cell is tightly coupled to an oscillatory release of Ca^{2+} from intracellular stores that leads to micromolar increases in $[Ca^{2+}]_i$. Each increase in $[Ca^{2+}]_i$ can trigger a burst of exocytosis [see (*17*)]. We suggest that each $[Ca^{2+}]_i$ elevation rapidly releases the most readily available secretory vesicles; while $[Ca^{2+}]_i$ is lowered, new vesicles are mobilized, and overstimulation of other Ca^{2+}-dependent processes is avoided. When $[Ca^{2+}]_i$ decreases, the plasma membrane depolarizes, a few action potentials are fired (3 to 5), and the attending Ca^{2+} influx (1.8×10^4 Ca^{2+} ions per action potential) (*5*) may compensate for the small amount of Ca^{2+} lost during each increase in $[Ca^{2+}]_i$. Oscillations in $[Ca^{2+}]_i$ have the advantage of reducing the toxic effects of Ca^{2+} yet maintaining (over a few minutes) a secretory output comparable to that attained by sustained elevation of $[Ca^{2+}]_i$. This may be ideal in endocrine cells such as gonadotropes, which are normally stimulated by GnRH in the portal circulation for several minutes every hour (*18*). Agonist-induced $[Ca^{2+}]_i$ oscillations in other secretory cells (*19*) may have a similar role.

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8. Gonadotropes were isolated from adult (35- to 45-day-old) male Sprague-Dawley rats and identified with the reverse hemolytic-plaque assay [P. F. Smith, L. S. Frawley, J. D. Neill, *Endocrinology* **115**, 2484 (1984)]. Details of experimental procedures have been described (*3*) [A. Tse and B. Hille, in *Pulsatility in Neuroendocrine Systems: Methods in Neurosciences*, J. E. Levine, Ed. (Academic Press, Orlando, FL, in press)]. The cells were voltage-clamped with the whole-cell, gigaseal method [O. P. Hamill, A. Marty, E. Neher, B. Sakmann, F. J. Sigworth, *Pfluegers Arch.* **391**, 85 (1981)]. Unless indicated otherwise, the pipette solution contained 120 mM potassium aspartate, 20 mM KCl, 0.1 mM sodium guanosine triphosphate, 2 mM $MgCl_2$, 2 mM sodium adenosine triphosphate, 20 mM Hepes (pH 7.4 with KOH), and 0.1 mM indo-1. The bathing solution contained 150 mM tetraethyl ammonium chloride (TEA), 2 or 5 mM $CaCl_2$, 1 mM $MgCl_2$, 8 mM glucose, and 10 mM Hepes (pH 7.4 with NaOH). A −10-mV junction potential was corrected throughout. In all experiments, GnRH (40 or 50 nM) with apamin (1 μM) was briefly applied with pressure from a puffer pipette while the bath was continuously perfused. We measured changes in membrane capacitance (ΔC_m) and alternating current conductance (ΔG_{ac}) with a two-phase lock-in amplifier by superimposing an 800-Hz sinusoid of 30 mV peak-to-peak amplitude onto the holding potential. The values ΔC_m and ΔG_{ac} were low pass–filtered with an RC circuit of 10-ms time constant. The value $\Delta C_m/\Delta t$ is the slope of successive 400-ms sections of ΔC_m. We measured $[Ca^{2+}]_i$ fluorometrically by introducing the Ca^{2+}-sensitive dye indo-1 into the cell through the patch pipette. Indo-1 was excited by light (365 nm) passing through an electronic shutter and a 40×, 1.3 numerical aperture oil objective. Fluorescence at 405 and 495 nm, collected by two photomultiplier tubes, was integrated over a duration of 5 to 20 ms and measured at 100- to 500-ms intervals. Background fluorescence measured after formation of a cell-attached seal was subtracted. We calculated $[Ca^{2+}]_i$ from the ratio R of fluorescence (405 nm/495 nm) with the following equation: $[Ca^{2+}] = K^* (R - R_{min})/(R_{max} - R)$ [G. Grynkiewicz, M. Poenie, R. Y. Tsien, *J. Biol. Chem.* **260**, 3440 (1985)]. R_{min} was the fluorescence ratio measured in gonadotropes loaded with 62 mM potassium aspartate, 50 mM Hepes (pH 7.4 with KOH), 50 mM EGTA, and 0.1 mM indo-1. R_{max} was measured in cells loaded with 136 mM potassium aspartate, 50 mM Hepes (pH 7.4 with KOH), 15 mM $CaCl_2$, and 0.1 mM indo-1. K^* was calculated from the equation with the ratio obtained from cells loaded with 20 mM potassium aspartate, 50 mM Hepes (pH 7.4 with KOH), 50 mM EGTA, and 39.5 mM $CaCl_2$, which has a calculated free Ca^{2+} concentration of 158 nM [J. R. Blinks, W. G. Wier, P. Hess, F. G. Prendergast, *Prog. Biophys. Mol. Biol.* **40**, 1 (1982)]. Values for R_{min}, R_{max}, and K^* were as follows: 0.35, 3.86, and 1882 nM for Fig. 1; and 0.35, 6.99, and 2433 nM for Figs. 2 to 4. All experiments were done at room temperature (20° to 25°C). Values given in the text are means ± SEM.
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