GnRH-Induced Cytosolic Calcium Oscillations in Pituitary Gonadotrophs: Phase Resetting by Membrane Depolarization

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ABSTRACT Cultured rat pituitary gonadotrophs under whole-cell voltage clamp conditions respond to the hypothalamic hormone GnRH with synchronized oscillatory changes in both cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and $[Ca^{2+}]_i$-activated, apamin-sensitive K^+ current ($I_{K(Ca)}$). We found, and report here for the first time, that in GnRH-stimulated cells a brief depolarizing pulse can elicit a transient $[Ca^{2+}]_i$ rise similar to the endogenous cycle. Furthermore, Ca^{2+} entry during a single depolarizing pulse was found to shift the phase of subsequent endogenous $[Ca^{2+}]_i$ oscillations, which thereafter continue to occur at their previous frequency before the pulse. Application of two consecutive depolarizing pulses showed that the size of the $[Ca^{2+}]_i$ rise evoked by the second pulse depended on the time lapsed between two consecutive pulses, indicating that each endogenous or evoked $[Ca^{2+}]_i$ rise cycle leaves the Ca^{2+} release mechanism of the gonadotroph in a refractory state. Recovery from this condition can be described by an exponential function of the time lapsed between the pulses (time constant of ca. 1 s). We propose that the underlying mechanism in both refractoriness after endogenous cycles and phase resetting by a brief pulse of Ca^{2+} entry involves the $InsP_3$ receptor-channel molecule presumed to be located on the cytosolic aspect of the endoplasmic reticulum membrane.

INTRODUCTION

Pituitary gonadotrophs secrete luteinizing hormone (LH) in response to the hypothalamic gonadotropin-releasing hormone (GnRH). Early studies on cultured rat gonadotrophs revealed that the pattern of agonist-induced LH secretion from a population of cells is biphasic, with an initial transient phase of release and a delayed phase of sustained secretion (Davidson et al., 1988; Naor et al., 1988; Izumi et al., 1989; Stojilkovic et al., 1990, 1992). Whereas the early transient phase of LH release is relatively independent of external $[Ca^{2+}]$, the sustained phase is inhibited in Ca^{2+}-deficient medium and thus depends on Ca^{2+} entry. Moreover, the delayed phase is profoundly diminished by nifedipine, a blocker of L-type Ca^{2+} channels (Izumi et al., 1989; Stojilkovic et al., 1990). Furthermore, early measurements of intracellular Ca^{2+} concentration changes after GnRH stimulation in dispersed gonadotrophs also show a biphasic $[Ca^{2+}]_i$ rise, suggesting a tight association between $[Ca^{2+}]_i$ and the LH release process (Tasaka et al., 1988; Naor et al., 1988; Izumi et al., 1989; Stojilkovic et al., 1990, 1992). In contrast, $[Ca^{2+}]_i$ measurements at the single isolated cell level revealed that GnRH evokes $[Ca^{2+}]_i$ elevations, and thus $[Ca^{2+}]_i$ measurements in a cell population represent the time average of nonsynchronized $[Ca^{2+}]_i$ oscillations (Shangold et al., 1988; Iida et al., 1991; Rawlings et al., 1992).

It is well established that GnRH receptor activation by the agonist is coupled to phospholipase C by a specific $G\alpha_q$ protein, and the ensuing production of second messengers $InsP_3$ and diacylglycerol (Morgan et al., 1987; Chang et al., 1988) in turn leads to the biphasic pattern of Ca^{2+} release from the ER, in which the second phase involves periodic elevations in $[Ca^{2+}]_i$ from its resting level (Stojilkovic et al., 1992). It is often assumed that these $[Ca^{2+}]_i$ oscillations sustain the second phase of LH secretion during prolonged GnRH stimulation (Stojilkovic et al., 1990, 1992). Because single cell $[Ca^{2+}]_i$ measurements revealed a tight relationship between Ca^{2+} entry and maintenance of the $[Ca^{2+}]_i$ oscillations (Stojilkovic et al., 1992; Kukuljan et al., 1994), it has been proposed that Ca^{2+} entry is required to reload the ER after each $[Ca^{2+}]_i$ oscillation cycle (Kukuljan et al., 1994).

Although randomly occurring action potentials can evoke small amplitude $[Ca^{2+}]_i$ elevations in nonstimulated single gonadotrophs, this spontaneous Ca^{2+} entry is insufficient to initiate $[Ca^{2+}]_i$ oscillations (unpublished observations). Furthermore, even though a sudden elevation in external $[K^+]$ can evoke a biphasic $[Ca^{2+}]_i$ rise both in cell populations (Iida et al., 1991) and in single isolated gonadotrophs, the sustained depolarization of the cells in high external $[K^+]$ is unable to initiate $[Ca^{2+}]_i$ oscillations.

Simultaneous measurements of $[Ca^{2+}]_i$ and the $[Ca^{2+}]_i$-dependent, apamin-sensitive K^+ current ($I_{K(Ca)}$) in cultured single isolated rat LH-secreting cells (Kukuljan et al., 1992) revealed that both membrane $I_{K(Ca)}$ and $[Ca^{2+}]_i$ oscillations are synchronous, i.e., the frequency of the $[Ca^{2+}]_i$ oscillations is identical to that of the plasma membrane $I_{K(Ca)}$ oscillations (Kukuljan et al., 1994) and, probably the amplitude of $I_{K(Ca)}$ follows the $[Ca^{2+}]_i$ near the intracellular mouth of the K^+ channel. These results suggest that the cellular systems responsible for both plasma membrane and cytosolic oscillations are tightly coupled (Li et al., 1994;

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1995), providing a mechanism to explain both initial and sustained $[Ca^{2+}]_i$ elevations.

This work was undertaken to study the kinetics of the interactions between the plasma membrane and the cytosolic oscillatory systems during the second phase of the GnRH-evoked response. Application of brief (100 ms) depolarizing voltage clamp pulses was used to induce Ca^{2+} influx at a desired time during a cycle of the sustained phase of GnRH-induced oscillations. To gather kinetic information on the dynamics of such interactions we applied two depolarizing pulses and measured the changes in the amplitude and frequency of the endogenous $I_{K(Ca)}$ oscillations, a measure of the $[Ca^{2+}]_i$ oscillations near the plasma membrane (Stojilkovic et al., 1993; Kukuljan et al., 1994). We found and report here that it is possible to shift the phase of the endogenous $[Ca^{2+}]_i$ oscillations by inducing Ca^{2+} entry during the application of a depolarizing pulse after a variable time interval from the peak of each endogenous cycle. This brief Ca^{2+} influx activated a premature cycle of $[Ca^{2+}]_i$ rise, the magnitude of which was found to increase with time lapsed between the peak of the previous endogenous cycle and the onset of the pulse. We propose that the amplitude of these elicited cycles defines the time course of the refractory state of the Ca^{2+} release mechanism immediately after the peak of $[Ca^{2+}]_i$ during each cycle. A preliminary presentation of the data in an abstract form has been made to the Biophysical Society (Vergara et al., 1994).

MATERIALS AND METHODS

Gonadotrophs were prepared by enzymatic dispersion of pituitary glands obtained from ovariectomized rats as described elsewhere (Stojilkovic et al., 1989).

Patch clamp experiments

Cells were plated on 35-mm dishes (250,000 cells/dish) and kept in culture for 2–3 days at 37°C in an atmosphere of 95% air and 5% CO_2. Experiments were carried out at room temperature (20–25°C). Measurements of whole-cell currents under voltage-clamp conditions were made using the nystatin-perforated membrane patch method (Horn and Marty, 1988) as described elsewhere (Kukuljan et al., 1994). Whole-cell membrane currents were recorded using an EPC-7 amplifier (List Electronics, Darmstadt-Eberstadt, Germany). Patch pipettes were pulled using a BB-CH-PC (Mecanex, Switzerland) from microhematocrit capillary tubes. Pipets filled with a high $[K^+]$ solution (in mM: 20 KCl, 120 K-aspartate, 10 KHepes, 3 $MgCl_2$, pH 7.2, and 200 μg/ml nystatin) had tip resistances in the range of 2 to 4 MΩ.

Simultaneous measurements of $[Ca^{2+}]_i$ and $I_{K(Ca)}$

Simultaneous measurements of $[Ca^{2+}]_i$ and $I_{K(Ca)}$ in single isolated gonadotrophs were made as previously described (Jaimovich and Rojas, 1994; Kukuljan et al., 1994). In brief, an inverted epifluorescence microscope (Carl Zeiss, ICM 405, Oberkochen, Germany) equipped with a photon counter system (Photon Technology International, Inc., Brunswick, NJ) was used to measure simultaneously the intensity at 405 ± 5 (F_{405}) and at 480 ± 5 (F_{480}) nm from the acid form of indo-1 (Molecular Probes, Inc., Eugene, OR) trapped inside the cells and excited at 365 ± 5 nm. The data were acquired using a PC provided with an acquisition board. The software (OSCAR) provided with the system allowed 1) corrections to be made for the background intensity of both the light emitted at 405 and 485 nm; 2) conversion of the values of the corrected ratio F_{410}/F_{485} into absolute values of $[Ca^{2+}]_i$. To calibrate the system we prepared different Ca^{2+}-buffered solutions (in mM: 5 NaEGTA, 140 KCl, 10 NaHepes, pH 7) by adding different amounts of $CaCl_2$. For each Ca^{2+}-buffered solution (1 ml), we added 0.025 ml of a 25% ethanol solution containing the acid form of indo-1 and proceeded to measure the corresponding F_{410}/F_{485} values.

For simultaneous measurements of $I_{K(Ca)}$ and $[Ca^{2+}]_i$, cells were plated on glass coverslips and cultured under standard conditions for 2–3 days (Stojilkovic et al., 1992). Cells were loaded with indo-1 AM at room temperature (20–25°C) by incubating them for 1–2 h in a loading solution containing indo-1 AM (2 μM) and pluronic acid (0.02%) of the following composition (in mM): 140 NaCl, 5 KCl, 2.6 $CaCl_2$, 1 $MgCl_2$, 5 NaHepes, 5 glucose, pH 7.4 (Jaimovich and Rojas, 1994). Coverslips with cells were mounted in a chamber that allowed rapid changes in external solution. Acquisition of the light signals emitted at 405 and 485 nm, and $I_{K(Ca)}$ were synchronized using a common external trigger logic pulse.

RESULTS

In cultured rat pituitary gonadotrophs, GnRH (0.1–100 nM) evokes a complex pattern of $[Ca^{2+}]_i$ signals. Because GnRH receptor occupancy is required for the initiation of the $[Ca^{2+}]_i$ response, the rate at which the agonist reaches the receptors determines the shape of the initial phase of the $[Ca^{2+}]_i$ signal, which depends, therefore, on the method used to present the agonist to the cells. Rapid applications of 100 nM GnRH always induce a biphasic pattern that includes an initial transitory $[Ca^{2+}]_i$ rise to a peak value followed by $[Ca^{2+}]_i$ oscillations (Stojilkovic et al., 1993). In contrast, the initial phase of the $[Ca^{2+}]_i$ signal in response to the addition of a small volume (100 μl) of external medium containing the peptide (1 μM) exhibits oscillatory changes in $[Ca^{2+}]_i$ despite the fact that the final GnRH concentration is the same in both situations. The second phase (a few minutes after the application of GnRH) is always oscillatory and, in the continued presence of the agonist, exhibits a gradual attenuation in the amplitude of the $[Ca^{2+}]_i$ oscillations (Stojilkovic et al., 1993).

GnRH-induced oscillations in $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$-activated K^+ current are highly synchronized

Typical $I_{K(Ca)}$ and $[Ca^{2+}]_i$ oscillations in response to the application of GnRH (100 nM) by the addition of a small volume (100 μl) of external medium containing the peptide are depicted in Fig. 1. Although the changes in both $I_{K(Ca)}$ (Fig. 1 *A*, upper record) and $[Ca^{2+}]_i$ (lower record) occurred simultaneously, the classical fast initial phase of the $[Ca^{2+}]_i$ rise is not apparent, probably reflecting the time required to achieve the final stable concentration of GnRH at 100 nM. Thereafter, $I_{K(Ca)}$ and $[Ca^{2+}]_i$ oscillations were perfectly synchronized and very regular. Because the main objective of the experiment illustrated in Fig. 1 *A* was to determine the phase relationship between $I_{K(Ca)}$ and $[Ca^{2+}]_i$ oscillations, we superimposed two cycles (denoted C_1 and C_2) taken at the time delineated by the dashed line box in Fig. 1 *A*. As illustrated in Fig. 1 *B* the apparent threshold for activation of

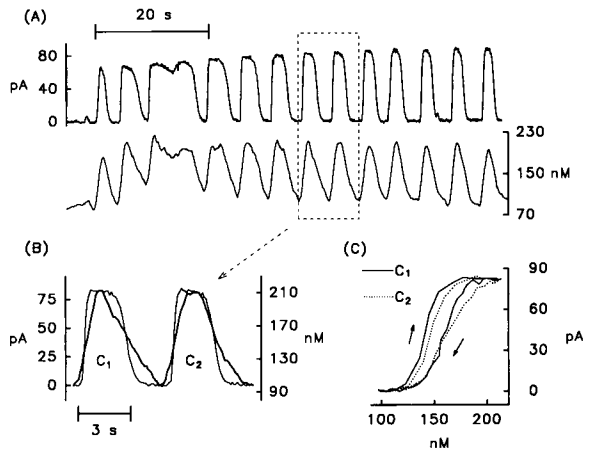


FIGURE 1 Simultaneous measurements of Ca^{2+}-activated K^+ current and whole cell $[Ca^{2+}]_i$. (*A*) Whole-cell $I_{K(Ca)}$ (upper record) and $[Ca^{2+}]_i$ (lower record) from a single isolated gonadotroph stimulated with 100 nM GnRH were measured simultaneously at room temperature (ca 25°C). The membrane potential was held at −50 mV. At time 0, 100 μl of extracellular solution containing GnRH (1 μM) was added gently. Two cycles of the $I_{K(Ca)}$ and $[Ca^{2+}]_i$ oscillations (outlined by the rectangular dashed line box) were used for the analysis depicted in *B* and *C*. (*B*) Superimposed $[Ca^{2+}]_i$ (thick trace) and $I_{K(Ca)}$ (thin trace) records. Vertical scales were adjusted arbitrarily. (*C*) Graph of $I_{K(Ca)}$ as a function of $[Ca^{2+}]_i$ from the two cycles in *B*. Arrows indicate the rise and the fall of the oscillation. Solid line corresponds to the first cycle and dotted line to the second cycle.

$I_{K(Ca)}$ (thin trace) corresponds to a level of whole cell $[Ca^{2+}]_i$ of ca. 145 nM (ordinate on the right). Thereafter $I_{K(Ca)}$ reached a maximum value of ca. 80 pA (ordinate on the left), remaining unchanged even though $[Ca^{2+}]_i$ continued to increase up to ca. 210 nM. It should be noted that during each cycle $[Ca^{2+}]_i$ decayed with a time constant of roughly 2.5 s, whereas the fall in $I_{K(Ca)}$ occurred in less than 1 s. The diagram $I_{K(Ca)}$ versus $[Ca^{2+}]_i$ shown in Fig. 1 *C* illustrates the phase relationship between the two signals. As expected, during the initial part of each cycle the $[Ca^{2+}]_i$ increase leads the plasma membrane event $I_{K(Ca)}$. During the rising phase, $[Ca^{2+}]_i$ activated $I_{K(Ca)}$ between 125 and 160 nM, whereas, toward the end of the cycle, $I_{K(Ca)}$ deactivation starts at a higher $[Ca^{2+}]_i$ (ca. 190 nM) and is complete at ca. 130 nM. Ca^{2+} gating of the apamin-sensitive K^+ channel probably occurs near the inner mouth of the channel. Because $[Ca^{2+}]_i$ represents whole-cell cytosolic free Ca^{2+}, the simplest explanation for this apparent change in $I_{K(Ca)}$ threshold is that the Ca^{2+} concentration near the K^+ channel is lowered before the overall cytosolic $[Ca^{2+}]_i$ because of the presence of a Ca^{2+} pump in the plasma membrane.

In two additional experiments, in which cells were superfused with external solutions allowing fast (<2 s) GnRH (100 nM) application, both $I_{K(Ca)}$ and $[Ca^{2+}]_i$ signals exhibited a rapid (<2 s) rise to a peak value (ca. 250 pA for $I_{K(Ca)}$ and ca. 1 μM for $[Ca^{2+}]_i$), which was followed by a slow decay toward a basal nonstimulated level. Furthermore, the delayed phase of the response exhibited highly synchronized oscillations in $I_{K(Ca)}$ and $[Ca^{2+}]_i$ of smaller amplitude, very similar to those shown in Fig. 1 *A* (data not shown).

Characteristics of $I_{K(Ca)}$ transients evoked by brief depolarizing pulses in resting gonadotrophs

Fig. 2 depicts two series of $I_{K(Ca)}$ records from a resting cell. For the first series (Fig. 2 *A*) the duration was kept constant (0.1 s) and the amplitude was increased in 15-mV steps, taking the membrane potential from −50 to a maximum of 45 mV. For the second series (Fig. 2 *B*) depolarizing pulses of constant amplitude (0 mV during the pulse) of decreasing duration from 100 to 10 ms were applied. Because the cell membrane was held at a potential positive to the K^+ reversal potential, the transient current elicited at the end of each pulse was in the outward direction. It may be seen that pulses taking the membrane potential below −15 mV failed to evoke the outward tail current (Fig. 2 *A*). At more positive potentials the peak of the tail current gradually increased, reaching a maximum level at ca. 30 mV. The figure also shows that the threshold for the tail current lies between −15 and 0 mV, close to the midpoint for the activation curve of the L-type Ca^{2+} inward current in these cells (−12.7 mV; Stutzin et al., 1989). Increasing the duration of pulses, taking the membrane potential from −50 to 0 mV, revealed that durations of 40 ms and greater were sufficient to induce a tail current (Fig. 2 *B*). Three additional experiments were carried out with the same protocol, but different cell preparations gave similar results.

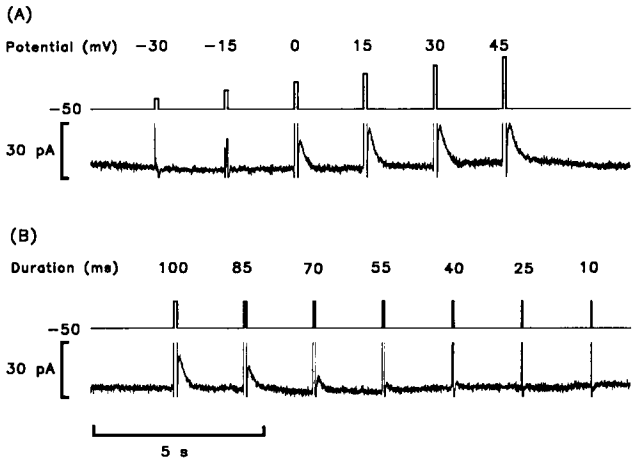


FIGURE 2 Effects of depolarizing pulses on $I_{K(Ca)}$ in resting cells. (*A*) Upper trace: Pulse protocol. Pulses of constant duration (100 ms) were applied from a holding potential of −50 mV. The amplitude of the pulses was increased in 15-mV steps. Membrane potential during the pulses is given in millivolts above the corresponding pulse. Noisy record below the pulse protocol represents $I_{K(Ca)}$. Records of the whole-cell currents during the pulses were omitted. (*B*) Same format as for *A*. A series of pulses of constant amplitude (to a membrane potential of 0 mV) and decreasing durations (from 100 to 10 ms) in 15-ms decrements were applied. Lower record represents $I_{K(Ca)}$. Here again records of the whole-cell currents during the pulses were omitted.

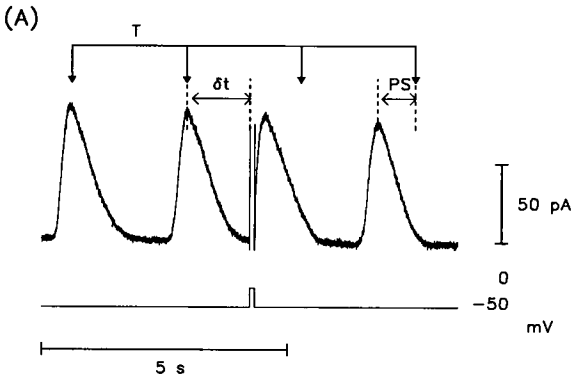
Transient $I_{K(Ca)}$ currents evoked by a depolarizing pulse in GnRH-stimulated gonadotrophs

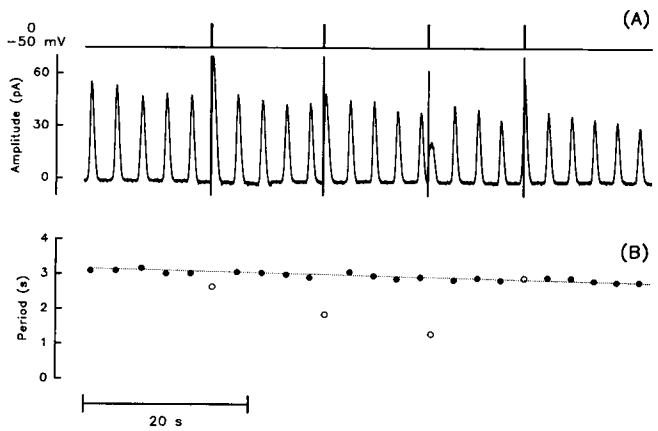
During the delayed phase of GnRH-induced current oscillations, the amplitude of the $I_{K(Ca)}$ cycles exhibited a gradual decrease (Fig. 3 *A*). In addition, random application of brief (100 ms) depolarizing pulses elicited transient $I_{K(Ca)}$ currents. Application of hyperpolarizing pulses was without effect (not shown). Usually the shape of the evoked transient $I_{K(Ca)}$ current was rather similar to the endogenous $I_{K(Ca)}$ cycles (Fig. 3 *A*, second pulse). Also, the amplitude of the evoked cycles augmented as the time lapsed between the peak of the endogenous $I_{K(Ca)}$ cycle and the onset of the pulse increased. In addition, evoked cycles exhibited a well-defined rising phase, reaching a peak $I_{K(Ca)}$ value in ca. 300 ms after the application of the pulse. Furthermore, the amplitude of the endogenous $I_{K(Ca)}$ cycles after the application of a pulse was not appreciably affected, and the time interval betwen the peaks of two consecutive cycles (i.e., the period) before and after the application of a depolarizing pulse remained essentially unchanged (Fig. 3 *B*, dotted line) and, therefore, for analysis of records including only a few $I_{K(Ca)}$ endogenous and evoked cycles, the period can be considered constant. In contrast, as depicted in Fig. 3 *B*, the time lapsed between the peak of the evoked and the preceding endogenous cycle was significantly reduced (Fig. 3 *B*). In contrast, when the amplitude of the endogenous $I_{K(Ca)}$ cycles had been reduced significantly (<10% initial amplitude), application of brief depolarizing pulses induced a transitory recovery of the amplitude of the oscillations lasting 3 to 5 $I_{K(Ca)}$ cycles (not shown).

From these data we conclude that a brief activation of Ca^{2+} influx through plasma membrane resident voltage-gated Ca^{2+} channels might be sufficient to reset the clock mechanism of the cytosolic $[Ca^{2+}]$ oscillator. The experiments described in the following sections lend further support to this idea.

Phase resetting of the GnRH-induced oscillations by single depolarizing pulses

To gather kinetic information on the clock resetting phenomenon of the $I_{K(Ca)}$ oscillations, brief (100 ms) depolarizing pulses were applied at random time intervals along the entire course of the $I_{K(Ca)}$ oscillations. Depending on the time lapsed between the peak of the cycle preceding a pulse and the onset of the pulse (δt in Fig. 4 *A*), the phase of the oscillations following the pulse was shifted (PS in Fig. 4 *A*).





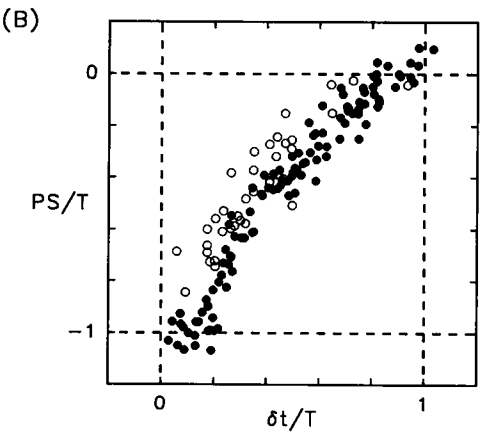


FIGURE 3 Phase shift of GnRH-stimulated $I_{K(Ca)}$ oscillations evoked by depolarizing pulses. (*A*) $I_{K(Ca)}$ record was made 4 min after the stimulation of the gonadotrophs with GnRH (10 nM). Single depolarizing pulses (100 ms) were applied from a holding potential of −50 mV (pulse protocol is outlined above the $I_{K(Ca)}$ record). Records of the whole cell currents during the pulses were omitted. (*B*) Filled circles correspond to the time interval between the peaks of two consecutive spontaneous $I_{K(Ca)}$ cycles. Open circles correspond to time interval between the peaks of consecutive spontaneous and elicited $I_{K(Ca)}$ cycles. The dotted line represents a linear fit to the time interval between the peaks of two consecutive spontaneous $I_{K(Ca)}$ cycles.

FIGURE 4 Analysis of the phase resetting induced by brief depolarizing pulses. (*A*) $I_{K(Ca)}$ records from a cell stimulated with 100 nM GnRH. The horizontal axis represents the peak to peak time interval between consecutive spontaneous $I_{K(Ca)}$ cycles (now on referred to as *T*). Arrows after the pulse indicate the expected peak time for the third and fourth spontaneous cycle. The time interval between the peak of the spontaneous cycle preceding a pulse and the onset of the pulse defines δt. The phase shift of the spontaneous $I_{K(Ca)}$ oscillation following the pulse defines PS. (*B*) Normalized phase shift data (*vertical axis labeled PS/T) as a function of normalized* δt (*horizontal axis labeled* $\delta t/T$) from two different experiments. Note that negative values for $\delta t/T$ represent an advance in the phase.

Normalized phase shift values (i.e., PS/T) are plotted in Fig. 4 *B* as a function of the corresponding normalized time interval δt (i.e., $\delta t/T$) for two different experiments (open circles, filled circles). Although the parameters appear to be linearly correlated, no attempts were made to find a model that could explain the data. From these data we conclude that phase resetting of the endogenous $I_{K(Ca)}$ oscillations can be achieved by activation of voltage-gated Ca^{2+} channels during the application of brief depolarizing pulses.

We have shown that $[Ca^{2+}]_i$ and $I_{K(Ca)}$ oscillations are highly synchronized cellular events (Fig. 1). Fig. 5 demonstrates that a brief depolarizing pulse can induce phase resetting not only of $I_{K(Ca)}$ oscillations but also of the whole-cell $[Ca^{2+}]_i$ oscillations. As depicted in Fig. 5, where the period for the endogenous $I_{K(Ca)}$ (upper record) and $[Ca^{2+}]_i$ (lower record) oscillations corresponds to the time lapsed between two consecutive dashed vertical lines, application of brief depolarizing pulses caused the same phase shift in both $I_{K(Ca)}$ (upper record) and $[Ca^{2+}]_i$ (lower record) oscillations. These results provide further support for the idea that the changes in $I_{K(Ca)}$ induced by brief depolarizing pulses occur in perfect phase with the changes in $[Ca^{2+}]_i$ and thus validate our previous studies of $[Ca^{2+}]_i$ in gonadotrophs that were based on $I_{K(Ca)}$ measurements (Kukuljan et al., 1994).

Characteristics of the refractory period after endogenous $I_{K(Ca)}$ cycles during the sustained phase of GnRH stimulation

We have shown that in nonstimulated gonadotrophs depolarizing pulses elicit highly reproducible tail $I_{K(Ca)}$ currents (Fig. 2). In contrast, during the sustained phase of the GnRH-evoked $I_{K(Ca)}$ oscillations, despite the fact that both the amplitude and the duration of the depolarizing pulses were kept unchanged, the amplitude of the evoked $I_{K(Ca)}$ cycles was phase dependent. Indeed, the superimposed $I_{K(Ca)}$ records shown in Fig. 6 *A* clearly demonstrate this property. When the time lapsed between the onset of the pulse and the peak of the preceding endogenous cycle was kept constant, not only the amplitude but the shape of the evoked $I_{K(Ca)}$ cycles was similar. Fig. 6 *A* also shows that the amplitude of the endogenous cycle used to align the evoked cycles varied from 30 to 70 pA. The reason for this variation is that the amplitude of the endogenous $I_{K(Ca)}$ cycles decreases with time. Fig. 6 *B* shows the relationship between the amplitude of the evoked cycle (ordinate in pA) and the time lapsed from the peak of the endogenous cycle to the onset of the pulse (abscissa in s). The curve represents a single exponential function of time drawn to fit the data. From the fit we estimated that the amplitude of the evoked $I_{K(Ca)}$ cycle increased toward a maximum size of 94.6 pA with a time constant of 1.3 s. The amplitude of GnRH-evoked $I_{K(Ca)}$

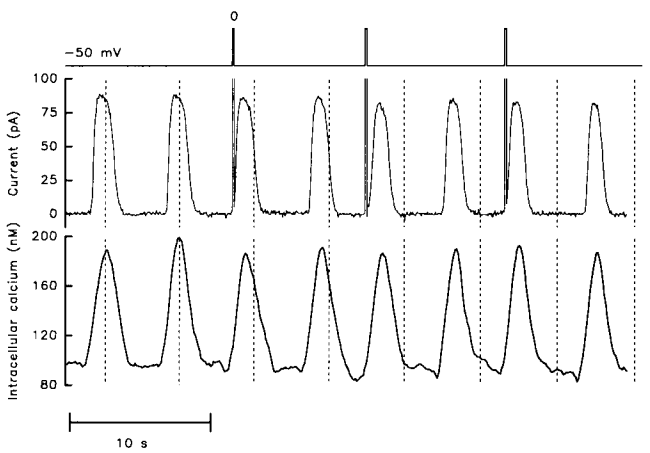


FIGURE 5 Simultaneous measurements of the effects of depolarizing pulses on $I_{K(Ca)}$ and $[Ca^{+2}]_i$. (*Upper trace*) Pulse protocol (pulse duration set at 100 ms). (*Middle record*) Whole-cell current $I_{K(Ca)}$. (*Lower record*) $[Ca^{+2}]_i$. Dashed vertical lines indicate the predicted peak time for the endogenous $I_{K(Ca)}$. This was estimated assuming a linear decay (as shown in Fig. 3 *B*).

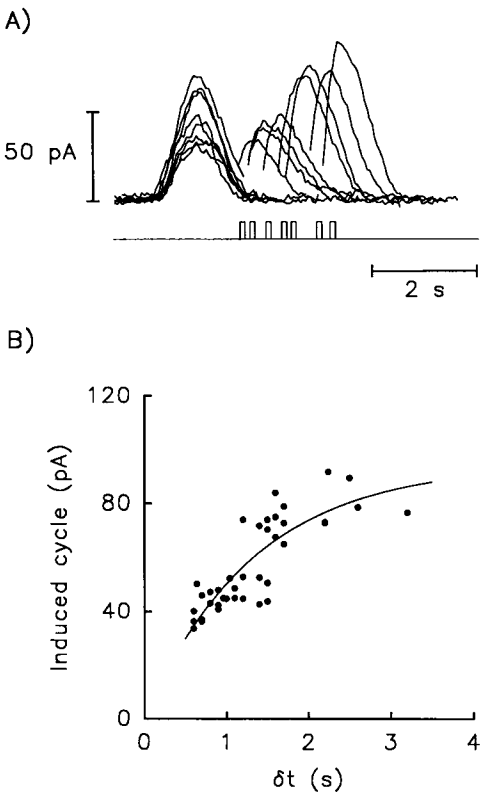


FIGURE 6 Absolute and relative refractory period after each endogenous $I_{K(Ca)}$ cycle in GnRH-stimulated gonadotrophs. (*A*) Superimposed $I_{K(Ca)}$ records in response to brief (50 ms) depolarizing pulses to 10 mV. The peak time of endogenous cycles preceding each pulse was used to align the records. Eight pulses were applied at different times along the experiment. Only seven are shown beneath the superimposed $I_{K(Ca)}$ records because the second pulse was repeated twice. (*B*) Amplitude of the evoked $I_{K(Ca)}$ cycles (●) as a function of the time lapsed between the peak of the endogenous cycle and the onset of the pulse (δt). Solid line represents the best fit of the data using a single exponential function (time constant, 1.3 s; maximum amplitude, 78.7 pA). Records were acquired at a holding potential of −50 mV, 14 min after the application of GnRH.

oscillations decreased progressively along the course of the experiment. In contrast, the amplitude of evoked $I_{K(Ca)}$ cycles elicited by single pulses applied after an endogenous cycle with delays that varied within a narrow range, i.e., from 0.8 to 1.3 s, was almost constant (not shown). The meaning of this result is that the amplitude of the elicited $I_{K(Ca)}$ cycle depends almost exclusively on the time lapsed between the peak of the endogenous cycle and the onset of the pulse. Thus, the amplitude of the evoked cycle is relatively independent of the size of the endogenous cycle preceding the depolarizing pulse.

Taken together the data presented in this section strongly suggest that a brief (50–100 ms) period of Ca^{2+} influx is sufficient to evoke a full-size $I_{K(Ca)}$ cycle, regardless of the amplitude of the preceding endogenous $I_{K(Ca)}$ cycle. We also conclude that each $[Ca^{2+}]_i$ rise cycle leaves the cell in a refractory period during which another complete $[Ca^{2+}]_i$ rise cycle is not allowed.

In GnRH-stimulated (0.1–10 nM) gonadotrophs, phase resetting by depolarizing pulses occurred in the majority of the cells examined at a holding potential of −50 mV. At this potential a significant fraction of the voltage-gated Ca^{2+} channels present in these cells will not be available for activation during the pulses (Stutzin et al., 1989). Because phase resetting was also observed in cells held at a membrane potential of −90 mV, negative to the midpoint potential for activation of the inactivating Ca^{2+} channel (data not shown), we conclude that the noninactivating L-type Ca^{2+} channel present in rat pituitary gonadotrophs (Stutzin et al., 1989) might play a predominant role in phase resetting.

Evoked $I_{K(Ca)}$ cycles are blocked by Cd^{2+} and cannot be elicited in Ca^{2+}-deficient medium

As mentioned before, we have observed that the amplitude of the evoked cycle is relatively independent of the size of the endogenous cycle preceding the depolarizing pulse. We also found that most of the effects of brief depolarizing pulses described so far required the presence of extracellular Ca^{2+} and were inhibited by the voltage-gated Ca^{2+} channel blocker Cd^{2+} (1 mM). In addition, we observed that pulses lasting less than 10 ms or taking the membrane potential to levels below the midpoint potential (−12.7 mV; Stutzin et al., 1989) for L-Type Ca^{2+} channel activation were unable to elicit phase resetting of the $I_{K(Ca)}$ oscillations (data not shown). Taken together these results suggest that Ca^{2+} entry through voltage-gated Ca^{2+} channels is necessary for phase resetting. Finally, because we have previously shown that the Ca^{2+}-ATPase inhibitor thapsigargin (1 μM) also inhibits endogenous $[Ca^{2+}]_i$ oscillations (Kukuljan et al., 1994), we tested the possibility that evoked $I_{K(Ca)}$ might be resistant to the drug with negative results (data not shown). Thus, in addition to Ca^{2+} influx, the integrity of the Ca^{2+} uptake mechanism is required for the generation of evoked cycles.

Refractory period for evoked cycles during the sustained phase of GnRH-evoked $[Ca^{2+}]_i$ oscillations

To gather quantitative kinetic information on the refractory period after each $I_{K(Ca)}$ cycle, pairs of brief (100 ms) depolarizing pulses were applied toward the end of the sustained phase, when the amplitude of the $I_{K(Ca)}$ cycles was less than 10% of its initial value. We noted that whereas the first depolarizing pulse always elicited a full-size $I_{K(Ca)}$ cycle, the amplitude of the cycle evoked by the second pulse was smaller and depended on the time lapsed between the peak of the cycle induced by the first pulse and the onset of the second pulse (Fig. 7 *A*; δt). As illustrated in Fig. 7 *B* (continuous and dashed curves), the amplitude of the $I_{K(Ca)}$ cycle induced by the second pulse increased exponentially with the time separation between the peak of the first evoked cycle and the onset of the second pulse. The set of superimposed records of two consecutive evoked $I_{K(Ca)}$ cycles depicted in Fig. 7 *B* shows that there is no overlapping between the two cycles. In contrast, Fig. 6 *A* shows some overlapping of the signals from the tail end of the endogenous $I_{K(Ca)}$ cycle and the start of the evoked cycle.

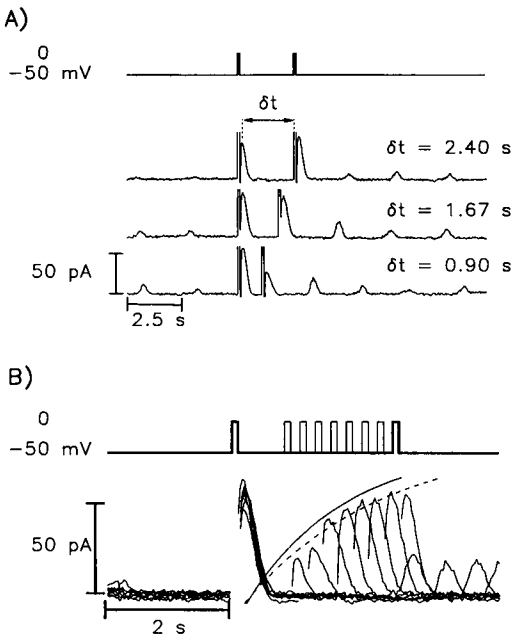


FIGURE 7 Recovery from inactivation after an evoked $I_{K(Ca)}$ cycle in GnRH-stimulated gonadotrophs. (*A*) Records were acquired at a holding potential of −50 mV. Records of $I_{K(Ca)}$ cycles elicited by two consecutive depolarizing pulses were made 6 min after the application of GnRH (100 nM), when the amplitude of the endogenous cycles reached about 5% of the initial value. Time interval (δt) between the pulses was augmented in 250-ms increments and the pulse duration was kept constant at 100 ms. (*A*) Upper trace: Pulse protocol. The time intervals between the two pulses (δt) are shown above tracings. (*B*) Upper trace represents the pulse protocol. Superimposed pairs of evoked $I_{K(Ca)}$ cycles aligned using the peak time of the first evoked cycle. Membrane currents in response to the first pulse were blanked. Dashed line represents the best fit of a single exponential function to the points representing the amplitude of the second evoked cycle. Solid curve represents the fit from Fig. 6 *B*.

From the superimposed records shown in Fig. 7 *B*, we estimate that the second evoked cycle reached a maximum amplitude of 78.7 pA with a time constant of about 1.8 s.

DISCUSSION

Simultaneous measurements of agonist-induced $[Ca^{2+}]_i$ and $I_{K(Ca)}$ oscillations in cultured rat pituitary gonadotrophs were made to further understand the underlying mechanisms. We found that GnRH-induced $[Ca^{2+}]_i$ and $I_{K(Ca)}$ oscillations occurred in perfect phase (Fig. 1), with the $[Ca^{2+}]_i$ rise always leading the activation of $I_{K(Ca)}$ during each cycle. These results validate the use of patch clamp $I_{K(Ca)}$ records to gather kinetic information on the $[Ca^{2+}]_i$ near the inner aspect of the cell membrane, where apamin-sensitive K^+ channels are located. The temporal association between $[Ca^{2+}]_i$ and $I_{K(Ca)}$ described here suggests that the membrane oscillations follow the clock of the cytosolic oscillator. We also found that, during the sustained phase of GnRH stimulation, brief depolarizing pulses can elicit evoked $[Ca^{2+}]_i$ and $I_{K(Ca)}$ cycles. Furthermore, the subsequent endogenous $[Ca^{2+}]_i$ and $I_{K(Ca)}$ oscillations exhibited a variable phase shift (Figs. 3, 4, 5, and 6) of up to 2 s, suggesting that Ca^{2+} entry through voltage-gated Ca^{2+} channels can reset the clock of the cytosolic oscillator. In addition, we found that after each cycle (endogenous or evoked) the Ca^{2+} release mechanism enters into a refractory phase, during which the amplitude of a second evoked cycle is diminished. This refractory phase reflects the time for recovery of the whole-cell cytosolic $[Ca^{2+}]$ signal (as in Fig. 1 *B*) rather than the $[Ca^{2+}]$ close to the inner aspect of the membrane, as indicated by $I_{K(Ca)}$ records.

Ca^{2+} entry induces phase-dependent shifts in subsequent $[Ca^{2+}]_i$ oscillations

During the sustained phase of GnRH stimulation, Ca^{2+} entry induced by a depolarizing pulse is able to reset the phase of subsequent $[Ca^{2+}]_i$ oscillations (Fig. 5). Furthermore, application of two consecutive depolarizing pulses during the late GnRH stimulation showed that the amplitude of the evoked cycle depends, in part, on the time lapsed between the two events (Fig. 7). The meaning of this result is that the size of the second evoked cycle probably depends on the Ca^{2+} load of the $InsP_3$-sensitive store. Support for this interpretation can be found in a recent report showing that gonadotrophs loaded with mag-indo-1, a fluorescent dye suitable to detect Ca^{2+} inside intracellular stores, exhibit significant decreases in the detectable Ca^{2+} (Tse et al., 1994).

Although more experiments are necessary to elucidate the mechanism responsible for the evoked $[Ca^{2+}]_i$ rise, it is likely that Ca^{2+} entry during a depolarizing pulse is sufficient to induce an elevation of the $[Ca^{2+}]_i$ near the Ca^{2+} regulatory domain of the $InsP_3$ receptor (Bezprozvanny et al., 1991), and this Ca^{2+} in turn modulates the activity of the $InsP_3$ receptor channel (Parker and Ivorra, 1990; Finch et al., 1991; Yao and Parker, 1992). Because $InsP_3$-induced Ca^{2+} release also depends on the Ca^{2+} load of the $InsP_3$-sensitive store (Missiaen et al., 1994), we speculate that the Ca^{2+} entering the cell during a depolarizing pulse potentiates the $InsP_3$-activated Ca^{2+} release from partly filled Ca^{2+} stores. Furthermore, free-running membrane potential experiments under current clamp conditions have also shown a tight correlation between periodic bursting and oscillations, presumably because of the opening of voltage-gated Ca^{2+} channels during the active phase followed by the activation of apamin-sensitive $[Ca^{2+}]_i$-dependent K^+ channels during the silent phase of the bursts (Kukuljan et al., 1994). An attractive feature of this mechanism is that it provides an alternative link between the plasma membrane Ca^{2+} entry and cytosolic Ca^{2+} release from the Ca^{2+} stores.

The Ca^{2+} release mechanism supporting $[Ca^{2+}]_i$ oscillations exhibits refractoriness

In the sustained phase of GnRH stimulation, after each endogenous $I_{K(Ca)}$ cycle, application of a depolarizing pulse elicits an $I_{K(Ca)}$ cycle, the amplitude of which depends on the time lapsed between the two events. This result indicates that the Ca^{2+} release system is left in a refractory state after each episode of Ca^{2+} release (Figs. 6 and 7). Recovery from the refractory state after an endogenous cycle occurs in ca. 1.7 s (Fig. 6). Furthermore, a brief pulse of Ca^{2+} influx can elicit a $[Ca^{2+}]_i$ cycle similar in shape to the endogenous cycles (Fig. 5), which also exhibit a refractory period (ca. 1.3–1.8 s) after each episode (see Fig. 7). These results suggest that the mechanism of refractoriness is likely to be the same in both endogenous and evoked cycles.

Taken together these results led us to propose that the rapid rise in $InsP_3$ concentration after the application of GnRH (0.1–100 nM) triggers Ca^{2+} release from Ca^{2+}-loaded stores even in the presence of low (<100 nM) $[Ca^{2+}]_i$. The ensuing $[Ca^{2+}]_i$ rise will activate $[Ca^{2+}]_i$-dependent K^+ channels, which will bring about the hyperpolarization of the cell membrane until $[Ca^{2+}]_i$ returns to its resting value. If the rate of desensitization is small (Stojilkovic et al., 1989), the concentration of $InsP_3$ would remain elevated. After these early events, during the initial part of the sustained phase of GnRH stimulation, $[Ca^{2+}]_i$ transiently returns to a resting level, causing the apamin-sensitive K^+ channels to shut. This in turn induces depolarization of the membrane and triggers bursts of electrical activity. Because the sensitivity to $[Ca^{2+}]_i$ of the $InsP_3$ receptor channel present in the membrane of partly depleted stores is greatly diminished (Missiaen et al., 1994), Ca^{2+} influx during the bursts of electrical activity would be required for Ca^{2+} release from $InsP_3$-sensitive Ca^{2+} stores. In contrast, fully loaded stores responded to $InsP_3$ even in the presence of low cytosolic $[Ca^{2+}]$ (<100 nM), and the release of Ca^{2+} from the stores was stimulated to a lesser extent by a $[Ca^{2+}]_i$ rise.

We have already mentioned that GnRH-induced $I_{K(Ca)}$ oscillations and Ca^{2+} decreases (amounting to ca. 10% of

the signal) within the intracellular stores occurred in synchrony (Tse et al., 1994). Furthermore, when the frequency of the $I_{K(Ca)}$ oscillations was ca. 5 cycles/min, the drop in stored Ca^{2+} during each cycle occurred in about 3 s; the duration of the refractory phase was about 1.5 s.

Different mechanisms for initiation and maintenance of $[Ca^{2+}]_i$ oscillations

A growing number of models have been proposed to explain the generation and the maintenance of cytosolic $[Ca^{2+}]$ oscillations in different cell types (Berridge, 1990; Harootunian et al., 1991; Keizer and De Young, 1992, 1993, 1994; De Young and Keizer, 1992; Berridge, 1993). We have shown here (Fig. 2) that, in resting gonadotrophs, the Ca^{2+} influx associated with brief depolarizing pulses is not sufficient to initiate the cytosolic Ca^{2+} oscillator. We now know that in gonadotrophs, as in nonexcitable cells operated by Ca^{2+}-mobilizing receptors, $InsP_3$ is responsible for the initiation and the maintenance of $[Ca^{2+}]_i$ oscillations (Tse and Hille, 1991; Stojilkovic et al., 1992; Tse et al., 1993; Li et al., 1994; Kukuljan et al., 1994). Thus, the mechanism of modulation of the $InsP_3$-driven Ca^{2+} mobilization process may prove to be a universal control mechanism in other excitable endocrine and neuroendocrine cells. In addition, in gonadotrophs exposed to GnRH, as in other cell types operated by $InsP_3$, refilling of the stores with Ca^{2+} from the extracellular medium is required for the $[Ca^{2+}]_i$ response. In the case of nonexcitable cells, several mechanisms have been proposed to explain the refilling of the Ca^{2+} stores. For example, Ca^{2+} store depletion-activated pathways have been described (Takemura et al., 1989; Bird et al., 1991; Lückhoff and Clapham, 1992; Hoth and Penner, 1992). By contrast, in gonadotrophs Ca^{2+} refilling exhibits a prominent dependence on membrane potential, suggesting that a different mechanism might operate in these cells. As shown previously (Kukuljan et al., 1994), holding the membrane potential at very negative values (−100 mV) reduced the duration of the sustained phase of $[Ca^{2+}]_i$ oscillations. This result suggests that voltage-independent pathways are either not expressed or are unable to refill the intracellular Ca^{2+} stores in cultured rat pituitary gonadotrophs. In conclusion, our results suggest a crucial role for the membrane potential in the Ca^{2+} refilling process and on the maintenance of $InsP_3$-controlled Ca^{2+} release in gonadotrophs, including resetting the clock of the cytosolic oscillator.

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