Modulation of the Kinetics of Inositol 1,4,5-Trisphosphate-Induced [Ca^{2+}]$_i$ Oscillations by Calcium Entry in Pituitary Gonadotrophs

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ABSTRACT   Inositol 1,4,5-trisphosphate (InsP$_3$) binds to its receptor channels and causes liberation of Ca^{2+} from intracellular stores, frequently in an oscillatory manner. In addition to InsP$_3$, the activation and inactivation properties of these intracellular channels are controlled by Ca^{2+}. We studied the influence of Ca^{2+} entry on the kinetics of InsP$_3$-triggered oscillations in cytosolic calcium ([Ca^{2+}]$_i$) in gonadotrophs stimulated with gonadotropin-releasing hormone, an agonist that activates InsP$_3$ production. The natural expression of voltage-gated Ca^{2+} channels (VGCC) in these cells was employed to manipulate Ca^{2+} entry by voltage clamping the cells at different membrane potentials (V_m). Under physiological conditions, the frequency of the GnRH-induced oscillations increased with time, while the amplitude decreased, until both reached stable values. However, in cells with V_m held at −50 mV or lower, both parameters progressively decreased until the signal was abolished. These effects were reverted by a depolarization of the membrane positive to −45 mV in both agonist- and InsP$_3$-stimulated gonadotrophs. Depolarization also led to an increase in the fraction of time during which the [Ca^{2+}]$_i$ remained elevated; this effect originated from both an increase in the mean duration of spikes and a decrease in the interval between spikes. The frequency and amplitude of spiking depended on the activity of VGCC, but displayed different temporal courses and voltage relationships. The depolarization-driven recovery of the frequency was instantaneous, whereas the recovery of the amplitude of spiking was more gradual. The midpoints of the V_m sensitivity curve for amplitude and duration of spiking (−15 mV) were close to the value observed for L-type Ca^{2+} current and for depolarization-induced increase in [Ca^{2+}]$_i$, whereas this parameter was much lower (−35 mV) for interval between spikes and frequency of oscillations. These observations are compatible with at least two distinct effects of Ca^{2+} entry on the sustained [Ca^{2+}]$_i$ oscillations. Calcium influx facilitates its liberation from intracellular stores by a direct and instantaneous action on the release mechanism. It also magnifies the Ca^{2+} signal and decreases the frequency because of its gradual effect on the reloading of intracellular stores.

INTRODUCTION

In many cell types expressing Ca^{2+}-mobilizing receptors, oscillations and waves in the cytosolic calcium concentration ([Ca^{2+}]$_i$) occur as a part of the signal transduction process for extracellular agonists. In several well-characterized systems such as *Xenopus laevis* oocytes (Lechleiter and Clapham, 1992; DeLisle et al., 1990), hepatocytes (Rooney et al., 1990), and pancreatic acinar cells (Zhao et al., 1990), the generation of oscillations in the [Ca^{2+}]$_i$ is explained as a consequence of Ca^{2+} release through inositol 1,4,5-trisphosphate (InsP$_3$) receptor channels. Current models yield the view that [Ca^{2+}]$_i$ oscillations arise as an outcome of the different kinetics of activation and inactivation intrinsic to the InsP$_3$ receptor (IP$_3$R) channel working in conjunction with endoplasmic reticulum (ER) Ca^{2+}-ATPase (Keizer et al., 1995). The critical element in these models is that a fluctuating Ca^{2+} signal originates in response to the continuous occupancy of plasma membrane receptors and a nonoscillatory production of InsP$_3$.

Calcium itself has been implicated to act as a coagonist with InsP$_3$ in the control of periodic Ca^{2+} release from the

ER (De Young and Keizer, 1992). [Ca^{2+}]$_i$ was found to have dual effects on InsP$_3$-induced Ca^{2+} release, facilitating it at low concentrations and inhibiting it at higher concentrations (Iino and Endo, 1992; Zhang and Muallem, 1992). These findings were confirmed by measurements of steady-state effects of InsP$_3$ and Ca^{2+} on isolated Purkinje IP$_3$R channels (Bezprozvanny et al., 1991). Parker and Ivorra (1990) showed that Ca^{2+}-dependent inactivation of InsP$_3$-induced Ca^{2+} release is a slower process than activation, and Payne et al. (1988) demonstrated that recovery from Ca^{2+}-mediated inactivation requires 10–30 s. In synaptosomes, Ca^{2+} triggers release within 50 ms, whereas inactivation is delayed for about 0.5 s (Finch et al., 1991). Such an action of Ca^{2+} on InsP$_3$-induced Ca^{2+} release is a key feature in the operation of the ER oscillator, a process termed by Lechleiter and Clapham (1992) and Keizer et al. (1995) as intracellular Ca^{2+} excitability and ER calcium excitability, respectively. Calcium serves as an excitation variable, i.e., provides the positive feedback that leads to a rapid increase in efflux to a maximum rate for a particular InsP$_3$ concentration (Parker et al., 1996).

To study the role of Ca^{2+} influx on InsP$_3$-induced Ca^{2+} release in intact cells, we explored the influence of voltage-gated Ca^{2+} entry on the kinetics of [Ca^{2+}]$_i$ oscillations triggered by the hypothalamic gonadotropin-releasing hormone (GnRH) in cultured rat gonadotrophs. These cells display robust [Ca^{2+}]$_i$ oscillations in response to GnRH (Shangold et al., 1988; Leong and Thorner, 1991), the

frequency of which depends on the $InsP_3$ concentration (Stojilkovic et al., 1993). Although agonists and $InsP_3$ can initiate Ca^{2+} oscillations in cells bathed in Ca^{2+}-deficient medium (Stojilkovic et al., 1992; Tse and Hille, 1992), Ca^{2+} entry through the voltage-gated calcium channels (VGCC) of the plasma membrane provides an important route for the replenishment of the ER stores (Rawlings et al., 1991; Stojilkovic et al., 1992; Kukuljan et al., 1994). In addition, transient Ca^{2+} entry, as observed during action potentials, can shift the phase of the $[Ca^{2+}]_i$ spikes (Vergara et al., 1995). Here we have evaluated the influence of sustained changes in the rate of Ca^{2+} entry on the pattern of agonist/$InsP_3$-induced Ca^{2+} spiking. The results indicate that $[Ca^{2+}]_i$ plays important roles in the control of the oscillatory mechanism and kinetics of Ca^{2+} release in pituitary gonadotrophs.

MATERIALS AND METHODS

Cell culture

Gonadotrophs were isolated from the pituitaries of ovariectomized Sprague-Dawley rats (200–250 g) by enzymatic dispersion and were maintained in primary culture as described (Stojilkovic et al., 1993). Briefly, rats were ovariectomized 14 days before each cell culture preparation. The tissue from four pituitaries was minced and incubated in phosphate-buffered saline (PBS) supplemented with 1.5 mg/ml trypsin (Sigma Chemical, St. Louis, MO) and 1 mg/ml DNase I (Boeheringer Mannheim, Indianapolis, IN) at 37°C for 1 h. After the tissue was mechanically dispersed and washed with fresh PBS, cells were suspended in Medium 199 (Gibco BRL, Grand Island, NY), plated in plastic culture dishes (Nunc, Roskilde, Denmark), and kept at 37°C in a 95% air/5% CO_2 atmosphere for 2–4 days before recording.

Electrophysiological recording

The perforated patch variation of the patch-clamp technique was used to measure the V_m and $I_{K\text{-}Ca}$ oscillations (Kukuljan et al., 1994). This approach provides an indirect measure of the $[Ca^{2+}]_i$. Before each experiment, the culture medium was replaced with a solution containing (in mM) 140 NaCl, 4 KCl, 2.6 $CaCl_2$, 1 $MgCl_2$, 10 HEPES (hydroxypiperazine ethanosulfonic acid, sodium salt), and 8 glucose. The pH was adjusted to 7.36 with NaOH. Patch-clamp electrodes were made of soft capillary glass (Blu-Tip) pulled with a BB-CH-PC puller (Mecanex, Geneva, Switzerland), adjusted to obtain a tip resistance between 2 and 4 MΩ. These electrodes were filled with a solution of the following composition (in mM): 70 potassium aspartate, 70 KCl, 3 $MgCl_2$, and 10 HEPES, and the pH was adjusted to 7.15. Nystatin was added from a stock solution to get a final concentration of 100–200 μg/ml. The electrode was mounted on the headstage of a EPC-7 amplifier (List, Darmstadt, Ebenstadt, Germany), and the tip resistance was monitored by applying a 1-mV square pulse of 10 ms at 10 Hz. Once a high-resistance seal (> 5 GΩ) was formed between the electrode and the cell membrane, the access resistance was monitored until values of under 30 MΩ were reached. The membrane potential was set at −50 mV, and in experiments with V_m measurements the holding current was set to zero. GnRH was directly added to the extracellular solution.

Measurements of $[Ca^{2+}]_i$

Simultaneous measurements of $[Ca^{2+}]_i$ and $I_{K\text{-}Ca}$ in single isolated gonadotrophs were made as previously described (Vergara et al., 1995). In brief, cells were plated on glass coverslips (250,000 cells/dish) and kept in

culture for 2–3 days under the conditions described above. Cells were loaded with indo-1 AM at room temperature (20–25°C) by incubating them for 30–60 min in a solution containing indo-1 AM (2 μM) and pluronic acid (0.02%) (Molecular Probes, Eugene, OR), of the following composition (in mM): 140 NaCl, 5 KCl, 2.6 $CaCl_2$, 1 $MgCl_2$, 5 NaHEPES, and 5 glucose, and the pH was adjusted to 7.4. Experiments were carried out at room temperature (20–25°C). Coverslips were mounted in an open chamber designed to fit in the stage of an inverted epifluorescence microscope (ICM 405; Carl Zeiss, Oberkochen, Germany). This microscope was equipped with a photon counter system (Photon Technology International, Brunswick, NJ) to simultaneously measure the intensity of light emitted at 405 ± 5 nm (F_{405}) and at 480 ± 5 nm (F_{480}) from the acid form of indo-1 trapped inside the cells and excited at 365 ± 5 nm. These data were acquired by using a PC equipped with an acquisition board. The system software (OSCAR; Photon Technology International) allowed corrections of background intensity at each emission wavelength. $[Ca^{2+}]_i$ values were derived from a standard curve that was constructed by the addition of increasing concentrations of Ca^{2+} (from 10 to 1500 nM to 15 μM indo-1) (Stojilkovic et al., 1993). The open recording chamber allowed access to cells for simultaneous electrophysiological studies. Acquisition of the fluorescence signals and $I_{K(Ca)}$ were synchronized with a common external trigger logic pulse.

Analysis of results

For the analysis of the kinetics of GnRH-initiated $[Ca^{2+}]_i$ oscillations, $I_{K(Ca)}$ current records were filtered at 50 Hz and digitized at 100 Hz, using the pClamp 5.5.1 software (Axon Instruments, Foster City, CA). The duration of each spike and the interval between spikes (time on and time off) were determined by using the single-channel analysis part of the pClamp package ("Fetchan"). Once the base line was established, the detection level for a spike was set at 10% of the total spike excursion. The list of events generated in this way was analyzed with pStat of the pClamp package. At least 30 spikes at each V_m and in every cell were considered for the calculation of the time constant for the time on and time off events.

RESULTS

Membrane potential influences the pattern of $[Ca^{2+}]_i$ oscillations

GnRH-induced Ca^{2+} spiking was analyzed in cultured gonadotrophs under several different experimental conditions: 1) in intact indo-1 AM-loaded cells by single-cell photometry (free-running cells); 2) in nystatin-perforated cells under a current clamp, by recording V_m oscillations; 3) in nystatin-perforated and voltage-clamped cells, by measuring the Ca^{2+}-activated potassium current ($I_{K(Ca)}$); 4) in indo-1 AM-loaded and nystatin-perforated patch-clamped cells, by simultaneous $[Ca^{2+}]_i$ and current measurements; and 5) in the whole-cell variant of the patch-clamp technique that was used for intracellular $InsP_3$ application. We (Kukuljan et al., 1992) and others (Tse and Hille, 1992; Tse et al., 1995) have previously shown that V_m and current measurements can be employed to estimate the $[Ca^{2+}]_i$ pattern, because the rise in $[Ca^{2+}]_i$ activates $I_{K(Ca)}$ channels. In non-voltage-clamped gonadotrophs, the activation of these channels is associated with V_m hyperpolarization. Using such varying experimental approaches, we addressed the role of Ca^{2+} entry in $InsP_3$-controlled Ca^{2+} release.

In free-running gonadotrophs exposed to a constant agonist concentration (1 nM), the frequency of Ca^{2+} spiking

increased and the amplitude decreased over a 100–200-s period (Fig. 1 A), reaching a stable level of frequency 0.22 ± 0.08 Hz and amplitude of 0.43 ± 0.11 μM (n = 20). The same evolution in frequency was observed by measuring the hyperpolarization episodes caused by the periodic activation of $I_{K(Ca)}$ channels in cells under a current clamp (Fig. 1 B). Thus the initial increase in the frequency of Ca^{2+} spiking at a constant agonist concentration was not a consequence of the presence of the fluorescent dye in the cells. However, after 5–15 min of spiking the stable mode of the oscillatory response in cells loaded with the fluorescent dye was usually replaced by a nonoscillatory increase in $[Ca^{2+}]_i$ to about 100 nM above the basal level. In contrast, the V_m
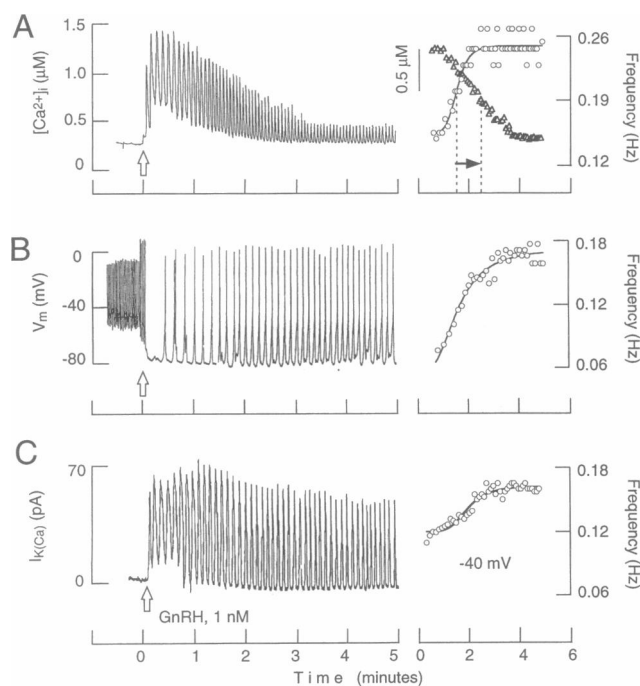

FIGURE 1   Time course of GnRH-evoked oscillations measured by three different experimental approaches. On each panel, the addition of the agonist is indicated by an upward arrow. (A) Left: GnRH-evoked $[Ca^{2+}]_i$ oscillations recorded by single-cell photometry. Right: Time course of instantaneous frequency (O) and amplitude ($\triangle$) of spiking. The dotted lines indicate the time at which frequency and amplitude reached half plateau levels (half-time). The horizontal arrow shows the shift between these two times. (B) Left: Agonist-induced modification of the electrical activity in an isolated gonadotroph. V_m was recorded under current clamp using the nystatin-perforated patch-clamp technique. After the addition of GnRH, there is a reduction in the frequency of the firing of action potentials that appears to be intercalated with hyperpolarization events. These events correspond to the periodic activation of calcium-controlled potassium channels by the agonist-induced $[Ca^{2+}]_i$ spikes. Right: Time course of the instantaneous frequency of the hyperpolarization events. (C) Left: Whole-cell current record obtained by the nystatin-perforated patch-clamp technique in a cell under voltage-clamp conditions. During this experiment the membrane potential was held at −40 mV. The oscillations of the whole-cell current observed after the addition of GnRH correspond to the periodic activation of $I_{K(Ca)}$ by the agonist-induced $[Ca^{2+}]_i$ spiking. Right: Time course of the instantaneous frequency of the GnRH-evoked $I_{K(Ca)}$ oscillations. In these and all other experiments shown in Figs. 2–9, gonadotrophs were stimulated with 1 nM GnRH.

oscillations were recorded for up to 1 h at a constant frequency, suggesting that the Ca^{2+}-buffering ability of indo-1 influences the pattern of signaling (see also Wagner and Keizer, 1994).

In gonadotrophs under voltage clamp through a nystatin-perforated patch, GnRH induced $I_{K(Ca)}$ oscillations similar to the $[Ca^{2+}]_i$ spiking observed by fluorescence measurements in intact free-running cells (Figs. 1 C and 2). In a cell clamped at −40 mV, an increase in the frequency of spiking was observed during the first 150 s of stimulation (Fig. 1 C). Such increases were also observed in gonadotrophs held at −50 and −30 mV (Fig. 2 B, upper panel), as well as in cells bathed in Ca^{2+}-deficient medium (not shown). These observations indicate that the initial increase in the frequency of spiking at constant agonist concentration is independent of the V_m and of Ca^{2+} entry. However, the V_m at which the cells were held had a significant impact on the sustained frequency of spiking. In a cell held at −30 mV, the frequency of Ca^{2+} oscillations reached a plateau during the first 2 min of stimulation and stayed unchanged during the next 15 min. In contrast, in a cell held at −50 mV the frequency of spiking reached a maximum value and then decayed continuously until the repetitive spikes disappeared (Fig. 2). The time course of the decrease in frequency and the total duration of the spiking varied widely among cells; in six cells held at −50 mV the time between the peak frequency and the last spike ranged from 200 to 1200 s. Similar results were obtained in cells held at −80 to −100 mV (not shown). These observations indicate that a V_m at or above −40 mV is required for sustained oscillatory Ca^{2+} release from the ER.

Fig. 3 illustrates the effects of transient change in V_m on the pattern of calcium spiking in two cells, one under voltage clamp at −50 mV (Fig. 3 A) and the other at −30 mV (Fig. 3 B). In the cell continuously clamped at −50 mV, Ca^{2+} oscillations were almost completely exhausted after 800 s of stimulation, whereas in the cell fixed at −30 mV no significant decrease in the frequency of Ca^{2+} spiking was observed over a similar time period. In both cells, a rapid change in the frequency of Ca^{2+} spiking occurred in response to the instantaneous change in V_m; an increase in the cell depolarized from −50 to −30 mV and a decrease in the cell hyperpolarized from −30 to −50 mV. The return of cells to their previous holding potentials was again associated with rapid changes in their frequencies. In 12 cells, steady depolarization from −50 to −30 mV resulted in an increase in the frequency of oscillations from 0.12 ± 0.03 to 0.23 ± 0.06 Hz. In both cases shown in Fig. 3, as well as in cases shown in Figs. 4, 7, and 9, the initial increase in frequency induced by the depolarizing transition was followed by a gradual decrease toward a steady-state level. The hyperpolarization-driven decrease in the frequency was also composed of two phases, a rapid drop followed by a further gradual decline.

Qualitatively similar results on the frequency of Ca^{2+} spiking were obtained when $[Ca^{2+}]_i$ was measured simultaneously with $I_{K(Ca)}$ in GnRH (1 nM)-stimulated cells (n =

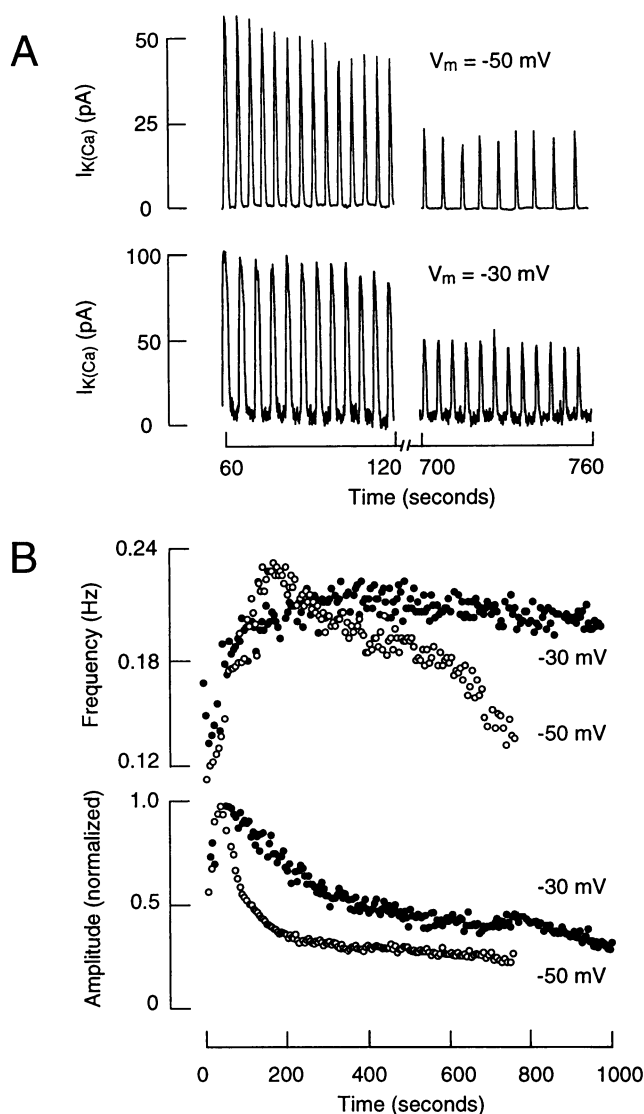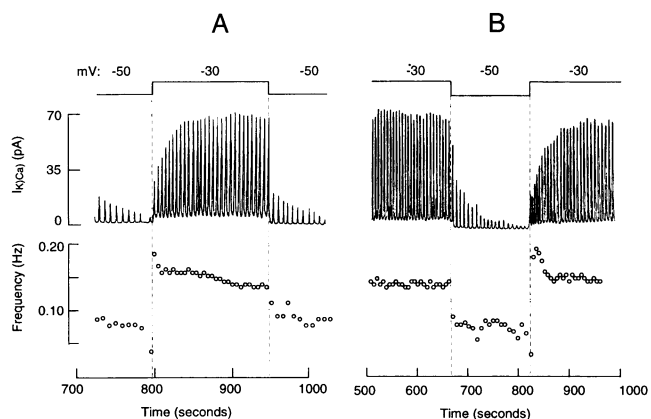FIGURE 3   Effects of step depolarization (A) and hyperpolarization (B) on the frequency and amplitude of $I_{K(Ca)}$ oscillations in agonist-stimulated gonadotrophs. Cells were stimulated with GnRH at time 0 and continuously held at -50 mV (A) or -30 mV (B).

lations, measured by $I_{K(Ca)}$ (Fig. 4 B). These data suggest that $I_{K(Ca)}$ is a good indicator of the $[Ca^{2+}]_i$, and that the V_m-induced changes in frequency of spiking also occur at "clamped" intracellular InsP$_3$ concentrations.

Difference in frequency and amplitude responses to changes in V_m

In free-running cells, the frequency and amplitude of the agonist-induced Ca^{2+} oscillations showed an inverse pattern; an increase in the frequency was associated with a decrease in the amplitude of spikes before the steady state of the signaling was reached (Fig. 1 A). This pattern was
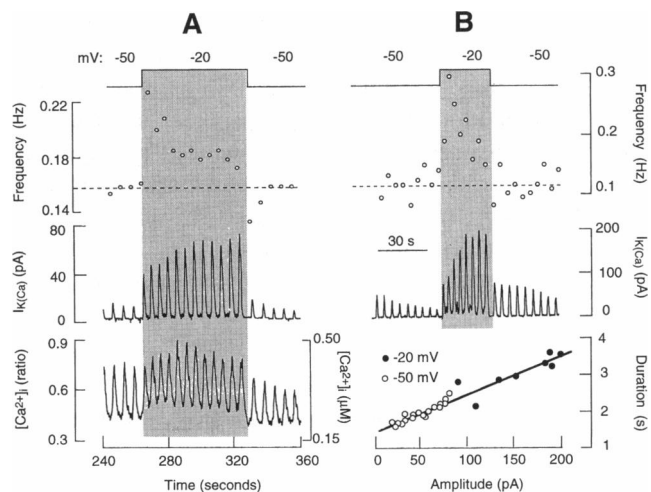

FIGURE 2   Effect of the holding potential on the time course of the amplitude and instantaneous frequency of $I_{K(Ca)}$ oscillations induced by GnRH in cells under voltage-clamp conditions. The experiments were performed using the nystatin-perforated patch-clamp technique. (A) Representative whole-cell current traces showing $I_{K(Ca)}$ oscillations recorded in cells held at two different V_m. Top traces: Comparison between the oscillatory patterns observed just after 1 min (left trace) and more than 11 min (right trace) after the addition of GnRH, in a cell voltage-clamped at -50 mV. Bottom traces: Comparison of the oscillatory patterns observed at the same times but in a cell held at -30 mV. (B) Temporal course of the instantaneous frequency (upper panel) and amplitude (lower panel) of the GnRH-induced $I_{K(Ca)}$ oscillations in a cell held at -50 mV (O) as compared to a cell held at -30 mV (●). The analyzed data are from the experiments illustrated in A.

FIGURE 4   Characterization of the depolarization-induced increase in the frequency of spiking. (A) Simultaneous measurements of $I_{K(Ca)}$ and $[Ca^{2+}]_i$ oscillations induced by GnRH in indo 1-AM-loaded and nystatin-perforated patch-clamped cells. (B) Effect of a step depolarization on the frequency of $I_{K(Ca)}$ oscillations induced by the injection of InsP$_3$ (1 μM) through the pipette (whole-cell patch-clamp configuration). The correlation between the amplitude and duration of spikes in this experiment is shown in the bottom panel.

2). Fig. 4 A shows that the depolarization of a gonadotroph from -50 to -20 mV induced a rapid increase in frequency and a gradual increase in amplitude of $[Ca^{2+}]_i$ and I_{K-Ca} oscillations. Similarly, when $[Ca^{2+}]_i$ spikes were initiated by direct intracellular application of InsP$_3$ (1 μM) through the patch pipette, depolarization from -50 to -20 mV was associated with an increase in the frequency of Ca^{2+} oscil-

detected in all ($n = 35$) analyzed records, but with a difference in the half-times required to reach the steady-state level (indicated by *horizontal arrow* in Fig. 1 *A*). In cells held at -30 mV, the frequency of $I_{K(Ca)}$ spiking reached a plateau after an initial increase, with a half-time of 85 s, whereas the amplitude of oscillations progressively decreased, with a half-time of 240 s (Fig. 2 *B*). In cells continuously held at -20 mV, the frequency of spiking reached a plateau within 120 s, and only a minor decrease in the amplitude of Ca^{2+} spiking was observed (not shown). In contrast, both the amplitude and frequency of spiking in the cell clamped at -50 mV decreased progressively after an initial increase, with calculated half-times of 80 and 475 s, respectively (Fig. 2 *B*). Thus clamping the cells at -40 to -30 mV was sufficient to mimic the temporal course of Ca^{2+} spiking observed under physiological conditions, although this pattern was modified by the hyperpolarization of the cells below -40 mV or depolarization above -30 mV.

In accord with this, an instantaneous change in V_m during the sustained phase of the oscillations had a differential effect on the amplitude and frequency of the signal. As shown in Fig. 3, a V_m transition had a rapid (within one cycle) effect on the frequency and a gradual effect on the amplitude of spiking. In all four cases shown in Fig. 3, a similar half-time dependence of the increase or decrease in the amplitude of spiking was observed (30 to 40 s). The instantaneous changes in the amplitude (first spike upon depolarization/hyperpolarization) are consistent with the effect of electrochemical gradient for K^+ across the plasma membrane on $I_{K(Ca)}$ (Tse and Hille, 1992; Kukuljan et al., 1992), and were not accounted for in calculations of half-times. These data suggest that even though the frequency and amplitude of Ca^{2+} oscillations are controlled by V_m, they show different time dependencies, the frequency being an instantaneous function of V_m and the amplitude of spiking presenting a much slower time constant of response. Furthermore, there is a linear correlation between the amplitude and duration of $[Ca^{2+}]_i/I_{K(Ca)}$ spikes. Fig. 4 *B* (*bottom panel*) illustrates a case of InsP$_3$-induced spiking. Similar levels of correlation were observed in GnRH-stimulated cells when $I_{K(Ca)}$ ($R = 0.98$) and $[Ca^{2+}]_i$ were measured ($R = 0.97$). Because the amplitude of $I_{K(Ca)}$ transients is also affected by the V_m, the duration of spikes was employed in further analysis.

Extracellular Ca^{2+} is required for the effects of V_m

In general, the effects of V_m on the frequency and amplitude of Ca^{2+} oscillations could be related to the modulation of Ca^{2+} entry through VGCC. They could also be related, however, to the modulation of other ionic fluxes like Na^+ or Cl^-, or to the effects of voltage per se. To address this problem, we studied the evolution of the GnRH-initiated $[Ca^{2+}]_i$ spikes under a voltage clamp in cells bathed in a Ca^{2+}-deficient and Na^+-containing medium. As in a Ca^{2+}-

containing medium, the frequency of GnRH-induced $[Ca^{2+}]_i$ oscillations in cells held at -50 mV initially increased to 0.24 $\pm$ 0.05 Hz ($n = 4$), and then decreased gradually until the spikes disappeared. However, in contrast to cells bathed in Ca^{2+}-containing medium, a steady depolarization to -20 mV was unable to modify the frequency of spiking in Ca^{2+}-deficient medium (Fig. 5 *A*; $n = 3$). Despite that inability, the reintroduction of Ca^{2+} to the extracellular medium recovered the sensitivity to V_m. As shown in Fig. 5 *B*, the rise in the extracellular Ca^{2+} concentration to 1 mM caused by the addition of a small
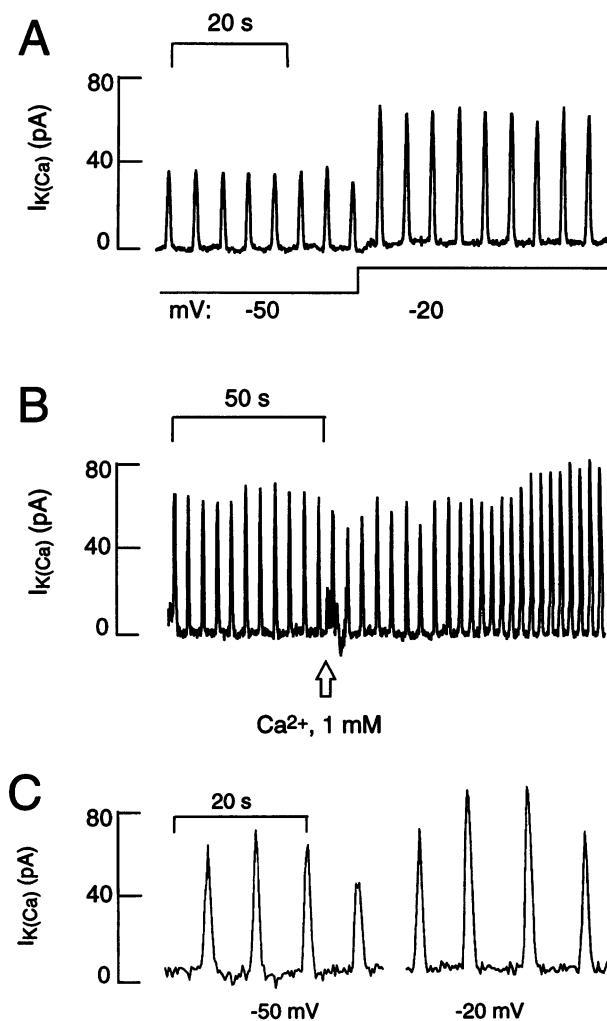

FIGURE 5 Extracellular calcium dependence of depolarization-induced changes in the pattern of spiking in agonist-stimulated cells. (*A*) The lack of effect of depolarization on the frequency response in GnRH-stimulated cells bathed in Ca^{2+}-deficient medium. The small increase in the amplitude of $I_{K(Ca)}$ spikes and base line immediately upon depolarization is consistent with the change in the electrochemical gradient for potassium across the plasma membrane. (*B*) Recovery of the sensitivity to V_m after the addition of calcium to the cell shown in *A*. The GnRH-stimulated cell was held at -20 mV, and Ca^{2+} was added in a 100-μl volume (1 mM final concentration) in a dish already containing 1 ml medium. The lack of effect of depolarization on the frequency of $I_{K(Ca)}$ oscillations in GnRH-stimulated cells exposed to dihydropyridine calcium channel antagonist nifedipine (5 μM).

volume (100 μl) to the dish was associated with a gradual increase in the frequency of spiking. The differences in the time course of this response compared to the response to voltage steps were probably due to the slow diffusion of Ca^{2+} in the bath.

In cells bathed in 2.6 mM Ca^{2+}-containing medium, the L-type calcium channel antagonist, nifedipine, abolished the depolarization-driven effects on the frequency and amplitude. Fig. 5 C illustrates the pattern of sustained Ca^{2+} spiking in a cell exposed to 5 μM nifedipine and held at two potentials, -50 and -20 mV. In addition, a V_m-dependent increase in $[Ca^{2+}]_i$ was confirmed in indo-1 AM-loaded and nystatin-perforated gonadotrophs. As shown in Fig. 6, a step depolarization induced a rapid and sustained increase in $[Ca^{2+}]_i$ in unstimulated gonadotrophs in a V_m-dependent manner. Thus all of this evidence indicates that Ca^{2+} entry through VGCC is required for the effects of V_m on the pattern of agonist-induced Ca^{2+} signaling in gonadotrophs.

Time and V_m dependence of depolarization on the frequency of spiking

The effect of depolarization on the frequency of GnRH-induced spiking was critically dependent on the time when the V_m pulses were applied. Fig. 7 A illustrates the lack of response to a depolarization to -20 mV applied only 100 s after the addition of GnRH. In general, no modulation in the frequency of spiking was observed when the V_m was changed from -50 to levels in the range -40 to 0 mV during the initial phase of the response (first 200 s). However, depolarizing pulses increased the frequency of spiking at any time point during the sustained phase of the response to the agonist (beyond 200 s). In the case shown in Fig. 7 B, depolarization of a cell held at -50 mV induced an increase in the frequency of spiking dependent on the magnitude of depolarization. As discussed above, the frequency of spiking decreased to the same plateau during the course of all four V_m steps after the initial increase. Furthermore, although the V_m-controlled Ca^{2+} entry differed in these cases (Fig. 6 B), the plateau frequency was similar for all voltage steps applied. As indicated by the dashed line in Fig. 7 B, such frequency did not differ from the initial maximum
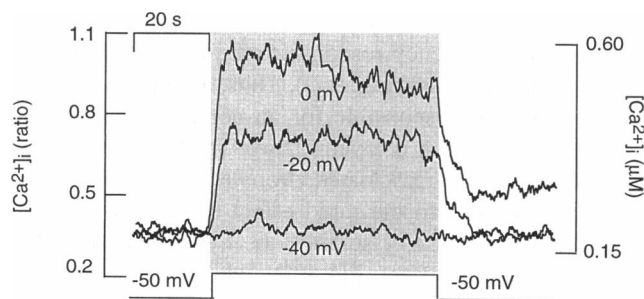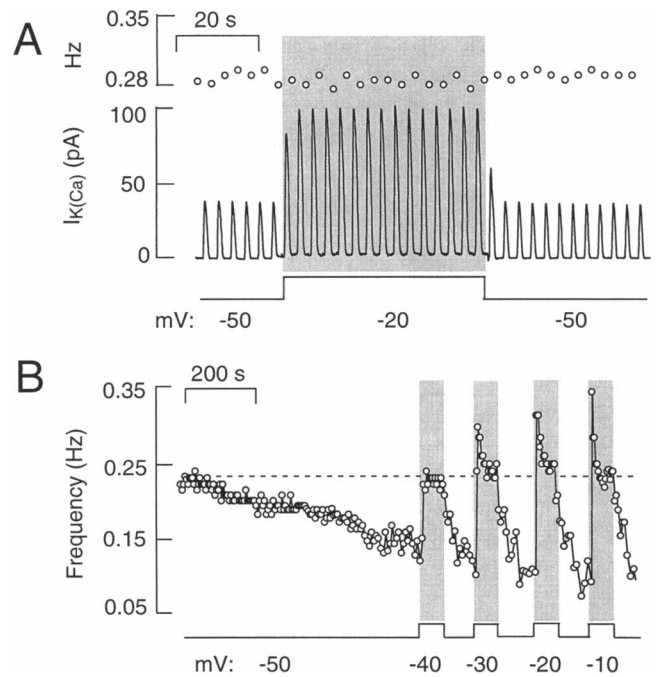

FIGURE 7 Time dependence of depolarization on the frequency of spiking. (A) The lack of effect of depolarization, applied 100 s after the addition of GnRH, on the frequency of oscillations. (B) V_m dependence of depolarization on sustained GnRH-induced Ca^{2+} oscillations. The dashed horizontal line indicates the maximum frequency response reached 150 s after the initiation of oscillations at the holding potential of -50 mV.

frequency determined by the exposure to GnRH. Thus, although the initial change in frequency of the GnRH-induced spiking is well correlated with the rapid V_m-dependent changes in Ca^{2+} entry, the secondary evolution of this parameter as well as the changes in amplitude of the spikes do not follow the changes in Ca^{2+} entry.

The effects of depolarization on the frequency of sustained oscillations was confirmed in experiments in which V_m was changed after an 80-s ramp from -70 to 0 mV. As shown in Fig. 8 A, depolarization of the cell above -45 mV was associated with a progressive increase in the frequency, reaching a maximum at -20 mV, whereas further depolarization was followed by a gradual decrease in frequency. The bell-shaped effect of V_m on the frequency of spiking was also observed in three other cells, with a maximum increase in frequency occurring between -20 and -10 mV. Such V_m dependence of the frequency in a depolarizing ramp protocol was similar to that observed in step depolarization, but it differs from the current-voltage relationship for VGCC and the V_m-derived rise in $[Ca^{2+}]_i$ in gonadotrophs. As shown in Fig. 8 A (bottom panel), there is a 20-mV shift in the V_m midpoints for frequency versus calcium current or frequency versus $[Ca^{2+}]_i$. Thus the bidirectional effect of depolarization on frequency is associated with a progressive increase in Ca^{2+} influx through VGCC.

FIGURE 6 Effects of depolarization on $[Ca^{2+}]_i$ in unstimulated cells. Gonadotrophs were loaded with indo-1 AM, and $[Ca^{2+}]_i$ was recorded in nystatin-perforated cells under voltage-clamp conditions.
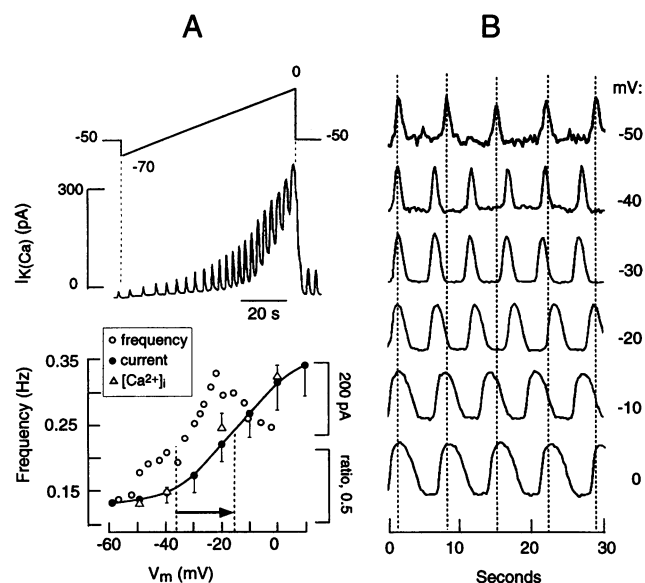
FIGURE 8 V_m dependence of depolarization on the frequency of sustained spiking. (A) Changes in the pattern of $I_{K(Ca)}$ response in GnRH-stimulated cell (middle panel) after an 80-s ramp from −70 to 0 mV (upper panel). The instantaneous frequency is shown in the bottom panel (O). Vertical dashed lines indicate V_m midpoints for frequency, calcium current (●), and [Ca^{2+}]$_i$ (△) in response to changes in V_m. Calcium current and [Ca^{2+}]$_i$ were examined by employing a depolarizing pulse protocol, and the results were expressed as mean ± SE. The horizontal arrow indicates a shift in the V_m midpoints for frequency versus calcium current and [Ca^{2+}]$_i$. (B) Effect of sustained depolarization on the pattern of $I_{K(Ca)}$ response in GnRH-stimulated cells. To avoid the driving-force-dependent changes in the amplitude of $I_{K(Ca)}$, the amplitudes of spiking were normalized. Before depolarization, the cell was held at −50 mV for 500 s.

Depolarization modulates frequency of spiking by two separate processes

To further characterize the bidirectional effects of membrane depolarization on the frequency of spiking, the pattern of spiking was analyzed in cells clamped at different potentials. Fig. 8 B shows the normalized records of sustained oscillations in a gonadotroph clamped at −50 to 0 mV. The depolarizing transitions from −50 to −40 and −30 mV led to an increase in the frequency of spiking, whereas the duration of individual spikes was not notably changed. However, a further increase in the depolarizing steps (−30 to 0 mV) was followed by an increase in the duration of the spikes and a progressive decrease in the frequency of oscillations. Therefore, although the membrane depolarization led to a bidirectional modulation of the frequency of spiking, it was associated with a progressive increase in the total time in which the [Ca^{2+}]$_i$ was elevated, with a V_m dependence comparable to that observed in calcium current and voltage-dependent increases in [Ca^{2+}]$_i$.

To better characterize this phenomenon, steady-state $I_{K(Ca)}$ records measuring the high and low calcium dwell times for a series of V_m were analyzed (Fig. 9 A). We found that an increase in $I_{K(Ca)}$ over a threshold during a record (P on) showed a strong dependence on the V_m (Fig. 9 C). Thus,
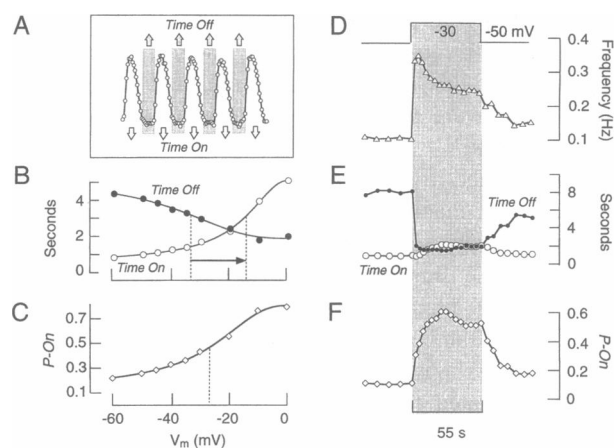

FIGURE 9 V_m and time dependence of depolarization effects on mean duration of spikes and interval between spikes in GnRH-stimulated cells. (A) The duration of spikes (time on), the interval between spikes (time off), and the fraction of the time during which the [Ca^{2+}]$_i$ remained elevated (P on) were determined as described in Materials and Methods. (B and C) V_m dependence of time on, time off, and P on as observed in step depolarization experiments. Vertical dotted lines indicate V_m midpoints for time off, time on, and P on, and the horizontal arrow indicates a shift in the V_m midpoints for time off and time on. (D–F) Time dependence of time on, time off, and P on during a 55-s depolarization pulse.

although the voltage-dependent increase in the frequency of spiking was smaller at more depolarized V_m, the cells spent more time with their [Ca^{2+}]$_i$ elevated. The increase in the P on as the membrane depolarized was a consequence of a V_m-dependent change in both the mean duration of the spike (time on) and the interval between spikes (time off) (Fig. 9 B). In the −50 to −20 mV range, a marked decrease in time off was observed. This interval approached a minimum value at V_m in the −10 to 0 mV range. In contrast, time on increased as the V_m depolarized. The arrow in Fig. 9 B illustrates the difference in the V_m midpoints for the time off and time on. In some cells stimulated with 1 nM GnRH as well as in the majority of those stimulated with 10 nM GnRH, the depolarization to V_m positive to −10 mV was associated with the loss of the oscillatory regime.

The P on, time on, and time off were also dependent on pulse duration at a certain V_m. As shown in Fig. 9 E, an instantaneous decrease in time off and a more gradual increase in the time on were observed upon depolarization. Such changes were in accord with the existence of the two phases in the frequency response to a step depolarization, transient and sustained (Fig. 9 D). Thus, a rapid decrease in the time off was responsible for an acute increase in the frequency, whereas a delayed change in the time on was accountable for the associated drop in the frequency response. In contrast to the rapid effect of the depolarizing step on the frequency of spiking, the hyperpolarizing step had a more gradual effect (Fig. 9 D), which was determined by a progressive increase in the time off and decrease in the time on (Fig. 9 E). Because the rise and the drop in [Ca^{2+}]$_i$ in response to depolarizing/hyperpolarizing steps occurred with similar half-times (Fig. 6 B), it is obvious that factor(s)

other than the change in the rate of Ca^{2+} entry influenced the frequency response. On the other hand, the lack of effect of V_m on frequency in cells bathed in Ca^{2+}-deficient medium suggests that Ca^{2+} entry during the depolarizing step is essential for the subsequent effect of hyperpolarization. Furthermore, the progressive and substantial increase in P on during the depolarizing pulse (Fig. 9 F) suggests that the increase in the fraction of the time with elevated $[Ca^{2+}]_i$ is associated with a substantial recovery of the ER calcium pool. Thus it is likely that the increase in the concentration gradient for Ca^{2+} across the ER membrane also influences the pattern of signaling.

DISCUSSION

A number of methodological considerations are critically important in the interpretation of these results: 1) To control Ca^{2+} entry through VGCC, gonadotrophs were voltage-clamped through nystatin-perforated patches or classic whole-cell patch-clamp. Because the nystatin-formed pores are not permeable to calcium (Horn and Marty, 1988), $[Ca^{2+}]_i$ is not influenced by the content of this cation or Ca^{2+} buffering compounds in the pipette solution. 2) Calcium release from intracellular stores was induced either through the extracellular application of GnRH, which leads to the activation of phospholipase C and $InsP_3$ release (Naor et al., 1986), or by directly applying $InsP_3$ to the intracellular compartment via a patch pipette (whole-cell recording). To avoid variations in the expression of GnRH receptors (Sealfon et al., 1990) and sensitivity to steroid hormones derived from the estrous cycle in gonadotrophs (Emons et al., 1991), cells from ovariectomized rats were used. 3) The kinetics of the $[Ca^{2+}]_i$ changes was followed my measuring $I_{K(Ca)}$ expressed by gonadotrophs (Tse and Hille, 1992; Kukuljan et al., 1992). This provides an indirect method for measuring the $[Ca^{2+}]_i$, and it follows only the changes of the $[Ca^{2+}]_i$ adjacent to the plasma membrane. However, it avoids the use of fluorescent dyes, which perturb the intracellular Ca^{2+} dynamics by increasing a non-regulated Ca^{2+}-buffering capacity.

Our results demonstrate that even though $InsP_3$-induced periodic Ca^{2+} mobilization can be initiated at any membrane potential (Stojilkovic et al., 1992; Kukuljan et al., 1994), V_m exhibits a significant influence on the pattern of sustained spiking. Thus, in cells held at hyperpolarized potentials, the oscillatory response occurs in a manner comparable to that observed in free-running cells bathed in Ca^{2+}-deficient medium (Stojilkovic et al., 1992). In both cases, the response is associated with a progressive decrease in the frequency and amplitude of spiking and abolition of oscillations, in contrast to the sustained rhythm achieved in free-running cells bathed in Ca^{2+}-containing medium. This result suggests that in addition to influencing the amplitude of the signal (Kukuljan et al., 1994), V_m also influences the kinetics of the intracellular Ca^{2+} mobilization.

The decrease in the frequency and duration of $[Ca^{2+}]_i$ spikes observed in hyperpolarized cells is rapidly reversed

by steady depolarization. Moreover, a graded increment in both parameters is observed at increasingly depolarized membrane potentials, which is reflected by an increase in the total fractional time the $[Ca^{2+}]_i$ remains elevated at constant agonist concentration. The full recovery of the frequency pattern was observed in cells continuously held at -40 to -30 mV, when the calculated Ca^{2+} entry is comparable to that in free-running cells. Furthermore, the oscillatory nature of Ca^{2+} response was preserved in voltage-clamped cells at -30 to 0 mV, when Ca^{2+} entry exceeds 3–8 times that calculated in free-running gonadotrophs. Only in some cells stimulated with 1 nM GnRH was the depolarization to V_m positive to -10 mV associated with the loss of the oscillatory regime. These observations indicate that 1) a steady-state Ca^{2+} entry in gonadotrophs can substitute for the periodic Ca^{2+} influx in free-running cells; and 2) the ER oscillator has the capacity to adopt a large and continued Ca^{2+} influx without disrupting the oscillatory response when operated at lower frequency.

It is unlikely that the effects of Ca^{2+} entry on the pattern of spiking were a consequence of the voltage and/or Ca^{2+} entry-dependent changes in $InsP_3$ production. The ability of $InsP_3$ and its nonmetabolized forms to induce oscillatory changes in $[Ca^{2+}]_i$ have been observed in several cell types (Wakui et al., 1989; DeLisle et al., 1990; Lechleiter and Clapham, 1992), including pituitary gonadotrophs (Stojilkovic et al., 1993). Fig. 5 A clearly shows that depolarization does not change the frequency of sustained Ca^{2+} spiking in cells bathed in Ca^{2+}-deficient medium. Furthermore, the increase in $[Ca^{2+}]_i$ in pituitary cells by high potassium-induced depolarization and by the addition of Bay K 8644 is not associated with an increase in inositol phosphate production (Stojilkovic et al., 1994), arguing against Ca^{2+}-dependent facilitation of phospholipase C activity in these cells. Stimulation and inhibition of voltage-insensitive Ca^{2+} entry in Xenopus oocytes were also found to modulate the frequency of $InsP_3$-induced Ca^{2+} spiking (Girard and Clapham, 1993), indicating that the nature of channels involved in Ca^{2+} entry as well as changes in V_m are not essential for the observed effects. Because the depolarization-induced modulation of sustained spiking was also observed in cells with "clamped" $InsP_3$ levels, our data support the hypothesis that changes in the $[Ca^{2+}]_i$ are modulating the oscillatory response by acting at the level of Ca^{2+} liberation from ER.

A closer analysis of the time course and voltage sensitivity of depolarization-induced changes in the pattern of spiking suggests that Ca^{2+} entry affects the kinetics of $InsP_3$-induced Ca^{2+} oscillations by at least two mechanisms. The first is instantaneous and is manifested as an increase in the frequency of spiking, which is correlated with a decrease in the silent interval between consecutive spikes. The other is a gradual amplificatory process, manifested by an increase in amplitude and duration of the spikes, which in turn leads to a reduction in the frequency of spiking, even though intervals remain shortened. Whereas

the rapid effect is apparent at lower depolarization, the gradual effect predominates at higher depolarized membrane potentials. The overall effect of such changes in the pattern of Ca^{2+} spiking is an increase in duration of $[Ca^{2+}]_i$ elevation.

The rapid effect of Ca^{2+} entry on sustained spiking is compatible with the activatory roles of this ion on IP_3R channels (Bezprozvanny et al., 1991), and the hypothesis of ER Ca^{2+} excitability (Lechleiter and Clapham, 1992; Keizer et al., 1995), which suggests that $InsP_3$ activates the IP_3R channels, sets the maximum rate of Ca^{2+} release and makes the IP_3R channel sensitive to Ca^{2+}. Thus, once the $InsP_3$-induced liberation of Ca^{2+} is initiated, the consequent rise in $[Ca^{2+}]_i$ provides a positive feedback signal that leads to a progressive increase in its own release up to the maximum rate determined by $InsP_3$ concentration (Keizer et al., 1995). Accordingly, the continuous depletion of the ER pool in hyperpolarized cells and the consequent reduction in the Ca^{2+} release gradient may be responsible for slowing down the response, i.e., the reduction in Ca^{2+} release can subsequently decrease the rate of activation of the receptor channel, even for a constant level of $InsP_3$. In addition, depolarization leads to a rapid increase in $[Ca^{2+}]_i$ that is sufficient for the instantaneous recovery of the initial frequency, but not of frequencies above it, as documented in Fig. 7.

On the other hand, the direct proportionality of the amplification of the signals with depolarization-induced activation of L channels and increase in $[Ca^{2+}]_i$ suggest that the gradual effects are a cumulative result of sustained Ca^{2+} entry. Thus it is reasonable to postulate that the increase in size and duration of spikes and the associated decrease in the frequency of spiking are the consequences of increase in the ER Ca^{2+} content. In support of this, it has been shown previously that depolarization-driven recovery in the amplitude of oscillations is a result of reloading of the ER pool (Kukuljan et al., 1994). In addition to that, several other factors may also participate in the observed effects on the pattern of Ca^{2+} signaling, such as stimulatory and inhibitory effects of Ca^{2+} on IP_3R channels, reuptake of Ca^{2+} by ER, and its exclusion from the cells. Together, these data indicate that at constant $[InsP_3]$, the frequency of Ca^{2+} spiking is not exclusively determined by the time needed for Ca^{2+} to activate the ER oscillator, but also by cellular Ca^{2+} homeostasis in general.

These effects of the depolarization-associated Ca^{2+} entry explain why the pattern of the signal in free-running cells during the sustained stimulation is so sensitive to the electrical activity of the plasma membrane. Action potential-driven Ca^{2+} entry not only protects the cells from complete depletion of the ER calcium pool during the sustained stimulation, but also provides the extra Ca^{2+} needed to reach the threshold for a rapid increase in the efflux of Ca^{2+} from ER, as occurs at the beginning of the rising phase of each spike. This establishes a highly regular periodic pattern of sustained spiking that can continue without alterations as long as $InsP_3$ levels remain elevated and the plasma membrane excitability is not altered.

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