0026-895X/94/051013-09$03.00/0

MOLECULAR PHARMACOLOGY, 45:1013-1021

Control of Calcium Spiking Frequency in Pituitary Gonadotrophs by a Single-Pool Cytoplasmic Oscillator

STANKO S. STOJILKOVIC, MELANIJA TOMIĆ, MANUEL KUKULJAN,[1] and KEVIN J. CATT

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

Received November 16, 1993; Accepted February 8, 1994

SUMMARY

The mechanisms by which the generation and frequency of cytoplasmic Ca^{2+} oscillations are controlled were investigated in pituitary gonadotrophs. In these cells, two Ca^{2+}-mobilizing receptors, the gonadotropin-releasing hormone and endothelin receptors, induce frequency-modulated Ca^{2+} spiking at the rate of up to 30 min^{-1}. The cytoplasmic oscillator is also activated by discharge of luminal Ca^{2+} (initiated by ionomycin, thapsigargin, or thimerosal) but not by increased voltage-sensitive Ca^{2+} influx or treatment with caffeine. The basic difference between these two types of Ca^{2+} oscillations is related to their requirement for inositol-1,4,5-trisphosphate ($InsP_3$). Thapsigargin-, thimerosal-, and ionomycin-induced spiking occurs without the rise in $InsP_3$ production that is essential for the generation of receptor-controlled oscillatory responses. The differential requirement for $InsP_3$ in the two types of Ca^{2+} spiking is indicated by two lines of evidence. First, agonist-induced Ca^{2+} spiking of frequency similar to that of non-receptor-mediated oscillations was accompanied by a significant increase in $InsP_3$, whereas none of the non-receptor-mediated oscillations was associated with measurable changes in inositol phosphate production. Second, agonist-induced $InsP_3$ formation and Ca^{2+} spiking were abolished by treatment with the phospholipase C inhibitors U73122 and neomycin sulfate, whereas non-receptor-mediated Ca^{2+} spiking was not affected by these agents. When the oscillator was activated by agents that do not increase $InsP_3$ formation, it operated only at the basal rate of ~5 min^{-1} and spiking frequency did not rise with increasing drug concentrations, in contrast to the situation in agonist-stimulated gonadotrophs. However, both types of oscillations were affected by depletion of luminal Ca^{2+} and by changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) but were not inhibited by ryanodine. These findings are consistent with the operation of a single-pool Ca^{2+} oscillator that is responsible for generation of both types of Ca^{2+} oscillations. The oscillator is controlled by the coagonist actions of $InsP_3$ and Ca^{2+} on the $InsP_3$ receptor channels and by the activation of Ca^{2+}-ATPase by rising $[Ca^{2+}]_i$. It can be induced to operate at low frequency without an increase in $InsP_3$ production by agents that reduce intraluminal $[Ca^{2+}]_i$, and it exhibits a dose-dependent increase in spiking frequency during agonist stimulation.

In rat pituitary gonadotrophs, the hypothalamic neuropeptide GnRH and the vasoactive peptide ET-1 induce prominent heparin-sensitive Ca^{2+} oscillations (1–5) that are initiated by release of luminal Ca^{2+} and sustained by Ca^{2+} entry through a voltage-sensitive Ca^{2+} entry pathway (6–8) (receptor-mediated oscillations). Similar Ca^{2+} oscillations are induced by injection of $InsP_3$ or its nonmetabolizable analog (5, 9), confirming that $InsP_3R$ channels are involved in the oscillatory response and demonstrating that oscillations in $InsP_3$ are not required for Ca^{2+} spiking. Although the rise in $InsP_3$ is necessary for the initiation of receptor-mediated oscillations, it is not essential for the maintenance of oscillations during the sustained phase of agonist occupancy (9). Oscillatory Ca^{2+} responses can also be induced by non-receptor-dependent processes, as observed during treatment with TG or ionomycin (7, 9, 10) (non-receptor-mediated oscillations). Such non-receptor-mediated Ca^{2+} oscillations have been observed in several other cell types operated by Ca^{2+}-mobilizing receptors (11–17).

The existence of non-receptor-mediated and agonist-induced Ca^{2+} oscillations in the same cell type can be explained by two models of the cytoplasmic Ca^{2+} oscillator that are based on the positive feedback effects of Ca^{2+} on $InsP_3$-induced Ca^{2+} release. One such model proposes that phospholipase C is activated by Ca^{2+} released from the $InsP_3$-sensitive store, with consequent amplification of the $InsP_3$ signal ($Ca^{2+}/InsP_3$ cross-coupling) (18–20); in this system, $InsP_3$ is the propagating messenger and oscillates in synchrony with the $[Ca^{2+}]_i$. The other model is based on a two-pool CICR process that does not require oscillations in $InsP_3$. In this system, $InsP_3$ initiates the release of Ca^{2+} from the $InsP_3$-sensitive pool and Ca^{2+} is responsible for the maintenance of spiking by activation of a ryanodine-sen-

M.T. is on leave from the Faculty of Sciences, University of Zagreb, Croatia.
[1] Present address: Centro de Estudios, Cientificos de Santiago, Casilla 16443, Santiago 9, Chile.

ABBREVIATIONS: GnRH, gonadotropin-releasing hormone; $InsP_3$, inositol-1,4,5-trisphosphate; $InsP_3R$, inositol-1,4,5-trisphosphate receptor(s); CICR, calcium-induced calcium release; ET, endothelin; TG, thapsigargin; TH, thimerosal; $[Ca^{2+}]_i$, intracellular calcium concentration; BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

sitive pool (21). Thus, an increase in $[Ca^{2+}]_i$ is sufficient to sustain spiking in both models.

Several lines of experimental evidence favor an alternative model for agonist-induced Ca^{2+} oscillations, in which Ca^{2+} release from a single $InsP_3$-sensitive pool is sufficient to generate Ca^{2+} oscillations. In this system, both $InsP_3$ and Ca^{2+} are required to maintain spiking by controlling the activity of the $InsP_3R$ channel (22–27). However, the current concept of the coagonist action of $InsP_3$ and Ca^{2+} does not provide a common mechanism for the generation of the non-receptor-mediated Ca^{2+} oscillations and their relationship to agonist-induced spiking. It is also not clear how the frequency of Ca^{2+} spiking is controlled by such coagonist actions of the two intracellular signals. Ca^{2+} may modulate $InsP_3R$ channel activity not only from the cytoplasmic but also the luminal aspects of the receptor; both facilitatory and inhibitory actions of luminal Ca^{2+} on $InsP_3$-controlled Ca^{2+} spiking have been proposed (9, 28, 29). In permeabilized smooth muscle cells, the efficiency with which $InsP_3$ releases $^{45}Ca^{2+}$ decreases with decreasing Ca^{2+} content (28). In contrast, passive Ca^{2+} loss in permeabilized nasal gland cells did not affect the ability of $InsP_3$ to release Ca^{2+} (30). In intact pituitary gonadotrophs, the frequency of Ca^{2+} spiking progressively increases with time during constant agonist stimulation (9), and such increases in frequency are temporally correlated with decreases in luminal Ca^{2+} content (6). Finally, Ca^{2+} entry can also participate in the regulation of $InsP_3$-dependent Ca^{2+} release (31, 32) through still uncharacterized mechanisms.

We have analyzed the coagonist actions of $InsP_3$ and Ca^{2+} in the generation of both receptor- and non-receptor-mediated Ca^{2+} oscillations in pituitary gonadotrophs. Our findings indicate that these two types of Ca^{2+} spiking are not dependent on $Ca^{2+}/InsP_3$ cross-coupling, i.e., Ca^{2+}-induced phospholipase C activation, or on CICR in a "two-Ca^{2+} pool" system. Rather, the data indicate that a single-pool Ca^{2+} oscillator is sufficient to explain both non-receptor- and receptor-mediated forms of Ca^{2+} spiking. We propose that modulation of CICR by $InsP_3$ accounts for the coagonist actions of $Ca^{2+}/InsP_3$ in gonadotrophs, in which a single-pool oscillator can be induced to operate at low frequency without an increase in $InsP_3$ production and generates progressively higher frequencies in the presence of increasing $InsP_3$ concentrations.

Experimental Procedures

Chemicals. Indo-1/acetoxymethyl ester was purchased from Molecular Probes (Eugene, OR) and Calbiochem (San Diego, CA); TG was from L.C. Services (Boston, MA). GnRH and ET-1 were obtained from Peninsula Laboratories, Inc. (Belmont, CA), and all other chemicals were from Sigma Chemical Co. (St. Louis, MO). U73122 and U73433 were provided by Dr. J. E. Bleasdale (The Upjohn Company, Kalamazoo, MI).

$[Ca^{2+}]_i$ measurements. Anterior pituitary glands from 2-week ovariectomized female rats were enzymatically dissociated and the dispersed cells were cultured as described previously (7). Pituitary gonadotrophs (0.25×10^6/dish) were plated in 35-mm Petri dishes (Falcon), containing 25-mm-diameter glass coverslips (Erie Scientific Co., Portsmouth, NH) coated with 0.01% poly-L-lysine. After culture for 2–3 days, the medium was replaced with 2 ml of Hanks' M199 phenol red-free incubation medium (0.1% BSA, 25 mM HEPES, 12.5 mM $NaHCO_3$, 1% penicillin/streptomycin) containing 2 μM indo-1/pentaacetoxymethyl ester. After washing, individual coverslips were transferred into a Leiden coverslip dish (Medical Systems Co., Greenvale, NY) containing 1 ml of M199, then mounted on the stage of an inverted Diaphot microscope attached to a dual-emission microscopic fluorometer (Nikon, Garden City, NY) and examined under a 40× oil immersion fluorescence objective. For excitation of indo-1, the light beam from a 100-W mercury arc lamp was reduced by a 1/16 neutral density filter. All $[Ca^{2+}]_i$ values were derived from a standard curve that was constructed by addition of increasing concentrations of Ca^{2+} (from 10 to 1500 nM) to 15 μM indo-1 (7). Measurements were taken each 0.36 sec. The frequency and amplitude of oscillations were determined from experimental records of $[Ca^{2+}]_i$ versus time.

[^{3}H]Inositol labeling and stimulation of pituitary cells. After cell culture for 1 day in four-well plates, the medium was changed to 0.5 ml of inositol-free M199 with Hanks' solution containing 25 mM $NaHCO_3$, 0.1% BSA, and 10 μCi of *myo*-[^{3}H]inositol (DuPont-New England Nuclear, Boston, MA). After a 48-hr incubation period, the cells were washed three times with inositol-free M199 containing 25 mM HEPES and 0.1% BSA. The cells were preincubated in the same medium for 15 min, in the presence or absence of 10 mM LiCl. In all experiments, the cells were stimulated for 15 min at 37°. Reactions were stopped by the addition of perchloric acid, and after extraction inositol phosphates were separated by anion exchange high performance liquid chromatography (33).

Calculations. The inverse value of the time period between two spikes (denoted as $1/T$) was calculated from experimental records. The position of each peak was determined by interpolation. A second-degree polynomial function ($[Ca^{2+}]_i = at^2 + bt + c$) was drawn through the point of the highest observed $[Ca^{2+}]_i$ value and its two closest neighboring points. The position of the maximum was determined as $t_m = -b/2a$. The period was determined with respect to the previous point where a maximum occurred, i.e., $T_i = t_{m,i} - t_{m,i-1}$. The inverse values of periods ($1/T$) are shown rather than periods, because $1/T$ corresponds to the spiking frequency. The width of the Ca^{2+} spikes was measured at their half-maximal amplitude during the first 60 sec of stimulation.

Results

Frequency of receptor- and non-receptor-mediated Ca^{2+} oscillations. It is well established that pituitary gonadotrophs express Ca^{2+}-mobilizing receptors for GnRH and ET receptors (2) and respond to both agonists with prominent base-line-type Ca^{2+} oscillations (1, 3, 4). These oscillations are characterized by a distinct threshold and a sharp rise in $[Ca^{2+}]_i$ (Fig. 1, A and B). The minimum agonist doses required to initiate long-lasting oscillations were 25–50 pM GnRH and 0.5–1 nM ET-1. Lower agonist doses induced only occasional spikes that did not persist for longer than 1–2 min (7). Ca^{2+} oscillations were also observed in cells treated with the endoplasmic reticulum Ca^{2+}-ATPase inhibitor TG (34) and ionomycin (7, 9), as well as the thiol reagent TH (Fig. 1D). Both receptor- and non-receptor-mediated oscillations were observed in normal and Ca^{2+}-deficient medium, indicating that extracellular Ca^{2+} is not essential for their initiation. We have previously reported that the amplitude of agonist-induced Ca^{2+} spiking decreases and the frequency increases during the initial phase of oscillations, before reaching the steady state level; in addition, this decrease is associated with a progressive decrease in luminal $[Ca^{2+}]$ (6). In the present study, the amplitudes of non-receptor-mediated Ca^{2+} oscillations also progressively decreased and the frequency of spiking increased with time.

The most obvious difference between these two types of oscillations is related to their rates of spiking (Fig. 2A). The frequency of GnRH- and ET-induced Ca^{2+} oscillations ranges between 5 and 25 min^{-1} and is determined by the agonist concentration (7, 35). The concentration dependence of agonist-induced Ca^{2+} spiking was mimicked by injection of $InsP_3$

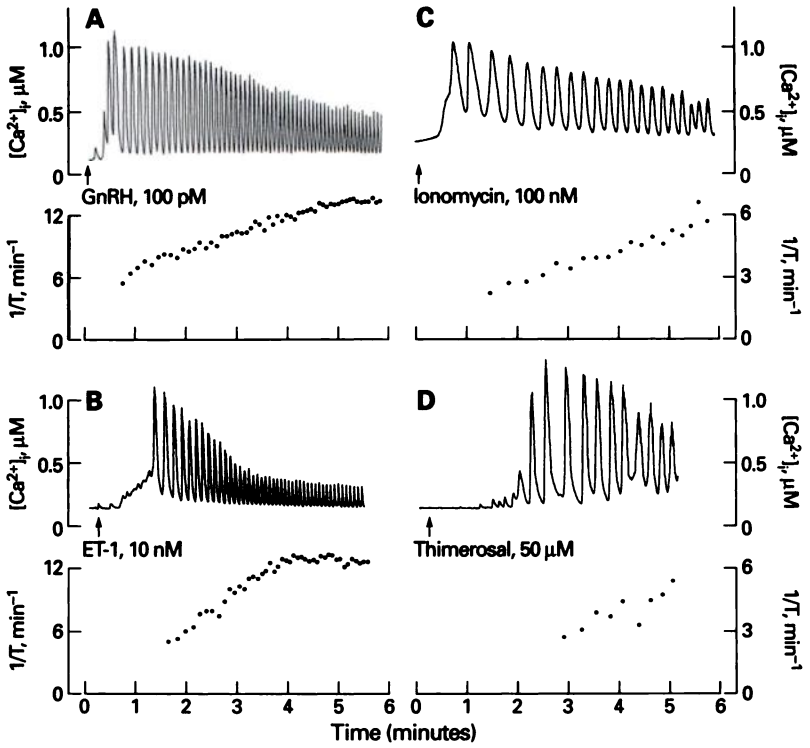

Fig. 1. Agonist-induced and non-receptor-mediated Ca^{2+} oscillations in gonadotrophs. A and B, GnRH- and ET-induced Ca^{2+} oscillations (*upper*) and frequency changes (*lower*). C and D, Ionomycin- and TH-induced Ca^{2+} spiking (*upper*) and frequency changes (*lower*). *Arrows*, moment of application of drugs.

(9). In contrast to the frequency-controlled oscillations observed in agonist-stimulated cells, the rate of non-receptor-mediated Ca^{2+} spiking was low and essentially similar for all agents studied [ionomycin, 5.9 ± 0.5 min^{-1} (36); TH, 5.3 ± 0.4 min^{-1} (67); TG, 4.9 ± 0.4 min^{-1} (26)]. Ionomycin-induced Ca^{2+} spiking occurred over a relatively wide dose range (1–1000 nM in 36 of 90 cells), and its frequency was unrelated to the concentration of the ionophore; at concentrations above 1 μM, ionomycin induced nonoscillatory biphasic responses in all 70 stimulated cells. Treatment with TH also induced Ca^{2+} oscillations without concentration-dependent modulation of spiking frequency. However, the percentage of cells showing such a basic frequency pattern increased progressively with rising TH concentrations [20 μM, four of nine cells; 50 μM, six of eight cells; 100 μM, 23 of 24 cells]. At concentrations above 500 μM, TH caused irregular nonoscillatory Ca^{2+} responses in which individual spikes were no longer distinguishable.

The frequency and the width of Ca^{2+} spikes were inversely correlated. As shown in Fig. 2B, the spike width decreased with increases in spiking frequency in agonist-stimulated cells. It is interesting that the spike width at low agonist concentrations was comparable to that observed during ionomycin-, TG-, and TH-induced spiking. This suggests that non-receptor-mediated and agonist-induced Ca^{2+} spiking share a common cytoplasmic oscillatory mechanism.

Effects of caffeine and ryanodine on Ca^{2+} oscillations. The ability of gonadotrophs to respond to TG, TH, and ionomycin with a low frequency of Ca^{2+} spiking could be consistent with the operation of CICR in a two-Ca^{2+} pool system. In such a system, $[Ca^{2+}]_i$ serves as an autocatalytic factor to provide the link between $InsP_3$-sensitive and $InsP_3$-insensitive but ryanodine/caffeine-sensitive pools during agonist stimulation (21). However, caffeine (1–20 mM) did not change the basal $[Ca^{2+}]_i$ in 19 of 24 gonadotrophs (Fig. 3A) and induced only low-amplitude (below 60 nM) $[Ca^{2+}]_i$ responses in the remainder. In addition, caffeine (20 mM) inhibited the agonist-induced Ca^{2+} transients at low agonist concentrations (0.01–1 nM) and this effect was overcome by addition of higher concentrations of GnRH (Fig. 3B). Such negative effects of caffeine were not observed in the physiological to high range of GnRH concentrations (1–100 nM) (Fig. 3C). Furthermore, caffeine did not inhibit TH-induced spiking (five of five cells) (Fig. 3D). Addition of ryanodine (50–500 μM) did not affect the basal $[Ca^{2+}]_i$ in 15 of 15 cells, did not mimic the inhibitory effects of caffeine on GnRH-induced spiking (10 of 10 cells), and did not inhibit TH-induced Ca^{2+} spiking (five of five cells) (Fig. 3, A-D, *right*).

We consistently observed that maneuvers that increased $[Ca^{2+}]_i$ without causing Ca^{2+} release from internal stores did not initiate oscillatory responses; these included K^+-induced depolarization, application of the Ca^{2+} channel agonist Bay K 8644 (7, 9), depolarization under voltage-clamp conditions, and injection of Ca^{2+} (data not shown). These findings indicate that ryanodine-sensitive channels are not present or are only a minor functional component in gonadotrophs, and argue against the CICR mechanism in a two-pool system. They are also consistent with an inhibitory effect of caffeine on $InsP_3$-dependent release of intracellular Ca^{2+} in agonist-stimulated gonadotrophs, as observed in other cell types (36, 37). However, the inability of caffeine to inhibit TH-induced spiking suggests that the thiol reagent initiates oscillations in an $InsP_3$-independent manner.

Ca^{2+} oscillations and $InsP_3$ formation. The occurrence of non-receptor-mediated Ca^{2+} oscillations in gonadotrophs could be attributable to Ca^{2+}/phospholipase C cross-coupling, in which elevations of Ca^{2+} activate phospholipase C and cause episodic phosphatidylinositol bisphosphate hydrolysis and generation of $InsP_3$ (18). However, two lines of evidence argue against this mechanism. (i) During long term stimulation (15 min), neither activation of Ca^{2+} entry by 50 mM K^+ nor the release of intracellular Ca^{2+} stimulated by TG (10 μM), TH

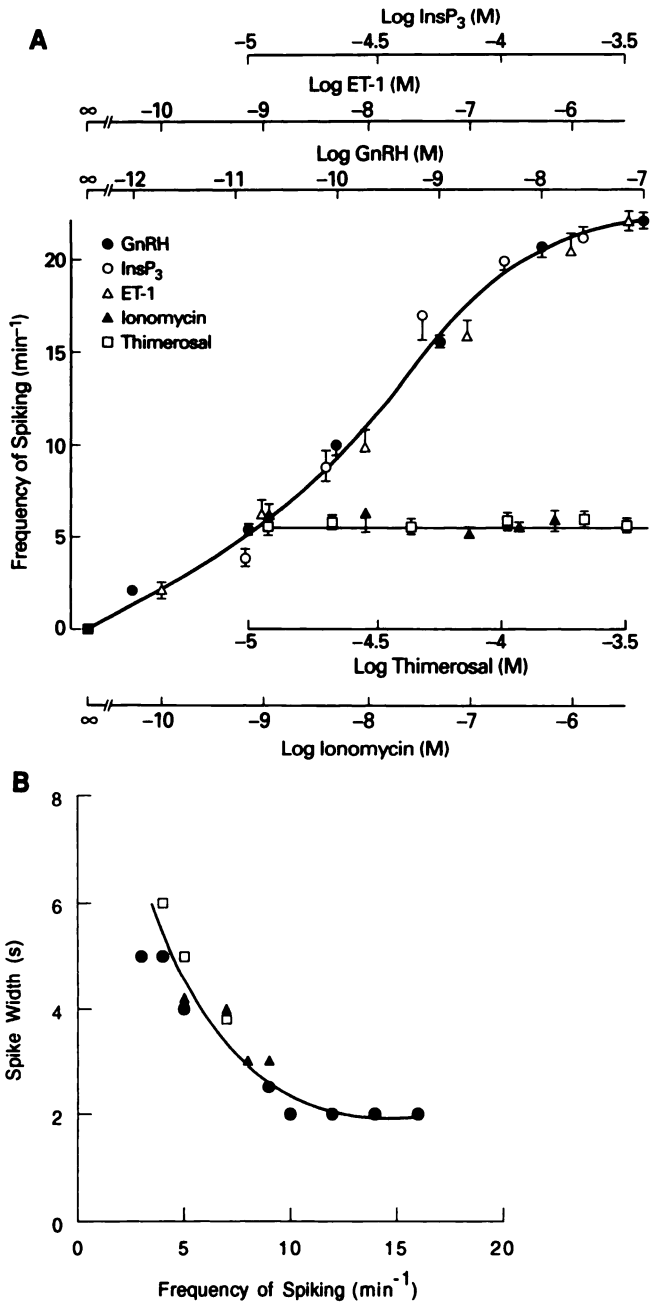

Fig. 2. Characterization of agonist- and non-receptor-induced Ca^{2+} spiking. A, Concentration dependence of Ca^{2+} spiking frequency elicited by GnRH, ET-1, $InsP_3$, TH, and ionomycin. The values shown are the mean ± standard error of three to 85 observations at each dose level. The Ca^{2+} spiking frequency represents an averaged value versus time. When $InsP_3$ was used as stimulus, Ca^{2+} spiking frequency was determined by measurement of Ca^{2+}-sensitive current oscillations. B, Relationship between width and frequency of Ca^{2+} spiking in gonadotrophs. The values are the means of the spike widths measured at the half-maximal spike amplitude during the first 60 sec of stimulation. Treatments were as shown in A.

(100 μM), or ionomycin (0.1 and 1 μM) had a measurable effect on phosphoinositide hydrolysis (Table 1). At high concentrations (5 and 10 μM), ionomycin caused only a minor increase in inositol phosphate production. In contrast, an ET-1 dose (1 nM, 15 min) that initiated spiking of frequency (~5 min^{-1}) similar to that observed in non-receptor-mediated oscillations (35) was associated with 2–4-fold increases in inositol monophosphate, inositol bisphosphate, and $InsP_3$ production. Stimulatory actions of agonists, but not high K^+, ionomycin, or TG, on inositol phosphate production were also observed in short term (60-sec) stimulated pituitary cells (Table 2). Thus, the non-receptor- and receptor-controlled Ca^{2+} spiking responses differ markedly in their requirements for $InsP_3$. (ii) ET-1 (10 nM)-induced inositol phosphate responses were attenuated by pretreatment of cells with the phospholipase C inhibitors U73122 and neomycin sulfate (38–40) (Table 1). Neomycin sulfate also attenuated the minor inositol phosphate response induced by 10 μM ionomycin (Table 1). In contrast, U73122 had no effect on the ionomycin-induced production, in accord with the proposal that the drug affects G protein coupling to phospholipase C (39). Both ET-1- and GnRH-induced oscillations were inhibited by pretreatment of gonadotrophs with U73122 (Fig. 4B) or neomycin sulfate (3 mM) (data not shown) but not with the inactive analog U73433 (data not shown). In contrast, U73122 did not prevent the Ca^{2+} spiking elicited by TG, ionomycin, and TH (Fig. 4, C and D). The inhibitory effect of U73122 on agonist-mediated Ca^{2+} spiking was evident within 50–75 sec, whereas TH-mediated oscillations could be elicited for up to 15 min after exposure to the drug. Treatment with 3 mM neomycin sulfate for 15 min also did not affect TG-, ionomycin-, and TH-induced oscillations (data not shown). The occurrence of such intrinsic oscillations during inhibition of inositol phosphate formation further demonstrates that Ca^{2+} release from the endoplasmic reticulum can elicit Ca^{2+} oscillations by a mechanism that does not involve positive feedback of $[Ca^{2+}]_i$ on phospholipase C and does not require a rise in $InsP_3$. However, these experiments do not exclude the possibility that the basal $InsP_3$ level could be sufficient to drive low-frequency Ca^{2+} spiking.

Evidence that $[Ca^{2+}]_i$ influences the frequency of non-receptor- and receptor-mediated Ca^{2+} oscillations. Although elevation of $[Ca^{2+}]_i$ by factors that promote Ca^{2+} entry did not initiate oscillatory responses in gonadotrophs, it was associated with marked changes in the pattern of agonist-induced Ca^{2+} signaling. The established Ca^{2+} spiking response in cells previously activated by GnRH was rapidly inhibited when $[Ca^{2+}]_i$ was further elevated by application of 50 mM K^+. However, when $[Ca^{2+}]_i$ fell from its initial high value to below 800 nM there was a return to the oscillatory mode (Fig. 5A), indicating that high $[Ca^{2+}]_i$ interferes with the oscillatory Ca^{2+} response. Similarly, the ability of GnRH to induce Ca^{2+} responses in cells previously exposed to high K^+ depended on the time at which the agonist was applied, i.e., on the prevailing $[Ca^{2+}]_i$. GnRH did not stimulate Ca^{2+} release when $[Ca^{2+}]_i$ was above 1 μM (Fig. 5B, *upper trace*) but began to elicit Ca^{2+} oscillations, albeit of lower frequency than in controls, when $[Ca^{2+}]_i$ was below 800 nM (Fig. 5B, *lower trace*). Such K^+-induced rises in $[Ca^{2+}]_i$ also interfered with TH-induced Ca^{2+} oscillations. As shown in Fig. 5C, such spiking was abolished when $[Ca^{2+}]_i$ was increased to about 500 nM. These data are consistent with the participation of $InsP_3R$ channels in TH-induced spiking (14) and with the bidirectional effects of $[Ca^{2+}]_i$ on their activity (24).

A more detailed analysis of the effects of $[Ca^{2+}]_i$ on spiking is shown in Fig. 6, which illustrates the relationship between the frequency of agonist- and TH-induced Ca^{2+} spiking and the initial $[Ca^{2+}]_i$. In unstimulated gonadotrophs, in which the basal $[Ca^{2+}]_i$ ranges from 120 to 300 nM, the frequency of Ca^{2+} spiking

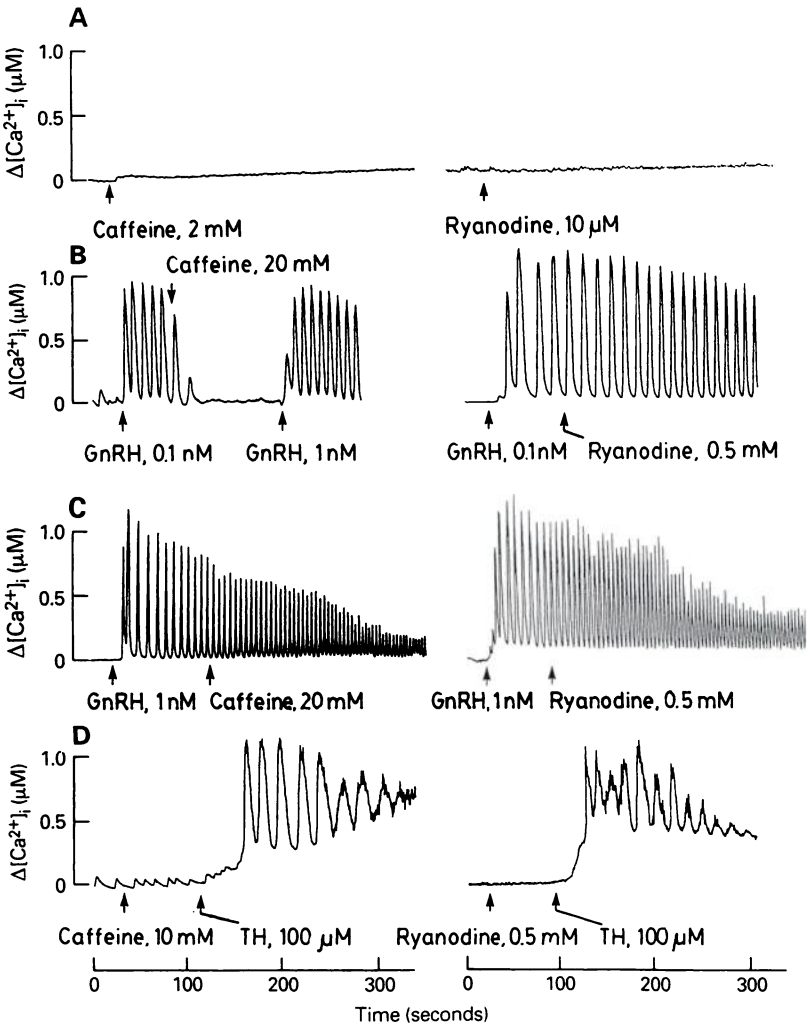

Fig. 3. Effects of caffeine and ryanodine on basal (A), GnRH-induced (B and C), and TH-induced (D) Ca^{2+} spiking. Inhibitory effects of caffeine were observed in 10 of 14 cells stimulated with 0.1 nM GnRH and in only one of 17 cells stimulated with 1 nM GnRH. All other tracings shown are typical of five or more experiments.

in response to a constant GnRH concentration reached a maximum between 200 and 220 nM, with a decrease at higher $[Ca^{2+}]_i$ values. When higher $[Ca^{2+}]_i$ levels were induced by elevating the extracellular K^+ concentration before agonist stimulation, further decreases in frequency and finally abolition of spiking were observed. Fig. 6, *curves a* and *b*, indicates that the range of $[Ca^{2+}]_i$ with inhibitory effects on signaling is larger for higher agonist concentrations. The TH-induced Ca^{2+} oscillations also showed dependence on $[Ca^{2+}]_i$, with reduction of spiking frequency over a relatively narrow concentration range (Fig. 6, *curve c*). Thus, non-receptor- and receptor-mediated spiking showed similar sensitivity to $[Ca^{2+}]_i$.

Discussion

The operation of both receptor- and non-receptor-mediated Ca^{2+} oscillations has been described previously in gonadotrophs (7, 9), as well as in other cell types activated by Ca^{2+}-mobilizing receptors (11–17, 41, 42). The present studies have revealed that non-receptor-mediated Ca^{2+} spiking shares many properties with the receptor-controlled responses observed in gonadotrophs, in addition to their similar oscillatory patterns. Both types of Ca^{2+} spiking can be elicited under extracellular Ca^{2+}-deficient conditions, indicating that release of intracellular Ca^{2+} is sufficient to drive the oscillations. Also, both show a decrease in amplitude and an increase in frequency during the initial phase of spiking. In further agreement with the existence of a common oscillator, ryanodine did not influence either type of Ca^{2+} oscillations. The basic difference between these two types of Ca^{2+} oscillations is related to their requirement for $InsP_3$ and their sensitivity to inhibition by caffeine. Thus, TG-, TH-, and ionomycin-induced spikings occur without a rise in $InsP_3$ production, whereas an increase in $InsP_3$ production is essential for the generation of receptor-controlled oscillatory responses. In the absence of $InsP_3$ formation, the oscillator operates only at the basal rate of ~5 min^{-1}.

The inability of TG, TH, and ionomycin to increase $InsP_3$ production and the differential requirement for $InsP_3$ in the two types of Ca^{2+} spiking argue against the possibility that Ca^{2+}-$InsP_3$ cross-coupling represents the mechanism for operation of the common oscillator. In addition, fluctuations in $InsP_3$ concentration are not required to induce oscillatory current responses, which were consistently observed in cells injected with a nonmetabolizable $InsP_3$ analog (9). The insensitivity of agonist- and non-receptor-mediated Ca^{2+} oscillations to ryanodine also argues against a two-Ca^{2+} pool model of CICR. Several additional lines of evidence also indicate that ryanodine channels are not present in gonadotrophs or are not a functionally significant component of their Ca^{2+} signaling system. For example, caffeine did not induce oscillatory Ca^{2+} release in gonadotrophs, in contrast to its action in cells expressing ryanodine channels (11, 17, 43); instead, caffeine in-

TABLE 1

Long term receptor- and non-receptor-stimulated inositol phosphate production

The cells were stimulated for 15 min in the presence of 10 mM LiCl (II-V) or without LiCl (I). In experiments with U73122, the inhibitor was added together with LiCl. Results were compared by a single-factor analysis of variance.

	Treatment	n[a]	Inositol phosphate production[b]			p[c]
			InsP	$InsP_2$	$InsP_3$	
			cpm			
I	Basal	3	3,324 ± 144	378 ± 51	161 ± 15	
	TG, 10 μM	3	3,572 ± 373	284 ± 3	168 ± 23	NS
	TH, 100 μM	5	3,339 ± 230	271 ± 29	132 ± 22	NS
II	Basal	4	33,894 ± 1,338	1,606 ± 75	692 ± 69	
	K^+, 50 mM	3	30,128 ± 677	1,433 ± 88	525 ± 26	NS
	Ionomycin, 0.1 μM	3	34,583 ± 1,439	1,650 ± 9	765 ± 38	NS
	Ionomycin, 1 μM	3	32,007 ± 2,198	1,867 ± 169	782 ± 80	NS
	Ionomycin, 5 μM	3	36,567 ± 290	1,987 ± 89	842 ± 39	<0.05
	Ionomycin, 10 μM	3	38,019 ± 1,077	2,384 ± 135	984 ± 50	<0.01
	ET-1, 1 nM	3	52,208 ± 2,138	6,169 ± 143	1,486 ± 11	<0.01
	ET-1, 10 nM	3	124,283 ± 1,463	20,187 ± 736	3,740 ± 296	<0.01
III	Basal	3	11,575 ± 559	401 ± 14	276 ± 109	
	ET-1, 10 nM	3	56,420 ± 4,801	10,910 ± 974	1,434 ± 108	
	ET-1, 10 nM, + U73122, 10 μM	4	11,376 ± 729	1,519 ± 85	537 ± 7	<0.05
IV	Basal	4	17,685 ± 1,542	697 ± 27	198 ± 62	
	ET-1, 10 nM	3	82,831 ± 1,600	10,194 ± 484	1,543 ± 44	
	ET-1, 10 nM, + neomycin sulfate, 3 mM	3	64,765 ± 3,207	5,553 ± 294	956 ± 52	<0.05
V	Ionomycin, 10 μM	3	26,464 ± 298	1,559 ± 68	208 ± 60	
	Ionomycin, 10 μM + U73122, 10 μM	3	26,265 ± 302	1,203 ± 193	298 ± 102	NS
	Ionomycin, 10 μM + neomycin sulfate, 3 mM	3	24,396 ± 253	1,263 ± 50	76 ± 13	<0.05

[a] n, number of experiments.
[b] InsP, inositol monophosphate; $InsP_2$, inositol bisphosphate. Inositol-1,3,4-trisphosphate and $InsP_3$ were measured separately, and the counts were added to indicate total production of $InsP_3$ and its inactive metabolite.
[c] I and II, basal versus treated; III and IV, ET-1 versus ET-1 plus U73122 or plus neomycin sulfate; V, ionomycin versus ionomycin plus U73122 or plus neomycin sulfate. NS, not significant.

TABLE 2

Short term receptor- and non-receptor-stimulated inositol phosphate production

Cells were stimulated for 60 sec.

Treatment	n[a]	Inositol phosphate production[b]			p[c]
		InsP	$InsP_2$	$InsP_3$	
		cpm			
Basal	3	4674 ± 145	481 ± 2	249 ± 19	
K^+, 50 mM	3	4550 ± 9	592 ± 26	201 ± 4	NS
Ionomycin, 100 nM	3	4351 ± 48	512 ± 9	219 ± 16	NS
TG, 1 μM	3	4324 ± 54	480 ± 21	216 ± 18	NS
ET-1, 100 nM	3	7385 ± 93	1262 ± 65	444 ± 30	<0.01
GnRH, 100 nM	3	5935 ± 152	975 ± 25	438 ± 19	<0.01

[a] n, number of experiments.
[b] InsP, inositol monophosphate; $InsP_2$, inositol bisphosphate.
[c] Versus basal. NS, not significant.

hibited agonist- but not non-receptor-mediated Ca^{2+} oscillations, presumably due to inhibition of phospholipase C or $InsP_3R$ channel conductance (24, 36, 37). Also, direct application of intrapipet Ca^{2+}, elevation of $[Ca^{2+}]_i$ by either K^+- or current-induced depolarization, and application of Bay K 8644 did not initiate Ca^{2+} release from the agonist-sensitive Ca^{2+} pool (9, 44).

On the other hand, the data are in general accord with the coagonist actions of $InsP_3$ and Ca^{2+} in the control of Ca^{2+} spiking by CICR in a one-pool system, with Ca^{2+}-ATPase and $InsP_3R$ channels as two essential elements of the spiking mechanism (45–48). In this system, $InsP_3$ is required for Ca^{2+} spiking but oscillations in $InsP_3$ are not essential because the bidirectional control of $InsP_3$-dependent channel activity by $[Ca^{2+}]_i$ is sufficient to drive the Ca^{2+} oscillations. This proposal is based on the bell-shaped Ca^{2+} dependence curve of $InsP_3$ channel activity, with a facilitatory effect at low $[Ca^{2+}]_i$ and a negative effect at higher concentrations (24), and has been supported by experimental observations in several systems (22, 23, 25, 27, 31, 49, 50). The role of $InsP_3R$ channels in Ca^{2+} oscillations was indicated by experiments in which agonist-induced Ca^{2+} oscillations were abolished in cells injected with an antibody against the $InsP_3R$ channels (51). The finding that TH-induced Ca^{2+} spiking in hamster eggs is blocked by the same antibody confirms that such channels are also involved in non-receptor-mediated Ca^{2+} oscillations (14).

The present data demonstrate that elevation of $[Ca^{2+}]_i$ has negative feedback effects on both types of Ca^{2+} signaling in gonadotrophs, as manifested by a decrease in spiking frequency and abolition of oscillations at higher concentrations. The increase in Ca^{2+} spiking frequency caused by raising $[Ca^{2+}]_i$ from 100 to about 250 nM supports the concept that $InsP_3R$ channels are under positive regulation by Ca^{2+}. Also, depolarization-driven elevations of $[Ca^{2+}]_i$ into the optimum range for $InsP_3R$ channel function transformed agonist-induced subthreshold responses into oscillatory responses. Thus, the bidirectional effects of $[Ca^{2+}]_i$ on $InsP_3R$ channel function provide an explanation for the actions of Ca^{2+} in non-receptor-mediated and agonist-induced Ca^{2+} spiking.

Because non-receptor-mediated Ca^{2+} spiking in gonadotrophs and other cell types (13) occurs without an increase in

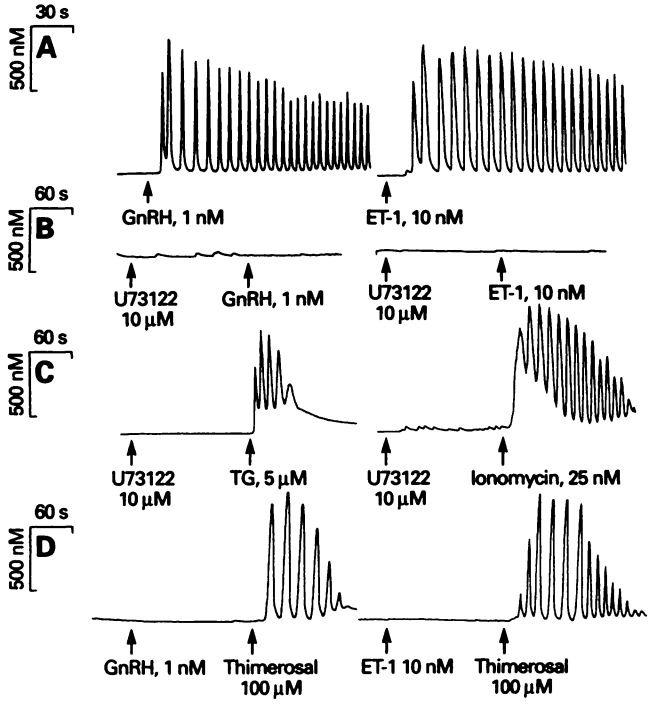

Fig. 4. Effects of the phospholipase C inhibitor U73122 on agonist- and non-receptor-mediated Ca^{2+} oscillations. A, Response to GnRH and ET-1 in control cells. B, Inhibitory actions of U73122 on GnRH- and ET-1-induced Ca^{2+} spiking. Similar effects were observed in 17 of 17 cells. C and D, Lack of effect of U73122 on TG-, ionomycin-, and TH-induced Ca^{2+} spiking. Of nine U73122-treated cells, nanomolar concentrations of ionomycin induced oscillatory responses in four cells, biphasic responses in three cells, and subthreshold responses in the remainder. Of 12 U73122-treated cells, TG (10 μM) induced oscillatory Ca^{2+} responses in five cells and subthreshold responses in the remainder. TH induced oscillatory responses in all 11 cells previously exposed to U73122, in which neither GnRH or ET-1 was effective. In the tracing shown in D, U73122 was applied 100 sec before agonist was added.

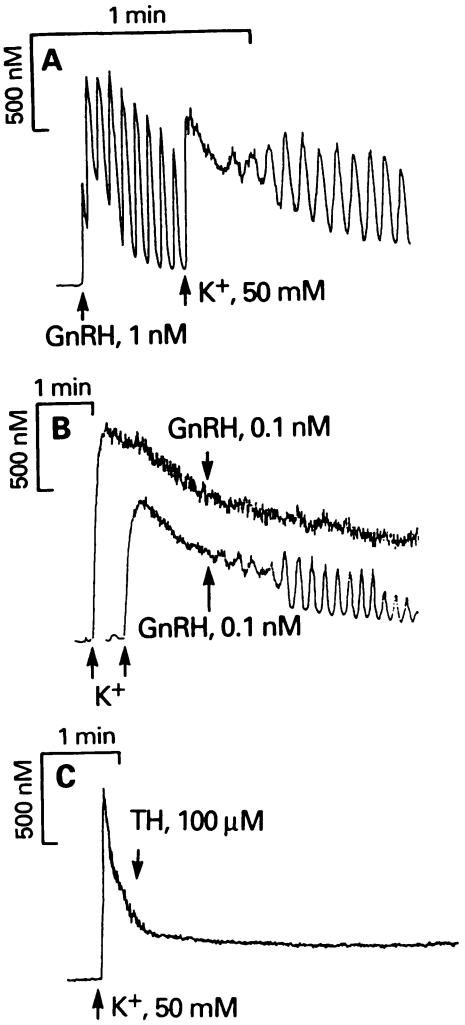

Fig. 5. Effects of K^+-induced depolarization on receptor- and non-receptor-mediated Ca^{2+} signaling. A, Transient inhibition of agonist-induced Ca^{2+} spiking by K^+-induced depolarization of gonadotrophs. B and C, Effects of high $[Ca^{2+}]_i$ (induced by K^+) on the subsequent responses to stimulation with GnRH (B) and TH (C).

$InsP_3$ production, it is possible that the sensitivity of $InsP_3R$ channels to basal $InsP_3$ is increased by treatment with TH, TG, and ionomycin. In the case of TH, there is experimental support for this hypothesis (13). However, interpretation of the effects of TH is difficult because the compound acts at two distinct sites, i.e., Ca^{2+}-ATPase and $InsP_3R$ channels (13, 52). Furthermore, the reported effects of TH on $InsP_3R$ channels are contradictory. For example, TH has been reported to facilitate $InsP_3$-induced Ca^{2+} release in permeabilized HeLa cells (13), to inhibit $InsP_3$-induced Ca^{2+} release from cerebellar microsomes without affecting binding of $InsP_3$ to $InsP_3R$ channels (52), to affect $InsP_3$ binding by decreasing the rate of dissociation without affecting the rate of association of $InsP_3$ with $InsP_3R$ channels (53), and to increase the rate of association of $InsP_3$ with its receptors (54). Also, TH induced heparin-insensitive Ca^{2+} oscillations in mouse oocytes (41). Finally, ionomycin and TG have not been found to affect $InsP_3R$ channel activity but induce Ca^{2+} spiking in gonadotrophs without a significant increase in $InsP_3$ concentration.

These findings suggest a common mechanism for the initiation of Ca^{2+} spiking by TG, TH, and ionomycin. Two obvious common factors during the initiation of Ca^{2+} spiking by agonists and drugs that impair the reuptake of Ca^{2+} by the endoplasmic reticulum, or increase its leakage, are an increase in $[Ca^{2+}]_i$ and a decrease in luminal $[Ca^{2+}]$. Because elevations of $[Ca^{2+}]_i$ caused by increased Ca^{2+} entry do not initiate Ca^{2+} oscillations, it is likely that a decrease in luminal $[Ca^{2+}]$ is responsible for the initiation of spiking. We have suggested that changes in luminal $[Ca^{2+}]$ are crucial events in the control of Ca^{2+} signaling (9). This proposal was based on observations that exposure of agonist-activated cells to a potent GnRH antagonist, or to the phospholipase C inhibitor U73122, did not alter the pattern of agonist-stimulated Ca^{2+} spiking during the initial, extracellular Ca^{2+}-independent, phase of the oscillatory response. In fact, the Ca^{2+} spiking established at the onset of GnRH action can continue for several minutes during inhibition of receptor-mediated $InsP_3$ production. Also, the initial increase in spiking frequency (9) that temporally corresponds to the decrease in the luminal $[Ca^{2+}]$ (6) is consistent with the tonic inhibitory effects of high luminal $[Ca^{2+}]$ on $InsP_3R$ channel activity in cells before treatment with agonist (9) or ionomycin and TH (present data). However, this proposal is in contrast to experimental observations in permeabilized cells, which suggest that decreases in luminal $[Ca^{2+}]$ inhibit (28) or do not affect $InsP_3$-induced Ca^{2+} release (30).

A recently developed mathematical model of Ca^{2+} oscillations

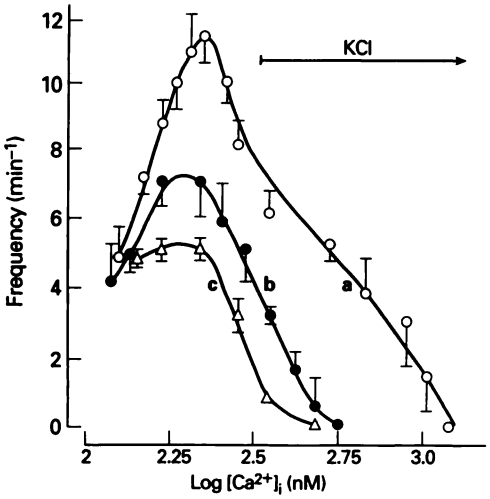

Fig. 6. Relationship between initial $[Ca^{2+}]_i$ and frequency of GnRH- and TH-induced Ca^{2+} spiking. Experiments were performed in cells with basal $[Ca^{2+}]_i$ of 120–260 nM or cells in which the initial $[Ca^{2+}]_i$ was increased by K^+-induced depolarization (to 260–1200 nM) (*arrow*). *Curve a*, GnRH, 100 pM; *curve b*, GnRH, 50 pM; *curve c*, TH, 100 μM. Values are the mean ± standard error of four to eight observations/point.

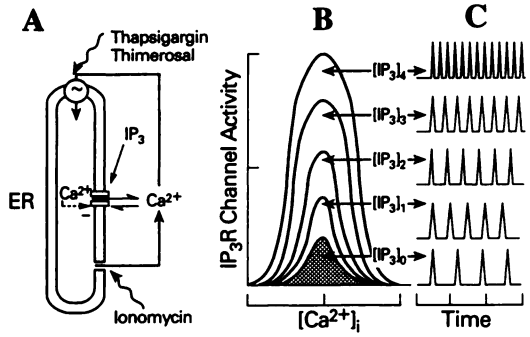

Fig. 7. Schematic illustration of the cytoplasmic Ca^{2+} oscillator in gonadotrophs. A, The oscillator is based on the autoregulatory actions of luminal and cytoplasmic Ca^{2+} and of $InsP_3$ on $InsP_3R$ channel activity. In the absence of $InsP_3$, a decrease in luminal Ca^{2+} is required to initiate Ca^{2+} oscillations by releasing the $InsP_3R$ channels from their tonically inhibited state. When a critical level is reached (due to release by $InsP_3$ under physiological conditions or by agents such as TG, TH, and ionomycin in pharmacological experiments), the cytoplasmic oscillator begins to generate Ca^{2+} transients. B and C, The oscillator can be induced to operate at its low basal frequency in the absence of $InsP_3$ production or at higher frequencies in the presence of $InsP_3$. The situation shown in C corresponds to the physiological condition in which the amplitude of Ca^{2+} spiking becomes stabilized after an initial rapid decrease and is sustained by the consequences of increased Ca^{2+} entry (9).

in gonadotrophs was based on the bidirectional effects of $[Ca^{2+}]_i$ on $InsP_3R$ channels, combined with reuptake by sarcoplasmic endoplasmic reticulum Ca^{2+} ATPase-type Ca^{2+} ATPase. This model predicted that ionomycin and TG, but not Ca^{2+} injection, would induce Ca^{2+} spiking without an increase in $InsP_3$ production when enhancement of channel opening by reduced luminal $[Ca^{2+}]_i$ was incorporated into the system (10). The present data provide two kinds of experimental support for this theoretical proposal, i.e., the ability of TG, TH, and ionomycin to operate at the basal level of $InsP_3$ and the bidirectional effects of $[Ca^{2+}]_i$ on the frequency of Ca^{2+} spiking. The proposed effects of these factors on Ca^{2+} signaling are illustrated schematically in Fig. 7. Under physiological conditions, $InsP_3$ initiates the threshold Ca^{2+} release that causes activation of the cytoplasmic oscillator and also determines the frequency of Ca^{2+} spiking. In our model, the ability of $InsP_3$ to induce dose-dependent increases in the frequency of Ca^{2+} spiking is attributable to its modulation of the sensitivity of the $InsP_3R$ channels to $[Ca^{2+}]_i$. However, the frequency of Ca^{2+} oscillations is not solely dependent on the $InsP_3$ concentration, because both pharmacologically and physiologically driven oscillations can be further modulated by changes in luminal $[Ca^{2+}]_i$. They are also influenced by changes in Ca^{2+} entry, which in turn affect both cytoplasmic and luminal $[Ca^{2+}]$.

Acknowledgments

We thank Dr. T. Balla for his help with measurements of inositol phosphates.

Note Added in Proof

Both GnRH- and TH-induced Ca^{2+} oscillations were abolished in the presence of low MW heparin (~6000, sigma), confirming that basal $InsP_3$ is sufficient to drive oscillations initiated by non-receptor mediated stimuli.

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Send reprint requests to: Stanko Stojilkovic, ERRB, NICHD, Building 49, Room 6A-36, National Institutes of Health, Bethesda, MD 20892.