Ba²⁺ stimulation of luteinizing-hormone release demonstrates two mechanisms of Ca²⁺ entry in gonadotrope cells

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Kinetic studies on gonadotropin-releasing-hormone (gonadoliberein, GnRH)-stimulated luteinizing-hormone (lutropin, LH) release in the cultured rat gonadotrope demonstrated a biphasic pattern of LH release. The first rapid phase of release was unaffected by the voltage-gated Ca²⁺-channel blockers methoxyverapamil (D600) and nifedipine [a dihydropyridine (DHP)], whereas the later second phase was partially inhibited by both drugs. These results suggested that the initial phase of LH release is independent of Ca²⁺ entry through dihydropyridine (DHP)-sensitive Ca²⁺ channels and might depend on entry of extracellular Ca²⁺ by another mechanism. These mechanisms were further studied by utilizing Ba²⁺ as a Ca²⁺ substitute. Ba²⁺, which freely permeates DHP-sensitive Ca²⁺ channels in the absence of GnRH, induced LH release which was sensitive to blockade by D600 and nifedipine. However, in the presence of the channel blockers, Ba²⁺-induced LH release could be elicited when GnRH was added to the system. This indicates that GnRH stimulates LH release by initially activating a DHP-insensitive Ca²⁺-entry mechanism and then a DHP-sensitive mechanism. The DHP-sensitive mechanism freely allows Ba²⁺ entry in the absence of GnRH-receptor occupancy, whereas the DHP-insensitive mechanism requires GnRH-receptor activation for Ba²⁺ entry.

INTRODUCTION

Previous kinetic studies on gonadotropin-releasing-hormone (GnRH)-induced luteinizing-hormone (LH) release from rat pituitary-cell cultures demonstrated a biphasic pattern of LH release (Hopkins, 1977; Bourne & Baldwin, 1980; Borges *et al.*, 1983; Smith *et al.*, 1987). The first secretory phase, which commenced within 30 s, was unaffected by the Ca²⁺-channel blockers methoxyverapamil (D600) and nifedipine [a dihydropyridine (DHP)], indicating that it was independent of Ca²⁺ entry through DHP-sensitive channels (Smith *et al.*, 1987).

The later second phase, which commenced at 2 min and continued for at least 20 min, was inhibited by the channel blocker D600 (Smith *et al.*, 1987). These results suggested that the initial phase of LH release was dependent on the mobilization of intracellular Ca²⁺ or on the entry of extracellular Ca²⁺ through another mechanism. This mechanism has been further investigated in the present study by utilizing Ba²⁺ as a Ca²⁺ substitute in LH release. In pituitary cells, Ba²⁺ acts as a secretagogue (Douglas *et al.*, 1983; Davidson *et al.*, 1987) by a mechanism which is dependent on its entry through DHP-sensitive Ca²⁺ channels (Davidson *et al.*, 1987).

The results demonstrate two mechanisms of Ca²⁺ entry into cultured rat gonadotropes when Ba²⁺ is used as a Ca²⁺ substitute in GnRH-induced LH release. One of these is permeable to Ba²⁺, in the absence of GnRH, and is sensitive to the Ca²⁺-channel blockers D600 and nifedipine. The second mechanism is insensitive to these agents and is dependent on GnRH binding to its receptor to allow Ba²⁺ influx.

MATERIALS AND METHODS

Adult male Long-Evans-rat pituitary cells were dispersed with collagenase as described previously (Smith *et al.*, 1987). The cells were suspended in Minimum Essential Medium (Gibco, Paisley, Renfrewshire, Scotland, U.K.) containing 10% (v/v) donor calf serum (Gibco), penicillin (60 mg/l), streptomycin (100 mg/l), neomycin (100 mg/l) and dispensed into six-well culture plates (Nunc, Copenhagen, Denmark) with a cell density equivalent to 1 pituitary/well [(5–7) × 10⁵ cells/pituitary]. Cells were cultured for 2 days at 37°C in CO₂/air (1:19).

Experiments were performed in Hepes buffer (NaCl, 140 mM; KCl, 4 mM; MgCl₂, 1 mM; glucose, 8.3 mM; Phenol Red (6 mg/l); and Hepes, 20 mM; pH 7.4), which contained bovine serum albumin (BSA) (0.03%; Pentex fraction V; fatty-acid-free; Miles Laboratories, Elkhart, IN, U.S.A.), but was free of added Ca²⁺ where indicated. Bivalent-cation chelators were not used, and nominally 'Ca²⁺-free' medium was estimated to contain 1–5 μM-Ca²⁺.

Ca²⁺ experiments

Before stimulation the wells were washed twice for 10 min at 37°C with Hepes buffer containing 1 mM-CaCl₂ and then preincubated for a further 10 min in 1 ml of Hepes buffer containing Ca²⁺ with or without 50 μM-D600 (the channel blocker Gallopamil; Knoll A. G., Ludwigshafen, Germany). The cells were stimulated with 1 ml Hepes buffer containing Ca²⁺ with or without 10 nM-GnRH (synthesized by R. C. deL Milton, University of Cape Town Medical School) in the presence or absence

Abbreviations used: GnRH, gonadotropin-releasing hormone (gonadoliberein); LH, luteinizing hormone (lutropin); DHP, dihydropyridine; BSA, bovine serum albumin; NIADDK, National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases; rLH, rat LH; nifedipine, 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester [4-(2'-nitrophenyl)-2,6-dimethyl-3,5-dicarboxymethoxy-1,4-dihydropyridine].

of the channel blocker. Medium in each well was collected at 1.5, 4 and 20 min after commencement of stimulation. The experiments utilized a complete-replacement technique in which the medium in each well was completely removed at the appropriate time interval and replaced with 1 ml of medium containing the substances being tested. The medium was collected into tubes on ice, centrifuged and stored at -20°C until radioimmunoassay.

Ba²⁺ experiments

The same procedure was followed as that in the Ca²⁺-experiments, except that the preincubation and stimulation media were nominally Ca²⁺-free. The cells were preincubated for 10 min with or without the appropriate channel blockers [50 μM -D600 and 1 μM -nifedipine (Bayer, Wuppertal, Germany)]. The cells were then stimulated for 20 min with HEPES buffer containing 2 mM-BaCl₂ with or without 10 nM-GnRH in the presence or absence of the channel blockers.

Each experimental treatment, which comprised triplicate wells, was repeated on at least three different occasions. Samples from each experiment were assayed in duplicate for rat LH (rLH) by the conventional double-antibody technique radioimmunoassay (NIADDK kit) using NIADDK-rLH-RP-2 as standard. The intra-assay coefficient of variation was 9%.

Each value shown represents the mean for three or more similar experiments, except where otherwise stated. Error bars represent the S.E.M.

RESULTS

Continuous GnRH stimulation of cultured rat gonadotropes demonstrated two phases of LH release (Table 1). The first rapid phase (0–1.5 min) was unaffected by the Ca²⁺-channel blocker D600, whereas the second later phase (4–20 min) was inhibited by 68% by this drug. In the absence of GnRH, millimolar concentrations of Ba²⁺ stimulated LH release (Fig. 1a). Extracellular Ca²⁺ had an inhibitory effect on Ba²⁺-induced LH release (Figs. 1a and 1b), which was competitive, since the

inhibitory effect could be overcome by increasing the dose of Ba²⁺. This suggests that Ba²⁺ competes with Ca²⁺ for a binding site on the external aspect of a Ca²⁺ channel and gains access to the secretory mechanisms within the cell by traversing these channels. Ba²⁺-stimulated LH release was blocked by D600 (Fig. 2a) and nifedipine (Fig. 2b), indicating that Ba²⁺ gains entry to the cell via DHP-sensitive Ca²⁺ channels.

When GnRH was added to the medium, Ba²⁺-stimulated LH release was enhanced by 26% (100%–74%; Table 2) compared with LH release induced by Ba²⁺ alone (Fig. 3). Moreover, in the presence of GnRH, Ba²⁺-stimulated LH release was not abolished by D600 at concentrations which completely blocked the effect of Ba²⁺ alone (Fig. 3). Similarly, nifedipine blockade was less complete in the presence of GnRH (Fig. 3). These results indicate that GnRH activates another mechanism of Ba²⁺ entry which is insensitive to the DHP Ca²⁺-channel blockers. The dependence of this mechanism on Ba²⁺ concentration was investigated by

Fig. 1. Effect of Ca²⁺ concentration on Ba²⁺-induced LH release

(a) Concentration-dependence of Ba²⁺-induced LH release in the absence (●) and in the presence of 1 mM-Ca²⁺ (○). (b) Effect of Ca²⁺ concentration on LH release with (●) and without (○) 2 mM-Ba²⁺. Cells were preincubated with indicated Ca²⁺ concentrations for 10 min before stimulation with Ba²⁺ for 20 min. Each point represents the mean result for two experiments. Error bars represent the S.E.M.

Table 1. Effects of D600 on the first and second phases of GnRH-induced LH release

Values are the mean $\pm$ S.E.M. for five similar experiments. Cells were preincubated for 10 min in HEPES buffer containing 1 mM-CaCl₂ with or without 50 μM -D600. Medium in each well was completely removed at the time intervals shown and replaced with medium containing Ca²⁺ with or without 10 nM-GnRH in the presence or absence of D600.

Treatment	[LH] (ng/pituitary equivalent)	
	First phase (0–1.5 min)	Second phase (4–20 min)
Buffer only	0.7 $\pm$ 0.05	3.5 $\pm$ 0.7
GnRH (10 nM)	1.7 $\pm$ 0.1	13.3 $\pm$ 0.5
D600 (50 μM)	0.5 $\pm$ 0.1	2.2 $\pm$ 0.3
GnRH/D600	1.6 $\pm$ 0.2	5.3 $\pm$ 0.5*

* 68% inhibition compared with GnRH without D600 [$P < 0.0001$ versus GnRH (t test)].

Fig. 2. Effect of Ca²⁺-channel blockers on Ba²⁺-induced LH release

Inhibition of Ba²⁺-induced LH release by D600 (a) and nifedipine (b) is shown. Cells were preincubated for 10 min with the indicated concentration of drug before stimulation with Ba²⁺ for 20 min. ○, Basal; ●, 2 mM-Ba²⁺.

Table 2. Comparison of GnRH-induced LH release in experiments utilizing either Ca^{2+} or Ba^{2+}

Cells were preincubated with or without $50\ \mu\text{M}$ -D600 for 10 min before 20 min stimulation with $2\ \text{mM}$ - Ba^{2+} and/or $10\ \text{nM}$ -GnRH. Basal secretion from cells incubated without Ba^{2+} or GnRH has been subtracted. Values are the mean $\pm$ s.e.m. The values in parentheses represent the percentage of the total LH release. Each experimental treatment comprised triplicate wells. n is the number of experiments.

Treatment	Ca^{2+} -entry mechanism	LH release (ng/pituitary equivalent)	
		Ca^{2+} present ($n = 5$)	Ba^{2+} present ($n = 3$)
A. GnRH	DHP-sensitive and DHP-insensitive	9.8 ± 0.9 (100 %)	26.1 ± 3.1 (100 %)
B. GnRH + D600	DHP-insensitive	3.1 ± 0.6 (32 %)	7.0 ± 1.5 (27 %)
C. A – B	DHP-sensitive	6.7 ± 1.1 (68 %)	19.1 ± 3.4 (73 %)
D. Ba^{2+} alone	DHP-sensitive		19.3 ± 3.9 (74 %)

Fig. 3. Effect of Ca^{2+} -channel blockers on Ba^{2+} -induced LH release in the presence and absence of GnRH

The effect of $50\ \mu\text{M}$ -D600 ($\square$), $1\ \mu\text{M}$ -nifedipine (hatched) and absence of channel blockers ($\blacksquare$) on basal LH release, $2\ \text{mM}$ - Ba^{2+} -induced LH release and $10\ \text{nM}$ -GnRH-stimulated LH release in the presence of $2\ \text{mM}$ - Ba^{2+} . Cells were preincubated with D600 or nifedipine for 10 min before stimulation for 20 min. * $P < 0.005$; ** $P < 0.01$ (t test).

stimulating cells with Ba^{2+} in the presence of both GnRH and D600 (Fig. 4). Half-maximal LH release was induced by concentrations of $1\text{--}2\ \text{mM}$ - Ba^{2+} (Fig. 4), which is similar to the concentration-dependence of the D600-sensitive channel (Fig. 1a). A similar Ba^{2+} -concentration-dependence was found when $1\ \mu\text{M}$ -nifedipine was used instead of D600 (results not shown).

DISCUSSION

The present findings support previous kinetic studies describing two phases of GnRH-stimulated LH release which differ in their susceptibility to the voltage-gated Ca^{2+} -channel blockers, D600 and nifedipine (Smith *et al.*, 1987; Davidson *et al.*, 1988; Tasaka *et al.*, 1988). The initial spike phase of GnRH-stimulated LH release was independent of Ca^{2+} entry through DHP-sensitive

Fig. 4. Effect of Ba^{2+} concentration on D600-insensitive LH release in the presence and absence of GnRH

The dependence of D600-insensitive LH release on Ba^{2+} concentration in the presence ($\bullet$) or absence ($\circ$) of $10\ \text{nM}$ -GnRH is shown. All media contained $50\ \mu\text{M}$ -D600, which was present for 10 min before stimulation for 20 min.

Ca^{2+} channels, but required Ca^{2+} entry through an alternative mechanism, possibly a receptor-operated Ca^{2+} channel. The later plateau phase of LH release is partially (68 %) dependent on DHP-sensitive channels (Table 1), suggesting that it comprises both the DHP-insensitive and the DHP-sensitive Ca^{2+} -entry mechanisms (Table 2).

Ba^{2+} has previously been shown to be a secretagogue in adrenal chromaffin (Douglas & Rubin, 1964), pituitary (Douglas *et al.*, 1983; Davidson *et al.*, 1987), mast (Foreman & Mongar, 1972) and colonic cells (Hardcastle *et al.*, 1985) and to induce smooth-muscle-cell contraction

(Hansen *et al.*, 1984). This stimulatory action of Ba^{2+} does not require the presence of extracellular Ca^{2+} (Douglas *et al.*, 1983; Hardcastle *et al.*, 1985; Kreye *et al.*, 1986), but is dependent on Ba^{2+} entry through DHP-sensitive Ca^{2+} channels (Davidson *et al.*, 1987; the present study). This type of channel is permeable to Ba^{2+} (unlike Ca^{2+}) in the absence of agonist or plasma-membrane depolarization, a property which may be due to the hydrated radius of Ba^{2+} being smaller than that of Ca^{2+} (Stokes, 1964).

In the present study the replacement of Ca^{2+} by Ba^{2+} has one very useful application. Since Ba^{2+} readily permeates the cell membrane through DHP-sensitive Ca^{2+} channels (Davidson *et al.*, 1987), the activity of these channels can be suppressed with specific blockers in order to reveal Ba^{2+} entry through other channels which are activated by GnRH.

In the present study, Ba^{2+} -stimulated LH release from rat gonadotropes in the absence of GnRH was blocked by D-600 and nifedipine as previously reported in chicken gonadotropes (Davidson *et al.*, 1987). This mechanism is identifiable with the DHP-sensitive component of the plateau phase of LH release elicited by GnRH in the presence of extracellular Ca^{2+} . Both comprised 68–74% of the total releasable LH (Table 2). Several different types of voltage-gated Ca^{2+} channels have been demonstrated and are widely distributed in a variety of cell types (Tsunoo *et al.*, 1984; Nowycky *et al.*, 1985; Fedulova *et al.*, 1985; Matteson & Armstrong, 1986; Childs *et al.*, 1987; Catterall *et al.*, 1988). A subset are DHP-sensitive, and, in neurons, these are designated 'L-type channels' (see Miller, 1987). The DHP-sensitive Ca^{2+} channels in the gonadotrope differ, however, from the L-type channels of neurons in being insensitive to ω -conotoxin (results not shown).

DHP-insensitive stimulation of LH release by Ba^{2+} occurred only in the presence of GnRH and might represent a receptor-operated process identifiable with the putative receptor-operated Ca^{2+} channel involved in the spike phase of GnRH-stimulated LH release in the presence of extracellular Ca^{2+} . Both constitute approximately one third (32 and 27%; Table 2) of total releasable LH, implying that Ba^{2+} and Ca^{2+} are entering the gonadotrope and activating LH-release mechanisms with similar facility. The GnRH-operated mechanism of Ca^{2+} entry is not a voltage-gated Ca^{2+} channel because it was not blocked by D600, which has been shown to totally block all LH secretion induced by K^+ depolarization of gonadotropes (Smith *et al.*, 1987; Davidson *et al.*, 1988). Receptor-operated Ca^{2+} channels have been described in sea-urchin eggs (Irvine & Moor, 1986) and T-lymphocytes (Kuno & Gardner, 1987), where they are activated by inositol 1,3,4,5-tetrakisphosphate and inositol 1,4,5-trisphosphate respectively. A 'capacitative' model has been proposed for receptor-activated Ca^{2+} entry regulated by inositol 1,4,5-trisphosphate (Putney, 1986). In neutrophils, a Ca^{2+} -activated Ca^{2+} channel has been described (Von Tscharner *et al.*, 1986) and, in smooth-muscle cells, extracellular ATP activates a Ca^{2+} channel without utilizing an intracellular second messenger (Benham & Tsien, 1987). GnRH has been shown to generate inositol phosphates in gonadotropes (Naor & Catt, 1981; Andrews & Conn, 1986; Davidson *et al.*, 1988) and to mobilize Ca^{2+} from intracellular stores (Limor *et al.*, 1987; Davidson *et al.*, 1988). However, these studies and the present work have not

established whether these events play a role in activating the putative receptor-operated channel.

In conclusion, the use of Ba^{2+} has enabled us to confirm the existence of two mechanisms of Ca^{2+} entry which are activated by GnRH binding to its receptor. In mechanism I (the 'receptor-operated channel'), Ba^{2+} is able to enter the gonadotrope only during GnRH occupancy of its receptor; Ca^{2+} -channel blockade by D600 and nifedipine is ineffective. The mechanism is activated by GnRH within 1 min and is operative during both spike and plateau phases. Mechanism II (the DHP-sensitive voltage-gated Ca^{2+} channel) allows Ba^{2+} entry without GnRH receptor occupancy, is inhibited by D600 and DHP, and is activated with a delay of at least 2 min after GnRH addition and thus operates in the plateau phase only.

We thank the South African Medical Research Council and the University of Cape Town for financial support, Ms. J. Poorter and Mr. J. Morta for technical assistance and Ms. L. Odes for manuscript preparation. We gratefully acknowledge gifts of research material from Knoll A.G. (Ludwigshafen, Germany) and the NIADDK.

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Received 20 June 1988/11 October 1988; accepted 20 October 1988