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The initial phase of GnRH-stimulated LH release from pituitary cells is independent of calcium entry through voltage-gated channels

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Kinetic studies on gonadotropin-releasing hormone (GnRH)-stimulated luteinizing hormone (LH) release were undertaken using rat and chicken pituitary cell cultures. In response to continuous GnRH stimulation, a biphasic pattern of LH release was demonstrated. The two phases showed different susceptibility to the voltage-gated Ca^{2+} channel blockers D600 and nifedipine. The first (transient) phase of LH release was unaffected by the Ca^{2+} channel blockers whereas the second (sustained) phase was inhibited by both drugs. These results indicate that the initial phase of LH release is independent of Ca^{2+} entry through voltage-gated Ca^{2+} channels and may depend on mobilisation of intracellular Ca^{2+} or entry of extracellular Ca^{2+} through another mechanism.

Kinetics; Gonadotropin-releasing hormone; Luteinizing hormone; Ca^{2+} channel; Ca^{2+} influx; Hormone release; (Pituitary cell)

1. INTRODUCTION

The specific binding of gonadotropin-releasing hormone (GnRH) to gonadotrope membrane receptors is a prerequisite for LH (luteinizing hormone) release [1,2]. This binding results in signal transduction across the plasma membrane causing an increase in cytosolic Ca^{2+} [2–5].

Previous studies have concluded that LH release occurs in a biphasic manner and that both phases are dependent on extracellular Ca^{2+} [6–8]. The present study has addressed the question of extracellular Ca^{2+} dependence and has shown by precise kinetic studies in rat and chicken pituitary cell cultures that the initial phase of LH secretion is independent of Ca^{2+} entry via voltage-gated Ca^{2+} channels whereas the second phase is partially dependent on Ca^{2+} influx through these channels.

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2. MATERIALS AND METHODS

2.1. *Pituitary cell culture*

Anterior pituitaries were removed from adult male Long-Evans rats immediately after decapitation, washed with Ca^{2+}/Mg^{2+}-free buffer [147 mM NaCl, 4 mM KCl, 10 mM Hepes (pH 7.4), 0.5 mM EDTA, 0.2% BSA (Pentex bovine serum albumin, fatty acid-free, fraction V, Miles, Elkhart, IN)], diced and incubated in collagenase solution [1% BSA containing 0.9% collagenase (155 U/mg, Worthington, Freehold, NJ) and

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1.8% DNase (Miles)] for 45 min at 37°C with continuous agitation by a magnetic stirrer and trituration with a 5 ml pipette at 10-min intervals. The dispersed pituitary cells were washed twice in Ca^{2+}/Mg^{2+}-free buffer, filtered through nylon gauze and suspended in minimum essential medium (Gibco, Paisley, Scotland) containing 10% donor calf serum (Gibco), penicillin (60 mg/l), streptomycin (100 mg/l), neomycin (100 mg/l) and dispensed into 6-well culture plates (Nunc, Copenhagen). Cell density was equivalent to 4 pituitaries/well ($5-7 \times 10^5$ cells/pituitary). Cells were cultured for 2 days at 37°C in 5% CO_2/95% air. Chicken pituitary cell cultures were established using the same procedure, from chicken heads obtained from a local abattoir.

2.2. *Kinetic studies*

The cells were washed twice for 10 min at 37°C with Hepes buffer (140 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1.4 mM Na_2HPO_4, 8.3 mM D-glucose, 20 mM Hepes, pH 7.4) and were preincubated for 10 min in 1 ml Hepes buffer with or without 20 μM D600 (Gallopamil, Knoll, Ludwigshafen, FRG) or 1 μM nifedipine (Bayer, Wuppertal, FRG). For K^+ stimulation studies, a modified Hepes buffer comprising 60 mM KCl and 90 mM NaCl was used throughout the 20 min period of stimulation. The kinetic studies utilised a complete replacement technique, i.e. the medium in each well was completely removed at each time interval shown and replaced with 1 ml medium. In all experiments substances being tested were present in the replacement medium throughout the study. Rat and chicken pituitary cells were stimulated with 10^{-8} M mammalian GnRH and 10^{-6} M chicken GnRH I (Gln^8GnRH), respectively. Peptides were from R.C.deL. Milton (UCT Medical School, Cape Town). Basal LH secretion was monitored for 5 min prior to stimulation. Control basal LH secretion throughout the entire 20 min period was monitored in parallel culture wells with or without Ca^{2+} channel blockers and these values were subtracted from values of LH secreted by GnRH-stimulated cells. Time intervals were based on information obtained from preliminary studies. The medium was collected into tubes on ice, centrifuged at 4°C and the supernatant was stored at −20°C prior to radioimmunoassay. Each experiment, comprising triplicate wells for each treatment, was repeated at least three times.

2.3. *LH radioimmunoassays*

Rat LH was measured in the sample media by the conventional double-antibody technique radioimmunoassay (NIADDK kit) using NIADDK-rLH-RH-2 as standard. RIA of chicken was as described in [7]. The intra-assay coefficient of variation was less than 10%. Each experiment shown is representative of 3–6 similar experiments.

3. RESULTS AND DISCUSSION

Continuous GnRH stimulation of cultured rat pituitary cells resulted in a biphasic pattern of LH release (fig.1). A rapid phase of release commenced within 30 s and declined by 2 min. This was followed by a sustained second phase. Similar kinetics of release occurred in chicken pituitary cells (not shown).

In both rat and chicken cells, D600 and nifedipine did not significantly inhibit the first phase of LH release, whereas the second phase was inhibited by between 52 and 68% (figs 1,2). In fig.2, data from an intermediate period between the two phases is not shown to avoid any possible overlap between the phases. The efficacy of D600 and nifedipine was established in rat and chicken pituitary cells by showing that they blocked LH release induced by 60 mM K^+ (85.4 ± 4.2% SE to 99.6 ± 9.2% SE inhibition).

Although GnRH is known to stimulate LH release in a biphasic manner [7–10] the pharmacological characteristics of the different phases of LH release have not been clearly characterised. Investigators have previously concluded that LH release is dependent on extracellular Ca^{2+} entry through Ca^{2+} channels [2,4,8,11]. Some of these conclusions were based on measurements of LH released over relatively prolonged periods (5–60 min) of stimulation, or on results from cells pretreated with EGTA, which is likely to have resulted in depletion of intracellular Ca^{2+} stores [5]. However, a recent study has shown that the initial rise in cytosolic Ca^{2+} is not dependent on the presence of extracellular Ca^{2+}, demonstrating mobilization of intracellular Ca^{2+} stores [5]. We therefore examined the early kinetics of GnRH-

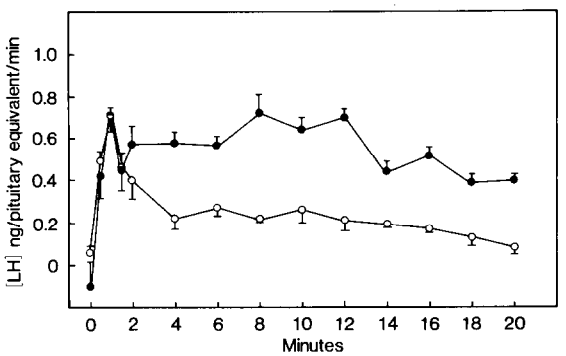

Fig.1. Stimulation of LH release from cultured rat pituitary cells by 10^{-8} M GnRH in the presence (○—○) and absence (●—●) of 2×10^{-5} M D600. Basal secretion of LH from cells incubated without GnRH has been subtracted. Each data point represents the mean ± SE of triplicate wells.

stimulated LH release to determine whether the initial phase of release required Ca^{2+} entry. The first phase of GnRH-induced LH release in rat and chicken pituitary cells occurs rapidly, commencing within 30 s and reaching completion within 2 min (rat) and 3 min (chicken). Our results indicate that this phase is independent of Ca^{2+} influx via voltage-gated Ca^{2+} channels, since LH release was not affected by D600 or nifedipine. Under the conditions of these experiments, D600 and nifedipine blocked K^+-induced secretion, demonstrating that all voltage-gated channels were blocked. In contrast, the GnRH-stimulated second phase of LH release was partially (59 ± 3.4% SE) blocked by D600.

It has recently been shown [12–14] that polyphosphoinositide hydrolysis is activated following GnRH binding to its receptor, resulting in the production of inositol trisphosphate. This intracellular messenger is known to trigger the release of Ca^{2+} from endoplasmic reticulum in a number of cell systems [15]. The results presented here are therefore consistent with the hypothesis that the first phase of LH release utilises Ca^{2+} from intracellular stores. However, it is possible that the first phase utilises extracellular Ca^{2+} entry by a mechanism which does not involve the voltage-gated Ca^{2+} channel. Experiments to address this possibility require the use of Ca^{2+}-free medium which is complicated by the possibility of concurrent intracellular Ca^{2+} depletion. Here, we

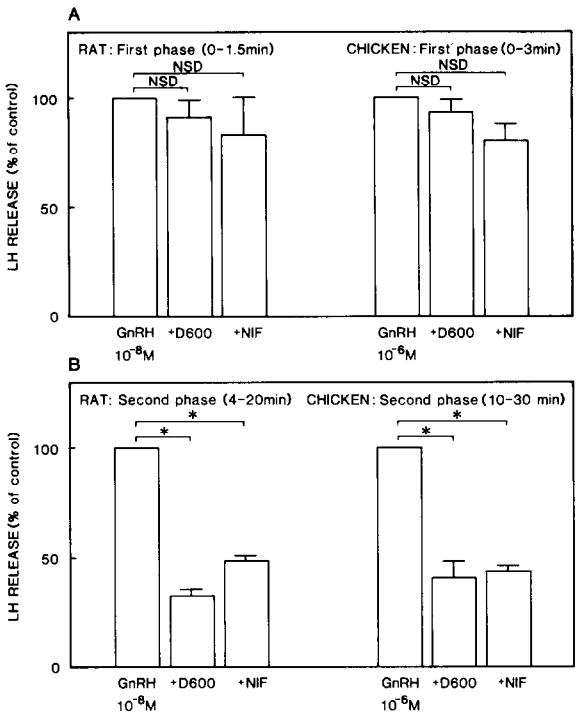

Fig.2. Effects of D600 (2×10^{-5} M) and nifedipine (10^{-6} M) on first-phase (A) and second-phase (B) LH responses to GnRH in rat and chicken pituitary cells. NIF, nifedipine; NSD, no significant difference ($p > 0.25$); * $p < 0.005$ (t-test).

therefore chose simply to use Ca^{2+}-channel blockers.

In conclusion, the first and second phases of LH release are clearly different in their dependence on Ca^{2+} entry through voltage-gated Ca^{2+}-channels. Although increased intracellular Ca^{2+} is a major mediator of GnRH-stimulated LH release [2] it is apparent that other intracellular events can modulate Ca^{2+} effects. Phorbol esters, which activate protein kinase C [16], have been shown to synergise with Ca^{2+} entering the cell through voltage-gated Ca^{2+} channels causing LH release, and this synergism is inhibited by calmodulin antagonists [17,18]. In addition, inhibitors of the lipoxygenase pathway of arachidonic acid metabolism are able to inhibit LH release [19]. GnRH-stimulated LH release may therefore be a combination of effects resulting from activation of the calmodulin system, protein kinase C and the arachidonic acid cascade.

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