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Gonadotropin Releasing Hormone Stimulation of Cultured Pituitary Cells Requires Calcium

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SUMMARY

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The requirement for Ca^{2+} during luteinizing hormone (LH) release stimulated by gonadotropin-releasing hormone (GnRH) was studied in dispersed cultured rat pituicytes. With maximal GnRH stimulation, LH release required 1 mM Ca^{2+} for optimal stimulation, although basal production was not calcium dependent. Stimulated release was inhibited when cells were incubated in media lacking Ca^{2+}, and by $LaCl_3$ (a potent inhibitor of Ca^{2+} action) which inhibited GnRH-induced LH release in the presence of Ca^{2+}. The requirement for Ca^{2+} is specific, since Mg^{2+} is not as effective, even at elevated concentrations. Two agents that block Ca^{2+} movement into and within cells, Ruthenium Red and D-600 (methoxy-verapamil), also blocked the release of LH from stimulated pituicytes. The present results demonstrate a specific dependence on calcium for GnRH stimulation of LH release from cultured gonadotrophs. Our observations satisfy some of the criteria require for a postulated role of Ca^{2+} in stimulus-secretion coupling in this system.

INTRODUCTION

The importance of extracellular Ca^{2+} in secretory processes is well known. Catecholamine release from the adrenal medulla (1, 2), vasopressin release from the neurohypophysis (3) and histamine release from mast cells (4-6) are all Ca^{2+}-dependent processes. Douglas (1, 2) and his colleagues initially proposed that calcium might serve to couple stimulus and secretion in the adrenal medulla. Subsequent to this suggestion, extracellular calcium has been shown to be required for secretory functions of a number of endocrine tissues, including insulin release from the pancreas (7), oxytoxin release from the neurohypophysis (8) and release of several anterior pituitary hormones from hemipituitaries (9-14). The active locus remains undefined.

The detection (15, 16) and characterization (17, 18) of a troponin C-like calcium binding protein (calmodulin), with similarities to troponin-C and with specific regulatory functions (19)—as well as kinases and ATPases that appear to be Ca^{2+} controlled (20-23)—have lent further credibility to the hypothesis that calcium plays an intermediate role in stimulated release mechanisms, particularly in mast cells (5, 6).

In the present work, we examined the dependence and specificity for calcium in gonadotropin releasing hormone (GnRH) stimulation of luteinizing hormone (LH) release from primary cultures of collagenase-dispersed rat pituicytes.

MATERIALS AND METHODS

Dispersion and culture of rat pituitary cells. Pituitaries were dispersed and cultured as described previously (24). Briefly, whole pituitaries were removed from fe-

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male weanling rats (21 days) (Zivic-Miller) and collected in sterile Medium 199 containing 0.3% BSA[1] (Fraction V, Armour), 10 mM HEPES, pH 7.4 (Sigma Chemical Co.) and 20 μg/ml gentamicin (Schering). Each pituitary was cut in 6–8 pieces in a Petri dish, using fine forceps and fine scissors. The cut pieces were allowed to settle in a 50 ml sterile conical centrifuge tube containing HEPES-buffered (10 mM) medium 199 (pH 7.4) with 0.3% BSA (199/BSA). This medium was decanted and replaced twice with fresh 199/BSA to remove lysed cells and their contents released during mincing of the tissue.

A 10 ml portion of 0.25% collagenase (Worthington) and 0.10% hyaluronidase (Sigma Chemical Co.) in 199/BSA was added to the decanted tissue fragments. The tube was then capped, placed on its side in a water bath (37°) and shaken (100 cycles/min). Every three minutes, the tube was removed and the contents gently drawn repetitively into and out of a disposable, sterile 10 ml pipette. After 15 min, the solution was filtered through organza cloth to remove residual tissue fragments. The filtrate, containing clumps of 5–10 cells, was brought to a volume of 50 ml with 199/BSA.

After centrifugation at 225 × *g* for 10 min, the supernate was discarded and the pellet was resuspended in 1–2 volumes (ml/pituitary) of 199/BSA containing 10% horse serum (Microbiological), 2.5% fetal calf serum (Microbiological) and 20 μg/ml gentamicin. One milliliter aliquots of the resuspended cells were plated in 2.2 ml wells of Costar well cluster dishes. The plates were covered and incubated at 37° in 95% O_2–5% CO_2 for two days.

Incubations of cultured cells. Following the 48 hour culture period, the culture medium was removed and the plated cells were rinsed twice with 2 ml/well of 199/BSA, to remove serum and nonadhered cells. In experiments which required media other than 199/BSA, cells were washed in the particular media prior to use. Each well was filled with a 1 ml aliquot of media, with or without GnRH (NIAMDD Hormone Distribution Program and the National Pituitary Agency), or various inhibitors, and the plates were incubated 3 hours at 37° under 95% O_2–5% CO_2. This length of time has been shown to provide maximum responsiveness from the gonadotrope (24) and from most endocrine culture systems. Some experiments included an incubation period prior to the addition of GnRH. When added, GnRH was present at a concentration of 1 μM, shown previously to provide maximal stimulation of LH release from this cell preparation (24). At the end of the 3 hour incubation period, the media was removed and stored frozen prior to assay of LH. When total cellular LH content was measured, cells were rinsed with 199/BSA prior to freezing and then solubilized in 1 ml of 1% Triton-X 100 (Rohm and Haas) by freezing and thawing twice and mechanical agitation.

For the Ca^{2+} dose-response and Mg^{2+} substitution studies, 0.3% BSA in Dulbecco's phosphate buffered saline (dPBS, Gibco) was prepared initially without $CaCl_2$. $CaCl_2$ and $MgCl_2$ were added back to the incubation media to obtain the indicated concentrations.

In the La^{3+} competition experiment, 136 mM NaCl, 5 mM KCl, 1.18 mM $MgCl_2$, 1 mM $CaCl_2$, 0.3% BSA, 10 mM HEPES, pH 7.4, was used containing the indicated concentrations of $LaCl_3$. This buffered salt solution was selected because phosphate, sulfate, and carbonate ions, normally present in other media, precipitate La^{3+} even at low concentrations. The cells were preincubated for 10 min with the La^{3+}-containing media prior to addition of GnRH (1 μM final concentration) as indicated in order to provide time for the ion to enter the cell.

Ruthenium Red (Sigma Chemical Co.) was dissolved, and cells were assayed in 199/BSA. The Ruthenium Red concentrations were determined using the assay data supplied by the distributor which indicated that 20% (by weight) of the commercial product was active. The washed cells were preincubated for 30 min with or without Ruthenium Red as indicated, then GnRH

[1] The abbreviations used are: BSA, bovine serum albumin; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; LH, luteinizing hormone; GnRH, gonadotropin-releasing hormone.

was added to a final concentration of 1 μM to stimulate cells. The preincubation period gave maximum inhibition of the Ca^{2+}-ATPases.

D-600 (a gift from Drs. Kirsten, Kleinsorge, and Oberdorf, Knoll Pharmaceutical Co.) was dissolved and cells assayed in 199/BSA without an incubation period since this was not found to be required to achieve inhibition.

Radioimmunoassay of luteinizing hormone (LH). Highly purified rat LH (NIAMDD rat LH I-4), antiserum to rat LH (NIAMDD anti-rat LHS-4) and reference preparation LH (NIAMDD rat LH-RP-1) were obtained from the NIAMDD Rat Pituitary Agency Hormone Distribution Program and Dr. A. Parlow. The RIA was performed as recommended in the NIAMDD kit instructions. Luteinizing hormone was labeled by the chloramine T procedure (25). Standard curves were prepared in the presence of Ruthenium Red, $LaCl_3$, and D-600, and these compounds did not interfere in the assay at the concentrations used in these studies. Typically, this assay detects 0.2 ng LH/ml, is specific, and produces duplicates that agree within 7% cv.

General. Data was derived as the mean ± SE of values obtained in three replicates. Luteinizing hormone released was normalized for the number of cells present by the diphenylamine procedure (26).

RESULTS

The effect of extracellular calcium concentration changes on cell responsiveness is shown in Fig. 1. Although basal release was not altered as a function of calcium, GnRH-stimulated release required 1 mM calcium for optimal release. Higher concentrations (up to 8 mM) afforded no additional responsiveness. Cells incubated in media containing less than 1 μM Ca^{2+} were unresponsive to stimulation by GnRH.

Mg^{2+} and La^{3+} were examined for their ability to substitute for calcium due to their molecular similarity to this ion. The addition of 10 μM $LaCl_3$ to the incubation media containing 1 mM Ca^{2+} inhibited GnRH-stimulated release, as seen in Fig. 2. Higher concentrations of $LaCl_3$ inhibited stimulated release further. There was no apparent inhibition of basal release. The effect of Mg^{2+} on LH release in the absence or presence of calcium is shown in Fig. 3. One millimolar $MgCl_2$ (equimolar to the optimal calcium concentration) allows slight (two-fold) stimulation of LH release by GnRH, but even a high concentration of Mg^{2+} (10 mM) does not render the cells as responsive as 1 mM Ca^{2+} (7-fold). One millimolar Mg^{2+} does not significantly block the action of 1 mM Ca^{2+}, consistent with studies in the adrenal (2).

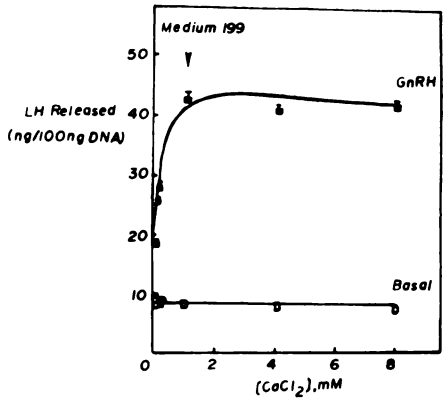

FIG. 1. *Effect of extracellular calcium concentration on LH release by cultured pituicytes*

Cells were incubated for 3 hours in the presence (■) or absence (□) of 1 μM GnRH at the indicated concentrations of Ca^{2+}. Released LH was assayed in the supernate as described in METHODS. The arrow shows the Ca^{2+} concentration contained in Medium 199 used in other studies herein.

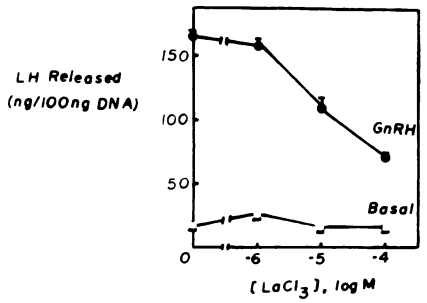

FIG. 2. *Inhibition of GnRH-stimulated LH release by $LaCl_3$ in the presence of 1 mM Ca^{2+}*

Cells were preincubated (10 min) in media containing the indicated concentrations of $LaCl_3$. GnRH (1 μM), (●) or carrier was added (○) and the cells incubated for 3 hr. Released LH was measured in the supernate as described in METHODS.

Ruthenium Red is a hexavalent-cationic inorganic compound that noncompetitively inhibits calcium transport (27) and blocks the Ca^{2+} ionophoric activity associated with Ca^{2+}-Mg^{2+} ATPase. Ruthenium Red's ability to inhibit stimulated LH release is shown in Fig. 4. While the lowest dose tested, 0.1 μM, gave a slight, yet significant potentiation of the effect of GnRH, higher concentrations inhibited in a dose-related manner. There was no effect on basal release.

Compound D-600 has been shown to antagonize the role of Ca^{2+} in excitation-contraction coupling (28), probably through blockade of Ca^{2+} channels (29). The effects of D-600 on LH release are seen in Fig. 5.

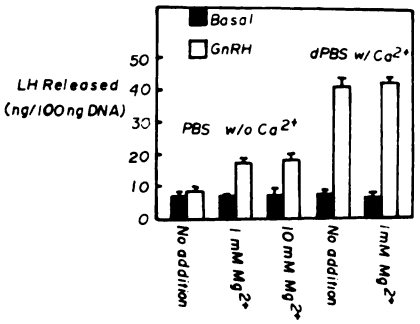

FIG. 3. *Effect of magnesium on LH release from pituicytes with or without calcium and in the presence (solid columns) or absence (unfilled columns) of 1 μM GnRH*

Released LH was measured in the supernate after 3 hr.

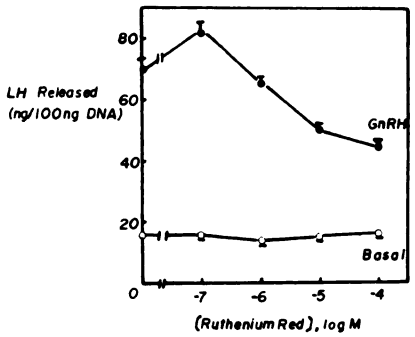

FIG. 4. *Effect of Ruthenium Red on LH release by cultured pituicytes*

Cells were preincubated for 30 min in the indicated concentrations of Ruthenium Red prior to the addition of 1 μM GnRH (●) or carrier (○). Released LH was measured in the supernate after 3 h.

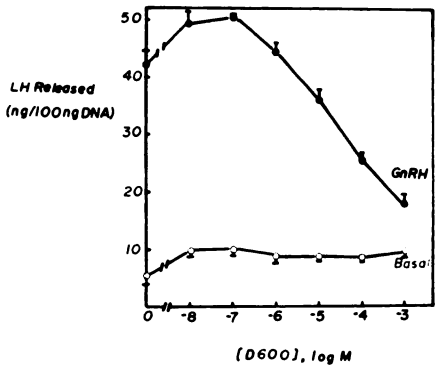

FIG. 5. *Effect of D-600 on LH release from cultured pituicytes*

Cells were incubated for 3 hr in media containing the indicated D-600 concentrations and in the presence (●) or absence (○) of 1 μM GnRH. Released LH was measured in the supernate after 3 hr.

At low doses of D-600, a slight potentiation of GnRH-induced LH release was seen, similar to that observed with Ruthenium Red. Progressive inhibition of stimulated, but not basal, LH release was observed at concentrations of D-600 above 0.1 μM.

The partial agonistic action of drugs that generally behave as calcium inhibitors at high concentrations may be due to their ability to block open the calcium channels at trace doses.

DISCUSSION

The data described in the present work suggest that gonadotrophs have a specific and dose-dependent requirement for physiological concentrations of Ca^{2+} during GnRH stimulation of LH release. Stimulated LH release is blocked in the absence of calcium, and responsiveness is inhibited by agents that alter calcium transport and distribution. Responsiveness is also blocked by a calcium antagonistic ion, La^{3+} (30).

The action of calcium is probably at the level of release rather than biosynthesis. It has been shown (14) in hemipituitaries that extracellular calcium does not play a role in LH biosynthesis. Moreover, we were able to account for LH released to the media by an equal cellular LH depletion (data not shown).

If the effect of calcium is limited to an involvement in the release mechanism only, several possibilities remain. First, calcium

may be required to maintain the functional integrity of the cell generally, via stabilization of membrane systems or other mechanisms. Calcium is bound by membranes and is required for their functional and structural maintenance (31). The effect of calcium on LH release may be permissive, in that functional membrane integrity is needed to effect release mediated through a granule-membrane interaction.

A second possibility is that calcium plays a specific role in the mechanism of GnRH action. By analogy to the criteria (32) that were developed to establish the role of cyclic AMP as a second messenger, a specific role of calcium as an intermediate in secretion-coupling would require rigorous proof that:

1. Removal of calcium from its site of action blocks the effect of GnRH on LH release.
2. LH release should occur as a consequence of inserting calcium ions into the gonadotroph, even in the absence of GnRH.
3. GnRH should stimulate the movement of calcium into specific sites which is a requisite for LH release.

The present findings partially satisfy the first criteria. The rapid movement of $^{45}Ca^{2+}$ in response to GnRH, but not to dibutyryl cyclic AMP, has been shown in pituitary slices (33). This observation is consistent with the finding by Conn *et al.* (24) that dibutyryl cyclic AMP does not effect LH release from pituicytes.

While extracellular calcium appears to be required for GnRH stimulation of LH release, we are not able to exclude altogether a role for intracellular calcium, since D-600, $LaCl_3$ and Ruthenium Red probably penetrate the plasma membrane.

The present data indicate that Ca^{2+} is specifically required for GnRH-stimulation of LH release. This study supports a role for Ca^{2+}, either permissive, that is, allowing release or intermediary and specific in GnRH-induced LH release from pituicytes.

Note Added In Proof: Since submission of this manuscript, we have shown the loss of responsiveness in calcium depleted cells is a reversible process (34).

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