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Effects of Various Secretagogues on Gonadotropin Release by Rat Anterior Pituitary Cell Cultures (41785)

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Abstract. Rat anterior pituitary cell cultures were utilized to compare LH and FSH release induced by LHRH, cAMP derivatives, cGMP derivatives, the calcium ionophore A23187, and elevated K^+. Of the cyclic nucleotide derivatives tested, only 8-Br-cAMP released significant quantities of both LH and FSH. A23187 and elevated K^+ released significant quantities of LH and FSH but supramaximal concentrations of these stimuli liberated only approximately 50% of the gonadotropins released by LHRH. Thus, none of the secretagogues tested was as efficacious as LHRH in releasing either LH or FSH.

Luteinizing hormone releasing hormone (LHRH)[3] is the normal physiological stimulus for luteinizing hormone (LH) and follicle stimulating hormone (FSH) secretion. Other secretagogues such as cyclic nucleotides (reviewed in (1, 2)) and agents which mobilize calcium (reviewed in (3, 4)) can also induce gonadotropin liberation. However, a study which compares the efficacy of these various stimuli on both LH and FSH release under similar experimental conditions is lacking. In the present study, we have utilized rat anterior pituitary cell cultures to compare gonadotropin release induced by cyclic adenosine monophosphate (cAMP) derivatives, cyclic guanosine monophosphate (cGMP) derivatives, the calcium ionophore A23187, and elevated K^+ concentrations to that induced by LHRH.

Materials and Methods. *Rat anterior pituitary cell cultures.* Anterior pituitaries from 40-day-old, female Long–Evans rats were dispersed with 0.125% (w/v) collagenase (Worthington, Freehold, N.J.) as previously described (5). Dispersed cells (100,000 per dish) were plated in 35-mm dishes in 1 ml Eagle's minimum essential medium (Earle's salts) containing 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone (MEM), and 10% fetal bovine serum (FBS). Cultures were maintained at 37°C under a humidified atmosphere of 95% air and 5% carbon dioxide. After 4 days, the cultures were rinsed three times with MEM and various secretagogues were added for the specified intervals. After removal of the medium, sodium phosphate (0.01 *M*, pH 7.4) buffered sodium chloride containing 1 m*M* disodium ethylenediaminetetraacetic acid was added to each dish. The cells were frozen, thawed, scraped from the dish, and vigorously homogenized. The media and cell extracts were clarified by centrifugation at 12,800*g* for 15 min and frozen until hormones were measured by radioimmunoassay. Each experimental group consisted of at least three cultures. The data presented in each figure were obtained from a group of cultures prepared from a single batch of cells and the group of cultures was stimulated during the same 6-hr period. All samples from a single experiment were assayed simultaneously. Results were expressed as nanograms hormone released (or present in the cell extract) per 100,000 cells initially plated for the specified time interval.

Tissue culture reagents were purchased from Gibco, Grand Island, N.Y. LHRH was pur-

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[3] Abbreviations used: LHRH, luteinizing hormone releasing hormone; LH, luteinizing hormone; FSH, follicle stimulating hormone; cAMP, cyclic adenosine monophosphate; 8-Br-cAMP, 8-bromo-cAMP; dbcAMP, dibutyryl cAMP; cGMP, cyclic guanosine monophosphate; 8-Br-cGMP, 8-bromo-cGMP; dbcGMP, dibutyryl cGMP; MEM, Minimum Essential Medium; MIX, 3-isobutyl-1-methyl xanthine; KRB, Krebs–Ringers biocarbonate buffer; DMSO, dimethyl sulfoxide; r, rat.

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chased from Beckman (Palo Alto, Calif.), dispensed in 0.1 *N* acetic acid, and lyophilized. Immediately before use, LHRH was reconstituted in MEM. Cyclic nucleotide derivatives were obtained from Sigma Chemical Co., St. Louis, Missouri. The 3-Isobutyl-1-methyl xanthine (MIX) was obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. Cyclic nucleotides and MIX were also added to cultures in MEM.

In experiments utilizing elevated K^+ concentrations, cultures were incubated in Krebs–Ringers bicarbonate buffer (118.5 m*M* sodium chloride, 4.7 m*M* potassium chloride, 2.5 m*M* calcium chloride, 1.2 m*M* potassium phosphate, 1.2 m*M* magnesium sulfate, and 24.9 m*M* sodium bicarbonate) supplemented with 10 m*M* glucose (KRB), MEM amino acid mixture (Gibco), and MEM vitamin mixture (Gibco) (KRB–MEM). Elevated K^+ concentrations were obtained by substituting appropriate amounts of potassium chloride for sodium chloride in the KRB–MEM. The calcium ionophore A23187 was obtained from Calbiochem (La Jolla, Calif.) and added to cultures in 10 μl dimethyl sulfoxide (DMSO). Corresponding control cultures received 10 μl DMSO.

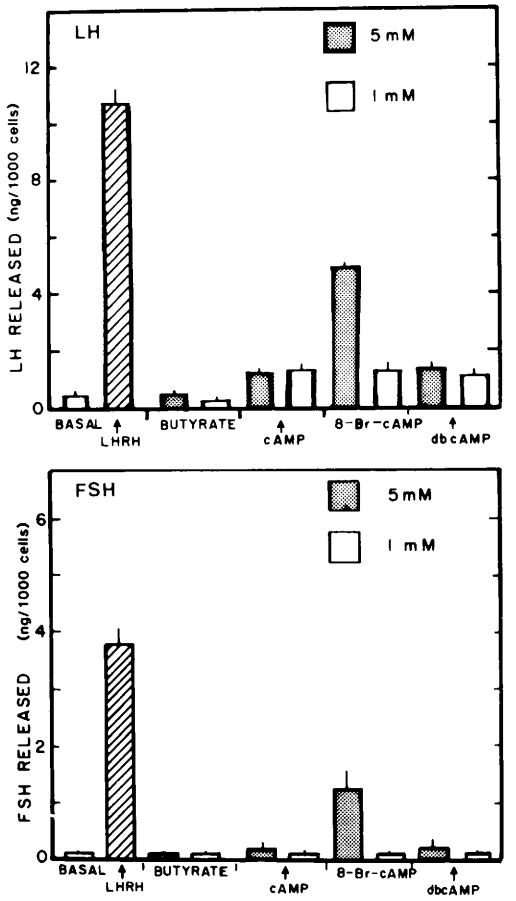

FIG. 1. LH and FSH release induced by a 6-hr exposure of rat anterior pituitary cell cultures to 10^{-7} *M* LHRH, sodium butyrate, or various cAMP derivatives. Sodium butyrate was included in this experiment to serve as an additional control for dbcAMP. Each bar illustrates the mean ± SEM of triplicate observations.

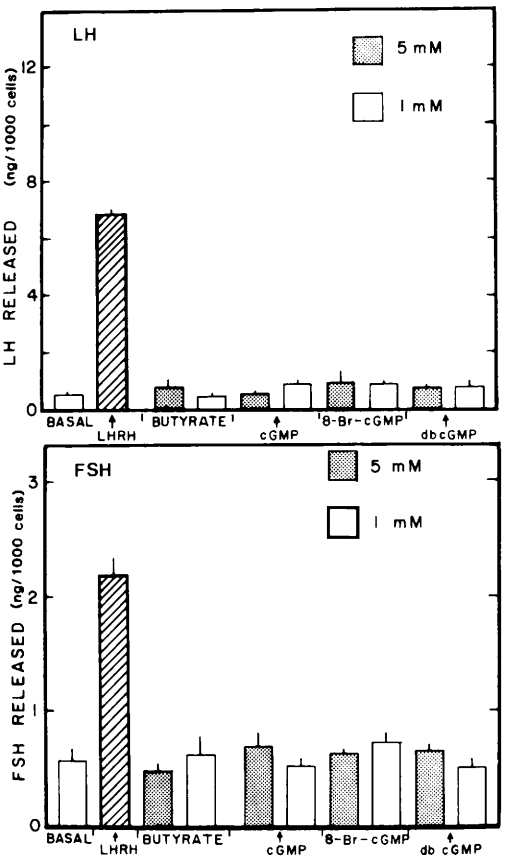

FIG. 2. LH and FSH release induced by a 6-hr exposure to 10^{-7} *M* LHRH, sodium butyrate, or various cGMP derivatives. Sodium butyrate was included in this experiment to serve as an additional control of dbcGMP. Each bar illustrates the mean ± SEM of triplicate observations.

Radioimmunoassays. Concentrations of LH and FSH were quantitated by double antibody radioimmunoassays utilizing kits provided by the NIH Pituitary Hormone Distribution Program. Directions supplied with the

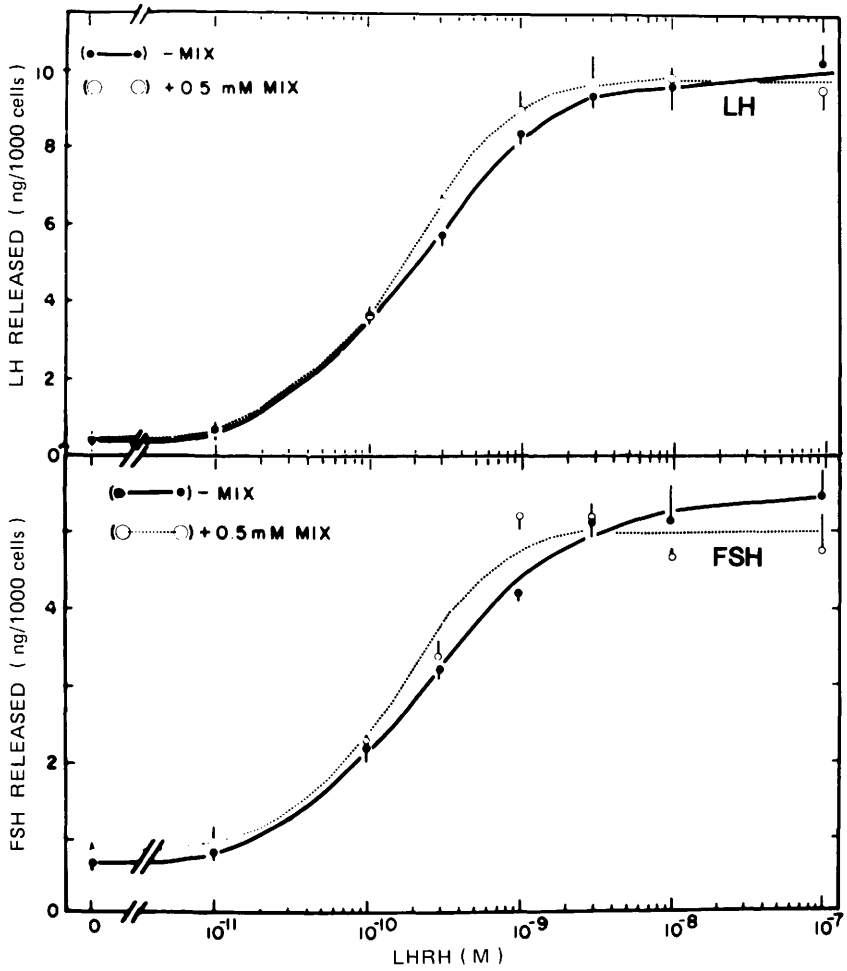

FIG. 3. The dose-response curves for LHRH-induced LH and FSH release in the presence and absence of 0.5 m*M* MIX, a phosphodiesterase inhibitor. In both cases the curves generated were statistically similar ($P \geq 0.05$) by ALLFIT (8). Each point illustrates the mean ± SEM of triplicate observations.

kits were followed except that a reduced quantity of carrier immunoglobulin (normal rabbit serum) was employed and the addition of labeled hormone was delayed 24 hr to slightly increase sensitivity. Reagents employed were: anti-rLH-S4, rLH-I5, rLH-RP1, anti-rFSH-S8, rFSH-I4, and rFSH-RP1. Iodinations were performed enzymatically using lactoperoxidase (6). Calculations were performed by a four-parameter logistic curve-fitting computer program (7).

Statistics. Statistical significance was established by one-way analysis of variance and Duncan's new multiple range tests. A probability of less than 0.05 was considered significant. LHRH dose-response curves (Fig. 3) were compared with ALLFIT (8).

Results. *LHRH.* The time-course and dose-response relationships for LHRH-induced LH and FSH release under the conditions utilized in this study have been published previously (5). Additional LHRH dose-response curves are presented in Fig. 3.

Cyclic nucleotides. Cyclic AMP (cAMP), 8-bromo-cAMP (8-Br-cAMP), and dibutyryl cAMP (dbcAMP) increased LH release over basal (Fig. 1, upper panel) but that induced by cAMP and dbcAMP was not dose related. The most effective cAMP derivative was clearly 8-Br-cAMP. With regard to FSH, only

8-Br-cAMP at a concentration of 5 mM significantly increased release over basal. For both gonadotropins, 5 mM 8-Br-cAMP was much less effective than 10^{-7} M LHRH. None of the cyclic guanosine nucleotides tested released significant quantities of LH or FSH (Fig. 2). Furthermore, the phosphodiesterase inhibitor MIX at a concentration of 0.5 mM did not alter the dose–response curves of LH and FSH to LHRH (Fig. 3).

Elevated K^+. LH and FSH release induced by 60 mM K^+ was approximately linear with time (Fig. 4) and the respective ED_{50}s were approximately 33 and 23 mM, (Fig. 5). K^+ concentrations of 60 mM were only approximately 50% as efficacious as 10^{-7} M LHRH (Figs. 4 and 5).

The calcium ionophore A23187. A23187 at a concentration of 10^{-4} M induced LH and FSH release in a time-dependent manner (Fig. 6). Under these conditions, LH and FSH release induced by A23187 was less than that induced by a 6-hr exposure to 10^{-7} M LHRH. A23187 also caused the cells to change shape and some detached from the dish.

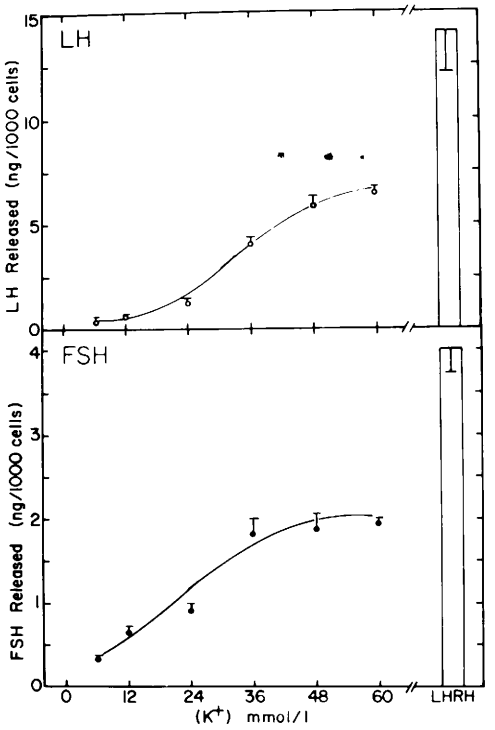

FIG. 5. Effect of increasing K^+ concentrations on the release of LH and FSH during a 6-hr incubation. Also illustrated is the gonadotropin released by a 6-hr exposure to 10^{-7} M LHRH. Each point or bar illustrates the mean ± SEM of triplicate observations.

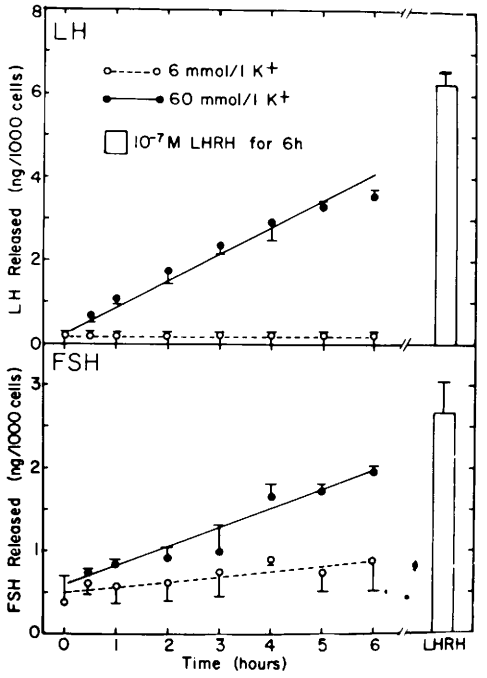

FIG. 4. Time-course of LH and FSH release induced by 60 mM K^+ or that induced by a 6-hr exposure to 10^{-7} M LHRH. Each point or bar illustrates the mean ± SEM of triplicate observations.

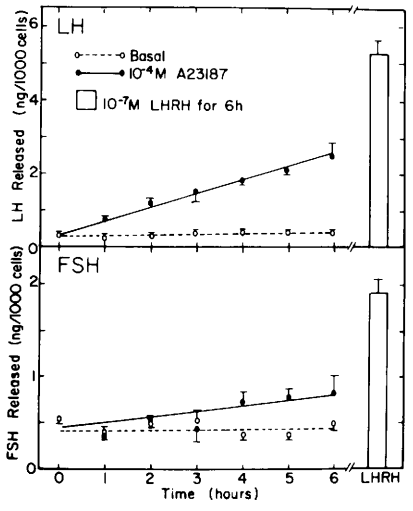

FIG. 6. Time-course of LH and FSH release induced by 10^{-4} M A23187, a calcium ionophore, or that induced by a 6-hr exposure to 10^{-7} M LHRH. Each point or bar illustrates the mean ± SEM of triplicate observations.

Cellular content and total gonadotropins. In each experiment, the LH and FSH in cell extracts was quantitated and the total amount of gonadotropins in each culture (medium plus cells) was calculated. With regard to LHRH, cyclic nucleotides and elevated K^+ the amount of LH and FSH found in the medium was associated with a corresponding reduction in cellular gonadotropins and the total amount of gonadotropins in each dish remained constant. Because some cells stimulated by A23187 detached from the dish and were removed from the medium by centrifugation, the apparent cellular content of LH and FSH was reduced (Fig. 7). This effect was associated with A23187 because the vehicle (DMSO) did not cause detachment or a similar reduction in apparent cellular gonadotropins.

Discussion. The normal physiological stimulus for gonadotropin release, LHRH, was consistently the most effective secretagogue for inducing both LH and FSH release from rat anterior pituitary cell cultures. In almost every case, the release of LH and FSH occurred in synchrony. The only exceptions were cAMP and dbcAMP which slightly increased LH release without significantly increasing FSH release. Thus, all of the secretagogues examined had qualitatively similar effects on LH and FSH release although none was as efficacious as LHRH.

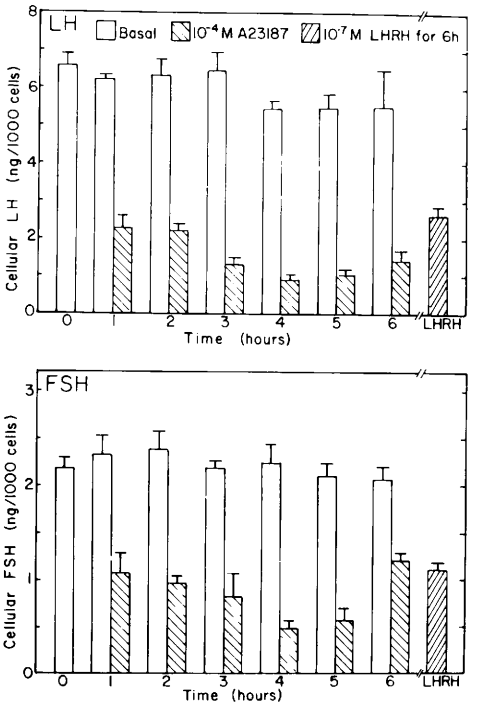

FIG. 7. Apparent cellular LH and FSH content after exposure of cultures to 10^{-4} *M* A23187 for various intervals. Also illustrated is the cellular content after a 6-hr exposure to 10^{-7} *M* LHRH. The corresponding media samples are presented in Fig. 6. Each bar illustrates the mean ± SEM of triplicate observations.

Cyclic nucleotides. The role of cyclic nucleotides in LHRH action remains to be clarified. Labrie and colleagues suggest that cAMP serves as an obligatory intermediate in LHRH-induced gonadotropin secretion (reviewed in (1,2)). A portion of the evidence used to reach this conclusion comes from Drouin *et al.* (9) who demonstrated that 8-Br-cAMP released LH and FSH from rat anterior pituitary cell cultures. However, they did not compare various cAMP derivatives nor the efficacy of 8-Br-cAMP and LHRH (9). Our data suggest that 8-Br-cAMP is more potent than other cAMP derivatives but is far less efficacious than LHRH. Moreover, there may be a difference in the response of fresh pituitary tissue and cultured cells. We and others (reviewed in (1)) have observed that rat hemi-pituitaries incubated *in vitro* or superfused (W.-S. A. Wun and H. E. Grotjan, unpublished observation) release LH and FSH in response to 1 m*M* cAMP, 8-Br-cAMP or dbcAMP. Thus, cultured cells may lose cAMP responsiveness. It is also possible that cAMP may alter LH synthesis while having a minimal effect on release (10, 11). Furthermore, Noar and Catt (12) have suggested that LHRH may induce changes in cGMP production not related to gonadotropin release.

Agents which mobilize calcium. A23187 and elevated K^+ can be considered as a class of secretagogues which act by mobilizing calcium, the latter presumably mediated by membrane depolarization (reviewed in (3, 4)). Although Conn *et al.* (13) presented evidence that 10^{-4} m*M* A23187 was as effective in releasing LH as 10^{-6} *M* LHRH, our data agree more closely with Noar *et al.* (14) who observed that supramaximal concentrations of A23187 were not as efficacious as LHRH. In our hands A23187 caused a change in cell shape and caused many cells to detach from the dish. This agent appears to have multiple

effects on cultured pituitary cells. Thus, even though calcium may play a critical role in gonadotropin release, neither elevated K^+ nor A23187 could mimic LHRH.

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