MOLECULAR PHARMACOLOGY, 27:532-536

Diacylglycerols and Protein Kinase C

Potential Amplifying Mechanism for Ca^{2+}-Mediated Gonadotropin-Releasing Hormone-Stimulated Luteinizing Hormone Release

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Received November 20, 1984; Accepted February 14, 1985

SUMMARY

The present study was designed to test the hypothesis that there is a functional interaction between the calcium-calmodulin system, which appears to mediate the action of gonadotropin-releasing hormone (GnRH), and activators of protein kinase C, which stimulate luteinizing hormone (LH) release by a mechanism which does not require extracellular calcium. We have examined a diacylglycerol and a phorbol ester, which both activate protein kinase C and stimulate LH release. These compounds show synergistic action with calcium ionophore A23187 as secretogogues. Pimozide (a calmodulin antagonist), methoxyverapamil (a calcium ion channel inhibitor), and Ac[D-pCl-Phe^{1,2}-D-Trp^{3}-D-Lys^{6}-D-Ala^{10}]GnRH (a potent gonadotropin-releasing hormone antagonist) were used to show that the diacylglycerol and phorbol ester can stimulate LH release in a manner that is independent of both Ca^{2+} and calmodulin and do not work by means of a direct action on the GnRH receptor. These observations, coupled with previously published reports that extracellular Ca^{2+} mobilization is both necessary and sufficient for initiation and perpetuation of GnRH-stimulated LH release, indicate that activation of protein kinase C by endogenous diacylglycerols may serve as an amplifier of the GnRH-stimulated signal which appears to be mediated independently by the Ca^{2+}-calmodulin system.

INTRODUCTION

Drugs that elevate intracellular Ca^{2+} levels can provoke gonadotropin release with the same efficacy as GnRH[1] (1). Recent studies (2) have shown that gonadotropin release in response to GnRH is terminated virtually immediately following removal of extracellular Ca^{2+} or blockade of its ability to enter the cell. In addition, calmodulin appears to be the intracellular receptor for Ca^{2+} mobilized in response to GnRH since GnRH provokes its redistribution (3) and GnRH action can be blocked by calmodulin inhibitors (4–6) in the same potency order with which they inhibit this Ca^{2+}-binding protein. These and related studies have made it possible to identify a role for Ca^{2+} as a second messenger for GnRH (7). We recently showed (8) that a family of 1,2-DAGs of differing length acyl groups stimulated pituitary gonadotropin release with the same structure-activity relationship with which they activated PKC. The most potent of these was *sn*-1,2-diC_8 and release occurred in a Ca^{2+}-independent fashion. Accordingly, it was of interest to determine the locus of activity of these compounds in relation to Ca^{2+}-mediated gonadotropin release. We have used inhibitors with well characterized sites of action in this system (4–9) in order to identify the molecular locus of action of the DAGs and PMA, another activator of PKC, relative to calcium and calmodulin. These data and studies showing synergistic effects with calcium ionophore A23187 have enabled us to identify a potential role for PKC in the molecular mechanism of GnRH action.

MATERIALS AND METHODS

Preparation and culture of dispersed rat pituitary cells. Pituitary dispersion was performed as previously described (9). Whole pituitaries (n = 30) were removed from female weanling rats (Harlan Sprague-Dawley, Indianapolis, IN) and collected into sterile HEPES-buffered (10 mM) Medium 199 (M. A. Bioproducts, Walkersville, MD) containing 0.3% BSA (fraction V Sigma, St. Louis, MO) at room temperature. When collection was completed, the medium was decanted and replaced with M199/BSA containing gentamycin (20 μg/ml) (Schering Corp., Bloomfield, NJ). The pituitaries were exposed to this solution for 15–20 min at room temperature.

This work was supported by National Institutes of Health Grant HD 19899.

[1] The abbreviations used are: GnRH, gonadotropin-releasing hormone; BSA, Cohn fraction V bovine serum albumin; D600, methoxyverapamil; DAG, diacylglycerol; diC_8, *sn*-1,2-dioctanoylglycerol; DMSO, dimethyl sulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LH, luteinizing hormone; M199/BSA, Medium 199 containing 0.3% bovine serum albumin; PKC, protein kinase C; PMA, 4β-phorbol-12-β-myristate-13α-acetate; RIA, radioimmunoassay.

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The pituitaries were finely minced in a Petri dish containing 40 ml of M199/BSA, transferred to a 50-ml centrifugation tube, and permitted to settle. The pieces were washed twice with 50 ml of M199/BSA to remove the remnants of lysed cells disrupted during mincing of the tissue.

A 10-ml portion of a freshly prepared solution containing 0.25% collagenase (CLSII, 126 units/mg, Worthington, Freehold, NJ) and 0.1% hyaluronidase (Sigma) in M199/BSA was sterilized by filtration through a 0.45-μm membrane (HAWP, Millipore, Bedford, MA) and added to the tissue fragments. The centrifuge tube was capped, placed on its side in a 37° water bath, and shaken at 100 cycles/min in a Dubnoff metabolic shaker. At 3-min intervals, the tissue suspension was gently passed five times through a 10-ml sterile disposable pipette. After 15 min, the solution was decanted through organza cloth, and the enzyme dispersion was repeated with the residual tissue fragments. The combined filtrate was brought to a volume of 50 ml with M199/BSA. Following centrifugation at 225 × *g* for 10 min, the pellet was resuspended in 4–6 volumes (milliliters/pituitary) of M199/BSA containing 10% horse serum and 2.5% fetal calf serum (M. A. Bioproducts). One-ml aliquots of the cell suspension were plated in 2.2-ml wells (Multiwell tissue culture plates, Costar, Cambridge, MA) and maintained for 2 days at 37°.

Incubations of cultured cells. After the culture period, plated cells were washed twice with 1–2-ml portions of M199/BSA to remove sera and unattached cells. They were then covered with 1.0 ml of M199/BSA containing the indicated treatments and incubated for 3 hr at 37°. At this time, the medium was removed from the cells by aspiration and assayed for LH by RIA.

Radioimmunoassay. The RIA for LH was performed as recommended in the kit instructions from the National Institute for Arthritis, Diabetes, and Digestive and Kidney Diseases except that antiserum (LH120), prepared and characterized as described previously (10), was used. RP1 LH standard and LH I-6 iodination protein were used. Bound and free hormones were separated with immobilized protein A (11).

Preparation and use of diacylglycerols. diC_8 was prepared as previously described (8). A stock solution of 20 mM in chloroform was maintained at −40°. For experiments, appropriate amounts were removed and evaporated to dryness in an Erlenmeyer flask and then dissolved in ethanol and M199/BSA to make a 1% ethanolic solution.

Preparation of PMA. PMA (Sigma, lot 34F0682) was prepared in DMSO as a 1 mg/ml stock solution immediately prior to bioassay. All solutions containing DAGs or PMA were brought to pH 11 after use to destroy the potential tumor-promoting activity.

Other drugs. The GnRH antagonist Ac[D-pCl-Phe^{1,2}-D-Trp^{3}-D-Lys^{6}-D-Ala^{10}]GnRH was a gift of Dr. David Coy, Tulane University School of Medicine (DC-12-119). D600 (Knoll Pharmaceuticals, Ludwigshafen am Rhein, F. R. G.), natural sequence GnRH (National Pituitary Agency, Baltimore MD), ionophore A23187 (Calbiochem, La Jolla, CA), and pimozide (Janssen Pharmaceutica, Beerse, Belgium) were obtained as indicated and used as previously described (1, 4, 9). D600 and pimozide were diluted from 10 mM stock solutions in DMSO. A23187 was diluted from a 1 mM stock solution in DMSO and kept shielded from light throughout use. The amount of DMSO never exceeded 1% (v/v) in the cell bioassay and had no measurable effect on basal or stimulated LH release.

Data analysis. Data are expressed as means showing the standard error ($n = 6$). Differences between uninhibited and inhibited LH release values for each secretogogue were determined using Student's *t* test. $p < 0.05$ was considered significant.

RESULTS

Fig. 1 shows LH release from cultured pituitary cells in response to GnRH, diC_8, or PMA. Since these secretogogues stimulate LH release with different efficacies, comparison of the dose-response curves was facilitated by expressing the data in terms of release in response to 10^{-6} M GnRH. This level was set to 100%. This correction was used in Fig. 1 as well as Figs. 3–5 and made it possible to pool data from different experiments, as cell cultures vary somewhat in their levels of responsiveness between preparations. Thus, it can be seen in Fig. 1 that diC_8 and PMA stimulate LH release with approximately 75 and 50% of the efficacy of GnRH, respectively.

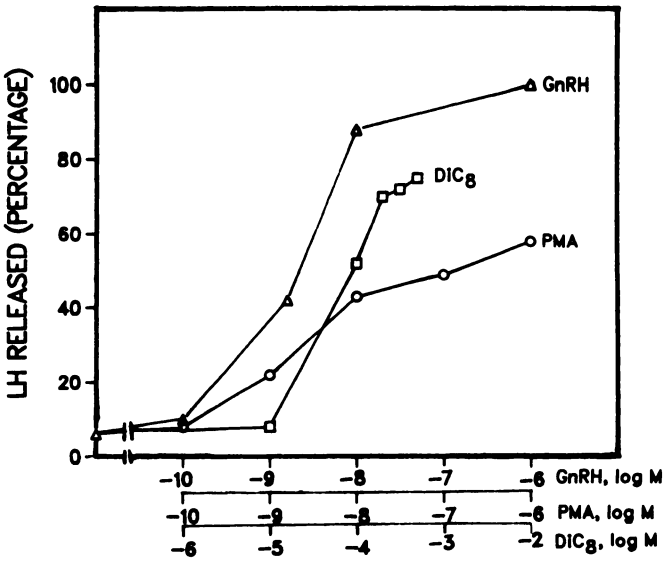

FIG. 1. *Stimulation of LH release from pituitary cell cultures in response to various secretogogues*

Two-day cultures were incubated for 3 hr in the indicated concentration of GnRH, diC_8, or PMA. LH release was determined by RIA as described in Materials and Methods. In order to facilitate comparison between these secretogogues with differing efficacy and in order to pool data from different experiments, the LH release values are normalized by comparison to release in response to 1 μM GnRH (100%). Standard errors between identically treated samples was <6% ($n = 6$), although, for graphic clarity, error bars are not shown.

The time course of LH release for concentrations of these secretogogues, which were chosen to give similar levels of release, is shown in Fig. 2. Qualitatively similar patterns of release were obtained for GnRH, diC_8, and PMA. A nearly linear rate of release was observed for the first hour. Maximal release was measured at 3 hr. Similar patterns of release were also seen with higher and lower concentrations of the secretogogues.

In order to compare the release requirements for the three secretogogues, LH release was measured in the presence of either a GnRH receptor antagonist, a calcium ion channel inhibitor (D600; Ref. 9), or a calmodulin inhibitor (pimozide; Ref. 4). These inhibitors were selected since the site of action of each has been characterized in this system and each is among the most potent inhibitors known at each site.

Fig. 3 shows the effect of the GnRH antagonist Ac[D-pCl-Phe^{1,2}-D-Trp^{3}-D-Lys^{6}-D-Ala^{10}]GnRH on LH release from cultured pituitary cells in response to 2 nM GnRH, 75 or 150 μM diC_8, 1.62, 4.86, or 16.2 mM (1, 3, or 10 ng/ml) PMA. The antagonist at 0.5 ng/ml was 50% effective at inhibiting GnRH-stimulated LH release. In contrast, LH release in response to either diC_8 or PMA was not inhibited at any concentration of the GnRH receptor antagonist examined (range, 0–1000 ng/ml).

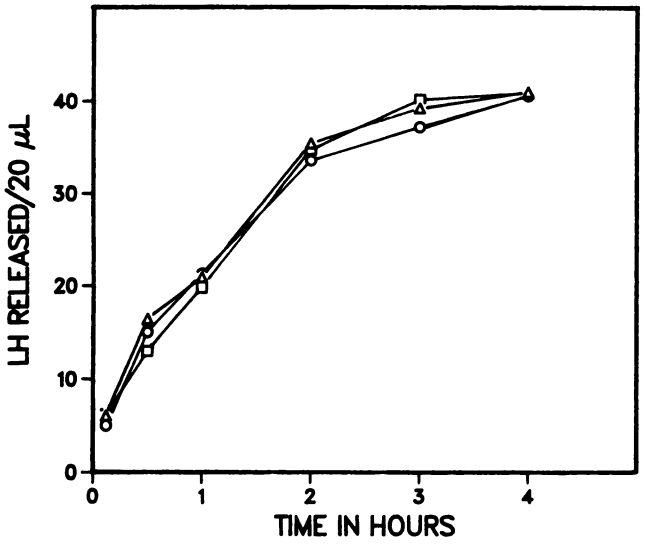

FIG. 2. *Time course for LH release in response to GnRH and protein kinase C activators*

Two-day cultures were incubated for the indicated time in the presence of 1 nM GnRH (○), 75 μM diC_8 (△), or 3.24 nM (2 ng/ml) PMA (□). LH release was measured by RIA. Standard error (not shown) was <10% ($n = 6$).

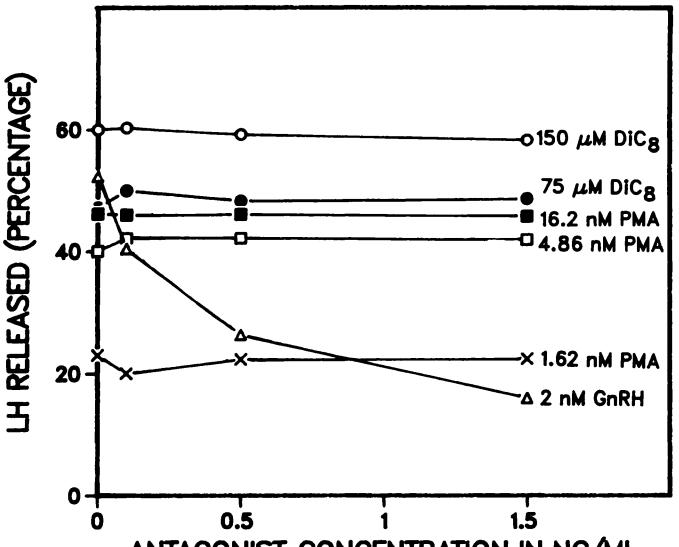

FIG. 3. *Effect of the GnRH antagonist Ac[D-pCl-Phe^{1,2}-D-Trp^3-D-Lys^6-D-Ala^{10}]GnRH on LH release in response to the indicated concentrations of GnRH, PMA, or diC_8*

Two-day cultures were incubated in the presence of the secretogogues and the indicated concentration of the antagonist. After 3 hr, released LH was determined by RIA. Values and standard error are expressed as described for Fig. 1 ($n = 6$).

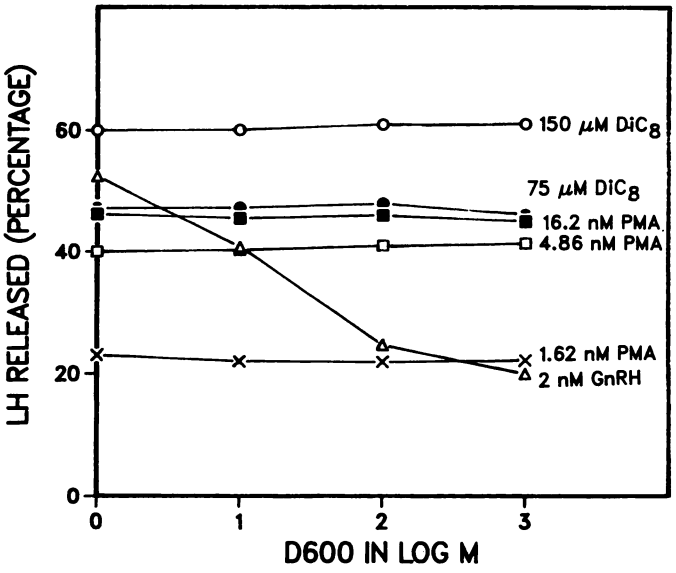

FIG. 4. *Effect of the calcium ion channel inhibitor D600 on LH release in response to the indicated concentrations of GnRH, PMA, or diC_8*

Two-day cultures were incubated in the presence of the secretogogues and the indicated concentration of the antagonist. After 3 hr, released LH was determined by RIA. Values and standard error are expressed as described for Fig. 1 ($n = 6$).

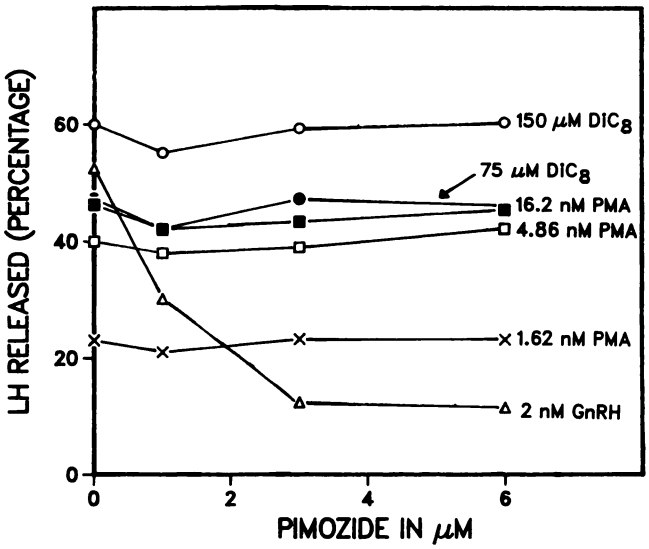

FIG. 5. *Effect of the calmodulin inhibitor pimozide on LH release in response to the indicated concentrations of GnRH, PMA, or diC_8*

Two-day cultures were incubated in the presence of the secretogogues and the indicated concentration of the antagonist. After 3 hr, released LH was determined by RIA. Values and standard error are expressed as described for Fig. 1 ($n = 6$).

In order to determine whether LH release in response to PKC activators requires either activation of calcium ion channels or calmodulin, agents which specifically block these actions were used. The calcium ion channel blocker D600 was an effective inhibitor of GnRH-stimulated LH release at the lowest concentration used (10^{-6} M, Fig. 4). Inhibition was dose dependent and was greater than 70% for 10^{-4} M D600. There was no significant change in the amount of LH released in the presence of D600 when cells were stimulated with either diC_8 (75 or 150 μM) or PMA (1.62, 4.86, or 16.2 nM (1, 3, or 10 ng/ml)].

The effect of the calmodulin inhibitor pimozide on cultured pituitary cells stimulated with GnRH (2 nM), diC_8 (75 or 150 μM), or PMA [1.62, 4.86, or 16 nM (1, 3, or 10 ng/ml)] is shown in Fig. 5. Significant inhibition of LH release was observed when the cells were stimulated with GnRH, with the IC_{50} for pimozide being ap-

proximately 1 μM. Such marked inhibition was not observed with LH release in response to diC_8 or PMA.

It appeared from the data above that LH release in response to PKC activators was not mediated by the GnRH receptor and did not require activation of either the receptor-regulated calcium ion channel or calmodulin. In order to study the relationship between activation of LH release by calcium and by PKC activators, the ability of these secretogogues to synergize in their actions was examined. Figs. 6 and 7 show the results obtained with PMA and diC_8, respectively. A dose-dependent stimulation of LH release with the Ca^{2+} ionophore A23187 was observed.

Concentrations of PMA and diC_8 which alone are minimally active in stimulating LH release show substantially more than an additive effect when added with the ionophore. Accordingly, activators of PKC show synergistic activity with the calcium ionophore A23187.

DISCUSSION

The present work provides evidence that activators of PKC (PMA, diC_8) (8, 12) stimulate LH release by a mechanism which is not inhibited by antagonists of GnRH (Ac[D-pCl-$Phe^{1,2}$-D-Trp^3-D-Lys^6-D-Ala^{10}]GnRH), Ca^{2+} ion channels (D600; Refs. 9–13), or calmodulin (pimozide; Ref. 4) at concentrations which inhibit LH release in response to the releasing hormone. While no action of the PKC activators at the receptor was expected based on structural dissimilarity to GnRH, the GnRH receptor antagonist study was undertaken since some molecules which appear dissimilar to GnRH activate the receptor by a poorly understood mechanism which can be inhibited by GnRH antagonists (14). D600 and pimozide were also not inhibitory to the actions of the PKC activators. A recent preliminary report (15) indicated that pimozide doubled the amount of LH released in response to PMA. It was necessary, however, to use a concentration nearly 20-fold higher than that which was examined in the present study and which was previously shown (4) to inhibit the action of GnRH by inhibition of calmodulin (16). Accordingly, the action of pimozide at higher concentrations may reflect effects on sites other than calmodulin or a general toxicity of the drug at high concentrations as previously noted (4).

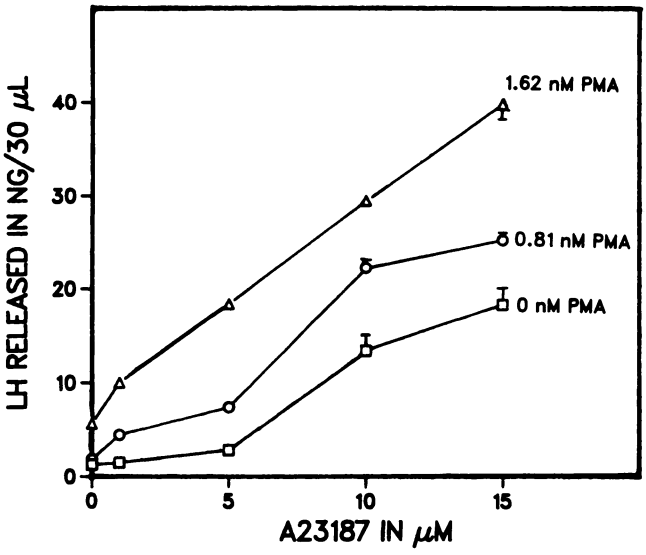

FIG. 6. *Synergistic actions of protein kinase C activator PMA with calcium ionophore A23187 on LH release*

Two-day cultures were incubated in the presence of A23187 with the PMA added at the indicated concentration. After 3 hr, released LH was determined by RIA. Values are expressed as described for Fig. 1 (n = 6). When the standard error is not shown, it is smaller than the symbol.

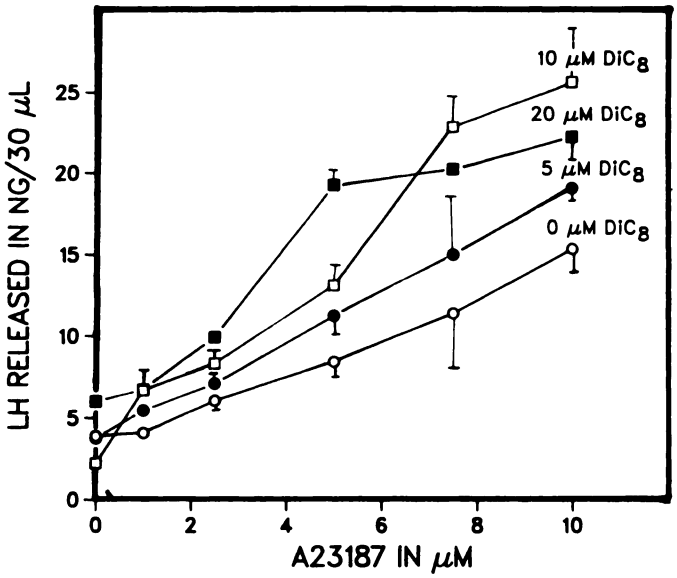

FIG. 7. *Synergistic actions of protein kinase C activator diC_8 with calcium ionophore A23187 on LH release*

Two-day cultures were incubated in the presence of A23187 with the diC_8 added at the indicated concentration. After 3 hr, released LH was determined by RIA. Values are expressed as described for Fig. 1 (n = 6). When the standard error is not shown, it is smaller than the symbol.

Despite the 3-hr incubations used in the present study, desensitization is not likely to be involved since gonadotrope cells take more than 6 hr to become markedly refractory to PMA (17) and, even at this time, remain fully responsive to stimuli which depend on Ca^{2+} mobilization to stimulate LH release (17). A similar lack of cross-desensitization has been noted in short pulse desensitization studies (18). In addition, the 3-hr data presented in the present study could be qualitatively reproduced at a 1-hr time (data not shown).

Previous studies have shown an absolute requirement for extracellular Ca^{2+} for GnRH stimulation or maintenance of LH release (2, 19). Indeed, the more recent study shows that stimulated LH release has a minute-to-minute dependence on extracellular Ca^{2+} and access to that pool. More recently (20), it has been possible to show, using the fluorescent probe Quin 2, that Ca^{2+} flux following gonadotrope receptor activation is extremely rapid. These studies, taken with the observations that A23187, but not PKC activators, can stimulate LH release with the same efficacy as GnRH support a primary role for Ca^{2+} in the mechanism of GnRH action. This ion fulfills the requirements of a second messenger for GnRH (7).

The present study also demonstrates that PKC activators synergize with the actions of the Ca^{2+} ionophore,

A23187. In numerous systems, PKC appears to be regulated by both Ca^{2+} and DAGs (21), although PKC activators do not appear to be able to cause Ca^{2+} mobilization (22). A recent study (23) also implicated DAGs as stimulators of prolactin release from a clonal line. It is notable that the concentrations of various DAGs needed to stimulate LH release compares to that required to maximally stimulate PKC. Thus, the lack of dependence on Ca^{2+} shown in the present work and suggested previously (8) may reflect the ability of these high levels of these lipids to synergize with small yet measurable (20, 22) concentrations of intracellular Ca^{2+} in the gonadotrope which has not been exposed to GnRH. Moreover, such synergy could allow low levels of Ca^{2+} to be functionally significant; this is especially important since the measured concentration of Ca^{2+} in GnRH-stimulated gonadotropes is lower (20) than would have been predicted from the relatively high concentration of ionophore A23187 (1) needed to provoke release. The recent observation (24) that increases in intracellular Ca^{2+} at levels too low to provoke LH release effectively up-regulates the GnRH receptor suggests the biological efficacy of such small increases.

In order to demonstrate a role for PKC in GnRH action, it will be necessary to show that the releasing hormone activates the enzyme, that specific inhibition of the enzyme blocks GnRH action, and that enzymatic activation by any means leads to GnRH-like actions. While only the third of these criteria has been met (in two GnRH-dependent systems; Refs. 8 and 25), the present data indicate a potential role for PKC since activators of the enzyme show synergism with drugs that elevate cellular Ca^{2+}. Accordingly, the possibility is presented that such lipids may serve an amplification role of the GnRH receptor-mediated Ca^{2+} signal.

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