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The phorbol ester-induced extracellular Ca^{2+}-independent release of LH is dependent on estradiol and de novo protein synthesis

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Phorbol 12-myristate 13-acetate (PMA) was used to determine whether the PMA-induced extracellular Ca^{2+}-independent release of LH was dependent on sex, estradiol and de novo protein synthesis. Infusions of gonadotropin-releasing hormone (GnRH) or PMA in a perifusion system stimulated a partial secretion of LH from diestrous II and ovariectomized + estradiol-treated female pituitaries (responses inhibited by cycloheximide). In contrast, PMA was ineffective in stimulating PRL secretion from these pituitaries, as well as LH secretion from male or ovariectomized female pituitaries. These results indicate that the PMA-stimulated extracellular Ca^{2+}-independent secretion of LH is a specific process which is dependent on sex, estradiol and de novo protein synthesis, and mimics the characteristics of the GnRH-stimulated responses.

Cycloheximide; Diestrus II; Estradiol; Phorbol 12-myristate 13-acetate; Protein kinase C; (Ovariectomized pituitary)

1. INTRODUCTION

Previous reports have demonstrated the manifestation of a gonadotropin-releasing hormone (GnRH)-stimulated extracellular Ca^{2+}-independent component of LH secretion [1,2]. Subsequent studies indicated an indirect role for cAMP [3], suggesting that the nucleotide interacts with some other intracellular mediator(s) which directly regulates this component of LH secretion. Interestingly, diacylglycerols (DAGs) and phorbol esters which can activate protein kinase C (PKC) without increases of intracellular Ca^{2+} [4,5], stimulated LH secretion in the absence of extracellular Ca^{2+} [6,7]. These observations suggest PKC as a possible direct mediator of the GnRH-stimulated extracellular Ca^{2+}-independent release of LH. Additionally, the GnRH-stimulated extracellular Ca^{2+}-independent secretion of LH was shown to be dependent on sex, estradiol and de novo protein synthesis [2,3]. Consequently, as a first approach to determining whether PKC directly mediates this component of LH secretion, initial studies were undertaken to determine whether the ability of the phorbol ester, phorbol 12-myristate 13-acetate (PMA) to induce LH secretion in the absence of extracellular Ca^{2+} mimics the characteristics of the GnRH response.

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2. MATERIALS AND METHODS

2.1. *Materials*

Cycloheximide, EGTA, 17β-estradiol and PMA were purchased from Sigma (St. Louis, MO). GnRH was obtained from Peninsula Labs (Belmont, CA).

2.2. *Perifusion system*

Adult males, ovariectomized (72 h) ± estradiol (OVX ± E_2) capsule implanted (24 h) females (cf. [2]), and regularly cycling diestrous II rats (determined by daily vaginal cytology) were used as pituitary donors in a previously described perifusion system [1]. Briefly, two quartered pituitaries from donor animals were placed in a Millipore Swinnex-13 filter holder (perifusion chamber), and superfused with Ca^{2+}-free Krebs Improved Ringer I medium (37°C, equilibrated with 95% O_2/5% CO_2) at 0.25 ml/min. Sequential effluent samples were collected every 10 min after discarding the perifusate from the first hour. The Ca^{2+}-free medium was prepared by omitting $CaCl_2$, and adding EGTA to chelate contaminating amounts of the

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cation as described [1]. At the end of an experiment, the samples were frozen (−20°C) until assayed for LH and PRL, using the NIADDK rat RIA kits for LH and PRL.

3. RESULTS

As shown in fig.1, PMA stimulated LH secretion from both diestrous II and OVX + E_2-treated female pituitaries perifused with Ca^{2+}-free medium. However, the PMA-induced secretion from OVX + E_2-treated pituitaries became apparent 30 min after the administration of PMA, while the secretion from diestrous II pituitaries was manifest only after 60 min. These temporal characteristics of the PMA-stimulated secretory profiles were similar to those obtained in response to GnRH, although the amounts of LH secreted in response to GnRH were greater (fig.1). Furthermore, the PMA-induced extracellular Ca^{2+}-independent component of LH secretion was inhibited by cycloheximide (fig.2), suggesting a dependency on de novo protein synthesis.

Although PMA stimulates PRL secretion from female pituitary cells perifused with normal medium [8], the phorbol ester was ineffective in stimulating PRL secretion from diestrous II or OVX + E_2-treated female pituitaries in Ca^{2+}-free medium (fig.3). These results suggest that the efficacy of PMA to act as a secretagogue in diestrous II and OVX + E_2-treated pituitaries perifused with Ca^{2+}-free medium is not a generalized phenomenon, i.e. it is not just a manifestation of the ability of PMA to activate PKC in the absence of increases of intracellular Ca^{2+}.

In contrast to the results obtained from diestrous II and OVX + E_2-treated female pituitaries, PMA, like GnRH (cf. [2]) was totally ineffective in stimulating LH secretion from male or OVX female pituitaries (fig.4).

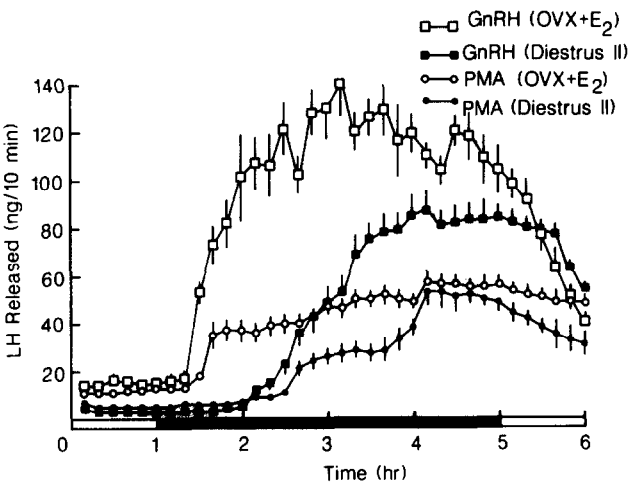

Fig.1. Secretory profiles of LH obtained from diestrous II and OVX + E_2-treated pituitaries in response to continuous infusions (4 h) of 1 nM GnRH or 1 μM PMA in Ca^{2+}-free medium. The periods of infusion of GnRH and PMA are indicated by the black bar. The results are the mean ± SE (n = 3 chambers). Absence of standard error bars is indicative of the fact that the standard errors are smaller than the size of the symbols. The silastic implants maintain estradiol serum concentrations at proestrous levels (cf. [2]).

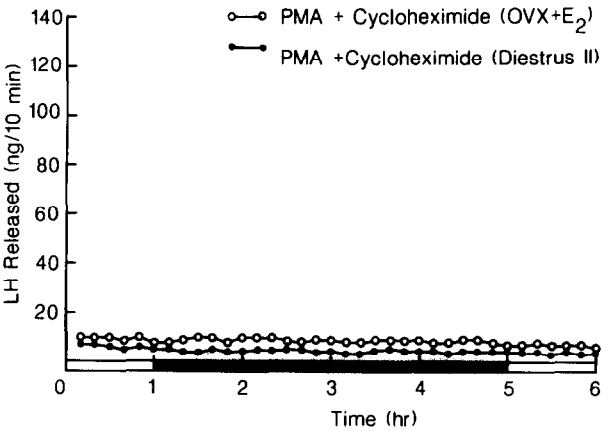

Fig.2. Secretory profiles of LH obtained from diestrous II and OVX + E_2-treated pituitaries in response to 1 μM PMA (indicated by the black bar) in Ca^{2+}-free medium containing 5 μM cycloheximide. Cycloheximide was present in the medium from the beginning of the experiment. For further details see legend for fig.1.

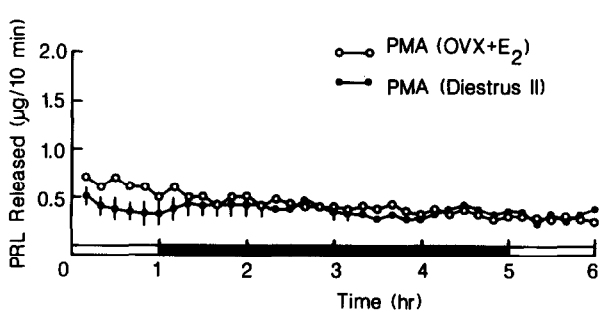

Fig.3. Secretory profiles of PRL obtained from diestrous II and OVX + E_2-treated pituitaries in response to continuous infusions of 1 μM PMA (indicated by the black bar) in Ca^{2+}-free medium. For further details see legend for fig.1.

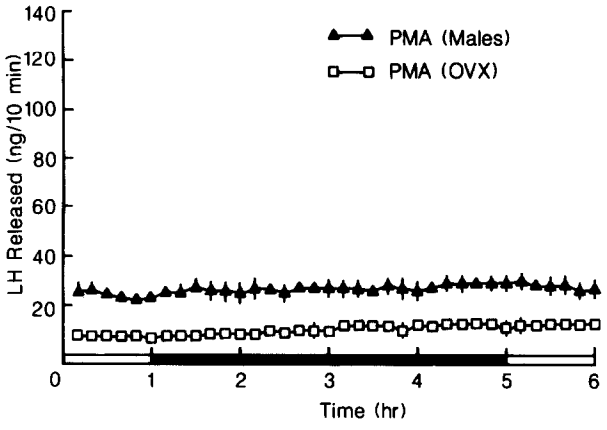

Fig.4. Responses of male or OVX female pituitary tissues to continuous infusions of 1 μM PMA (indicated by the black bar) in the absence of extracellular Ca^{2+}. For further details see legend for fig.1.

4. DISCUSSION

PMA was used instead of the endogenous activators (DAGs) of PKC because exogenous DAGs were shown to stimulate the production of inositol phosphates (IP) in the anterior pituitary [9], raising the possibility of increased IP_3 formation, and its mobilization of Ca^{2+} from intracellular stores [10]. This in turn would have complicated data interpretation. Fortunately, PMA does not suffer from this potential disadvantage since it does not directly increase IP production [9], or affect phospholipid turnover [11].

The present results extend earlier reports on the ability of activators of protein kinase C to stimulate LH secretion in the absence of extracellular Ca^{2+} [6,7], by demonstrating that the efficacy of PMA to stimulate LH secretion in Ca^{2+}-free medium is not a generalized phenomenon, but appears to be a very specific process which in gonadotrophs depends on sex, estradiol and de novo protein synthesis.

In addition, for the first time this study clearly demonstrates that the characteristics of the responses induced by PMA mimic those of the responses stimulated by GnRH. In the first instance, both secretagogues were effective stimulators of LH secretion from pituitaries obtained from diestrous II and OVX + E_2-treated females, but not from male or OVX female pituitaries. Secondly, the onset of the PMA-induced secretion duplicates that of the GnRH-stimulated responses, reflecting the delayed manifestation of the extracellular Ca^{2+}-independent secretion [1–3], and its dependency on estradiol [2]. Finally, both the GnRH [1,3] and PMA-induced extracellular Ca^{2+}-independent secretion appear to be dependent on de novo protein synthesis. Taken together, these observations suggest that PMA is duplicating the effects of GnRH with respect to the induction of the extracellular Ca^{2+}-independent secretion of gonadotropins, although the magnitudes of the responses differ (cf. fig.1).

Although an earlier report had questioned the essentiality of PKC as a mediator of GnRH responses [12], recent studies have provided evidence for a role of the enzyme in mediating some aspects of GnRH-stimulated LH secretion [13–15], including its activation by GnRH [16]. These observations coupled with the present demonstration that the PMA-induced secretion mimics the GnRH-induced responses is consistent with the possibility that PKC might mediate (at least in part) the GnRH-stimulated extracellular Ca^{2+}-independent secretion of LH. This suggestion is further supported by the fact that GnRH-stimulated activation of phospholipase C (which generates DAGs) in anterior pituitary cells is independent of extracellular Ca^{2+} [9,11,17]. Obviously, this hypothesis has to be tempered by the realization that PMA (at the concentrations used) could be exerting its effects via mechanisms other than PKC activation. As a result, experiments using inhibitors of PKC are currently underway to determine whether the enzyme does in fact mediate this component of LH secretion.

While the present results are consistent with a role for PKC as a mediator of the GnRH-stimulated extracellular Ca^{2+}-independent component of gonadotropin secretion, they do not exclude a role for inositol 1,4,5-triphosphate (IP_3). During the initiation of the GnRH-stimulated extracellular Ca^{2+}-independent secretion of LH, the generation of DAGs by GnRH activation of phospholipase C would result in the simultaneous liberation of IP_3 [11]. Since IP_3 was implicated in the mobilization of Ca^{2+} from intracellular stores [10], it is conceivable that increases of intracellular Ca^{2+} subsequent to the generation of IP_3 might also be involved in mediating some aspect(s) of the

GnRH-stimulated extracellular Ca^{2+}-independent secretion of LH. Thus, the GnRH component may actually represent a composite of secretory mechanisms involving a PKC component (the one presumed to be stimulated by PMA), interacting with a component involving Ca^{2+} mobilization and subsequent activation of the calmodulin system.

As stated in section 1, we had previously reported cAMP as an indirect mediator of the GnRH-stimulated extracellular Ca^{2+}-independent secretion of LH [3]. These observations, coupled with the present results, and the study by Turgeon and Waring [13], strongly suggest interactions between the adenylate cyclase-cAMP system and PKC. The fact that cAMP appears to be obligatory for the manifestation of the GnRH-stimulated extracellular Ca^{2+}-independent secretion of LH [3], suggests that the PMA-induced secretion should also be associated with increased cAMP production if PMA does in fact mimic the GnRH-stimulated secretion. In this regard it was interesting to note that phorbol esters were shown to increase basal cAMP production in anterior pituitaries [18,19]. Obviously, these hypotheses have to be rigorously tested, and preliminary experiments to delineate putative interactions between cAMP, PKC and IP_3 are currently underway.

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