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Involvement of protein phosphatases in gonadotropin releasing hormone regulated gonadotropin secretion

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Abstract

The role of persistent protein phosphorylation upon gonadotropin releasing hormone (GnRH) stimulated luteinizing hormone (LH) release was investigated by the use of the selective inhibitors of protein phosphatase type 1 (PP1) and 2A (PP2A), okadaic acid (OA) and calyculin A. Pre-incubation of cultured rat pituitary cells with OA (24 h) or calyculin A (30 min) resulted in inhibition of GnRH-stimulated LH release with significant inhibition being detected at 10 nM and 30 nM for OA and calyculin A, respectively. The inactive OA analog norokadone and the protein tyrosine phosphatase inhibitor vanadyl hydroperoxide had no significant effect on GnRH-induced LH release. The stimulatory effects of the protein kinase C (PKC) activator 12-*O*-tetradecanoylphorbol 13-acetate (TPA, 50 ng/ml) or the Ca^{2+} ionophore, ionomycin (1 μm), upon LH release were also abolished by pretreatment with OA (10–20 nM) or calyculin A (30 nM). Stimulation of LH release by high K^+ (28 mM) or residual LH release stimulated by GnRH in Ca^{2+}-free medium were also blocked by OA. These observations indicate that protein dephosphorylation is involved positively in GnRH-stimulated LH release. The site of action of the protein phosphatases PPI and PP2A is most likely downstream to Ca^{2+} elevation and PKC activation by GnRH.

Keywords: Okadaic acid; Calyculin A; Gonadotropin releasing hormone; Pituitary; Gonadotropin; Exocytosis; Protein phosphatases

1. Introduction

Gonadotropin releasing hormone (GnRH) regulates the synthesis and release of the pituitary gonadotropin luteinizing hormone (LH) and follicle stimulating hormone (FSH). Following its binding GnRH stimulates phosphoinositide turnover resulting in Ca^{2+} mobilization and influx and activation of protein kinase C (PKC) (Naor, 1990; Naor et al., 1993). Indeed Ca^{2+} and PKC have been implicated in GnRH-stimulated gonadotropin release (Naor, 1990; Naor et al., 1993, for reviews). Nevertheless, the role of protein phosphorylation/dephosphorylation in GnRH action is not known.

Protein phosphatases are classified as serine/threonine or tyrosine phosphatases according to substrate selectivity. There are four major classes of serine/threonine phosphatases (PP-1, PP-2A, PP-2B and PP-2C) with different isozymic forms and two major types of tyrosine phosphatases, either receptor-like or non-receptor-like (Cohen, 1989; Fischer et al., 1991). Elucidation of the role of protein serine/threonine phosphatases in the regulation of cellular systems has been made possible by the introduction of the selective protein phosphatase inhibitors okadaic acid (OA) and calyculin A, which are two structurally different, non-phorbol tumor promoters isolated from marine sponges (Tachibana et al., 1981; Kato et al., 1986; Ishihara et al., 1989; Cohen et al., 1990).

Okadaic acid (OA), a polyether derivative of a fatty acid, was first isolated from the black sponges *Halichondria okadai*, is a selective inhibitor of protein phosphatase-1 (PP1) and protein phosphatase-2A (PP2A) (Tachibana et al., 1981; Cohen et al., 1990). Calcyculin-A was isolated from the sponge *Discoderma colyx*, and like OA is a selective inhibitor of PP1 and PP2A (Kato et al., 1986; Ishihara et al., 1989). We report here the surprising inhibitory effect of OA and calyculin A on GnRH-induced LH release in cultured rat pituitary cells.

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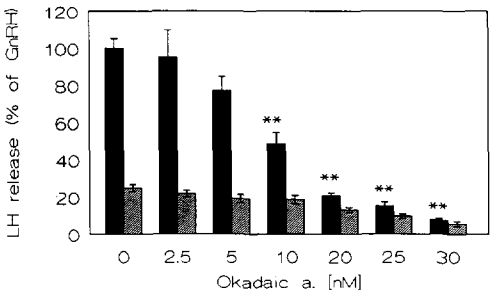

Fig. 1. Effect of okadaic acid on GnRH-induced LH release. Cultured rat pituitary cells (3×10^5/well) were incubated with increasing concentrations of okadaic acid for 24 h. Cells were then washed three times and further incubated with (black bars) or without (dashed bars) GnRH (10 nM) for 4 h in the absence of the drug and medium was collected for determination of LH by RIA. Results are mean ± SEM of samples from three experiments ($n = 9$) expressed as percent of the GnRH response. $^{**}P < 0.01$ GnRH + drug versus GnRH (Student's t-test).

2. Materials and methods

2.1. Materials

Trypsin, soybean trypsin inhibitor, DNase and TPA were from Sigma. Okadaic acid, norokadone and calyculin A were from Biomol (PA, USA). GnRH was purchased from Peninsula Labs. (San Carlos, CA, USA); ionomycin was purchased from Calbiochem (Los Angeles, CA, USA). All cell culture media, sera and antibiotics were purchased from Beit-Ha'Emek, Israel. RIA reagents for rat LH were kindly provided through the National Hormone and Pituitary Program, the NIDDK, NIH, Bethesda, MD and the US Department of Agriculture.

2.2. Methods

Pituitary glands from Wistar derived female rats (28 days old) were used for cell preparation as previously described (Limor et al., 1987). Pituitary glands were finely minced and incubated consecutively at 37°C in 5-ml volumes of medium 199 with 0.3% BSA, containing 5 mg/ml, trypsin, for 15 min, then 2 μg/ml DNase for 5 min, followed by 1 mg/ml soybean trypsin inhibitor for 5 min. Two additional incubations were then performed in Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate buffer with 0.3% BSA, containing, respectively, 2 mM EDTA (5 min) and 1 mM EDTA (15 min). The tissue fragments were then transferred to a 17 × 100-mm polycarbonate tube, washed in Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate buffer, and dispersed into single cells by gentle aspiration and extrusion through a plastic Pasteur pipette in 5 ml of the same buffer. The cell suspension was filtered through nylon gauze in medium 199 with 10% horse serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) for culture. After 3 days of culture, pituitary cells (3×10^5 cells/well) were washed three times in medium 199 containing BSA (0.1%) and incubated in the same medium with the inhibitors and stimulants as indicated in the figure legends. Vanadyl hydroperoxide [$V^{(4+)}$–OOH] was prepared by mixing equimolar concentrations of sodium orthovanadate and H_2O_2 (10 mM) followed by incubation for 15 min at 22°C and removal of residual H_2O_2 by catalase (200 μg/ml). Aliquots of vanadyl hydroperoxide were used after addition of catalase (Pumiglia et al., 1992). Upon termination of each incubation the medium was removed and stored at –20°C for LH-RIA as previously described (Limor et al., 1987).

3. Results

3.1. Effect of okadaic acid on GnRH action

Incubation of cultured rat pituitary cells with increasing concentrations of the protein phosphatase inhibitor okadaic acid (OA) inhibited GnRH-stimulated LH release; significant inhibition was detected at 10 nM or higher concentrations of OA (Fig. 1). Inhibition of basal LH release was noticed at concentrations above 20 nM of the drug. OA was as effective in inhibiting LH release induced by higher doses of GnRH (up to 100 nM) as in inhibiting LH release induced by submaximal doses of GnRH (100 pM, not shown). The specificity of the inhibitory effect of OA was confirmed by the use of the non-active analog norokadone, which had no effect on basal or GnRH-stimulated LH release (results not shown).

3.2. Effect of calyculin A on GnRH action

GnRH-stimulated LH release was inhibited by another protein serine/threonine phosphatase inhibitor, calyculin A, but higher concentrations were required compared to OA (30 nM versus 10 nM, respectively, Fig. 2). On the other hand, while OA required a long pre-incubation time (24 h) to exert its inhibitory effect, calyculin A was inhibitory after 30 min of pre-incubation. The LH release over the effective dose range of GnRH was markedly inhibited by calyculin A (30 nM, Fig. 3). The protein tyrosine phosphatase inhibitor vanadyl hydroperoxide (500 μM) (Pumiglia et al., 1992) had no significant effect

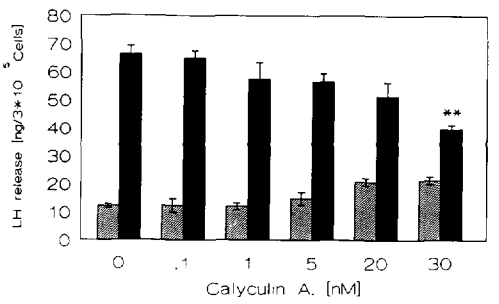

Fig. 2. Effect of calyculin A on GnRH-induced LH release. Cultured pituitary cells were incubated with increasing concentrations of calyculin A for 30 min. Cells were then further incubated with (black bars) or without (dashed bars) GnRH (10 nM) for 4 h. Medium was collected for LH determination by RIA. Results are mean ± SEM of triplicate dishes from a representative experiment. Similar results were observed in two other experiments. $^{**}P < 0.01$.

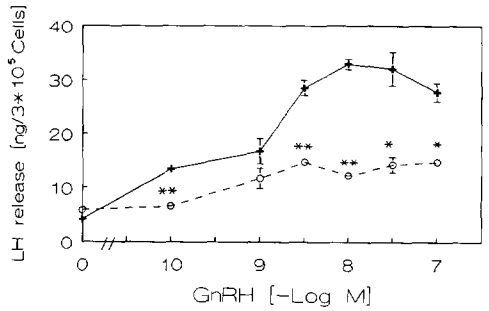

Fig. 3. Effect of calyculin A on GnRH-induced LH release. Cultured pituitary cells were incubated with (broken line) or without (full line) calyculin A (30 nM) for 30 min. Cells were then further incubated with increasing doses of GnRH for 4 h. Medium was collected for LH determination by RIA. Results are mean ± SEM from triplicate dishes of a representative experiment. Similar results were obtained in two other experiments. $*P < 0.05$; $**P < 0.01$.

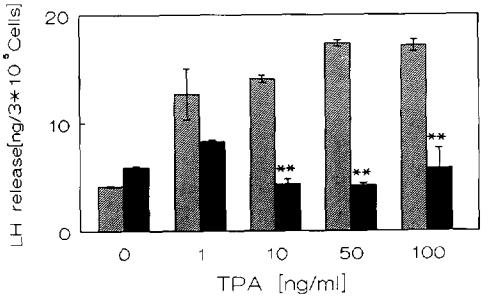

Fig. 5. Effect of calyculin A on TPA-induced LH release. Cultured pituitary cells were pre-incubated with calyculin A (30 nM) for 30 min. Increasing doses of TPA were then added for 4 h in the presence (black bars) or absence (dashed bars) of the drug. The medium was collected for LH determination by RIA and results are mean ± SEM of triplicate dishes from a representative experiment. Similar results were obtained in two other experiments. $**P < 0.01$.

on the response of pituitary cells to increasing doses of GnRH (results not shown).

3.3. Site of action for the protein phosphatase inhibitors

To follow the potential site of OA and calyculin A inhibition of GnRH action, we tested the effect of the drugs on TPA stimulation of LH release (Fig. 4). Incubation of cultured pituitary cells with TPA (50 ng/ml) resulted in enhanced release of LH which was inhibited by OA; significant inhibition was detected at 2.5 nM, while complete inhibition was found at 10 nM of OA (Fig. 4). Similarly, stimulation of LH release by TPA was blocked by 30 nM of calyculin A (Fig. 5). To further follow the site of action of OA, we stimulated the cells with the Ca^{2+} ionophore ionomycin in the presence of OA which blocked stimulation of LH release at 5 nM (Fig. 6). In another approach, LH release was induced by high K^+ (28 mM) and the effect was inhibited by OA with complete inhibition of the response and return to basal levels of LH release at 10 nM of OA (Fig. 7). Calyculin A (30 nM) blocked stimulation of LH release by ionomycin (1 μM) or TPA (50 ng/ml) and markedly inhibited the additive response induced by ionomycin + TPA (Fig. 8). Similar magnitude of inhibition of the additive response was exerted by 10 nM of OA (not shown). To distinguish between Ca^{2+} mobilization and influx (Limor et al., 1987), we incubated the cells with GnRH in Ca^{2+}-free medium (Fig. 9). A fraction of the exocytotic response of LH remained intact and was abolished by OA (20 nM).

4. Discussion

Following the binding to specific receptors, GnRH activates phosphoinositide turnover resulting in Ca^{2+} elevation and PKC activation which participate in gonadotropin secretion (Naor, 1990; Naor et al., 1993, for reviews). Protein phosphorylation is the most common post-translational modification of cellular proteins and plays an important role in all known pathways of signal transduction including exocytosis. Net phosphorylation is the balance between the activities of protein kinases and phosphatases. While protein kinases were widely explored, little is known about the role of protein phos-

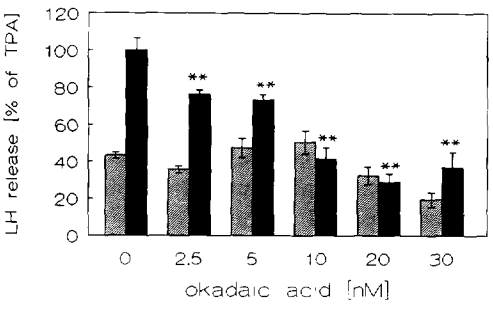

Fig. 4. Effect of okadaic acid on TPA-induced LH release. Cultured pituitary cells were pre-incubated with increasing doses of okadaic acid for 24 h. Cells were then washed and further incubated with (black bars) or without (dashed bars) TPA (50 ng/ml) for 4 h. The medium was collected for LH determination by RIA. Results are mean ± SEM of samples from three experiments ($n = 9$) expressed as percent of the TPA response. $**P < 0.01$ versus TPA alone.

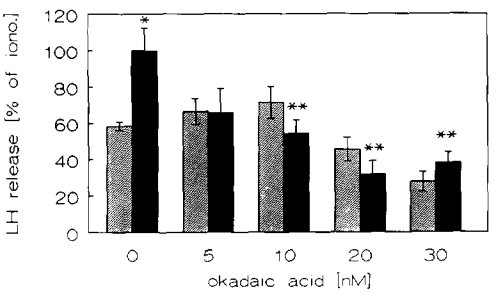

Fig. 6. Effect of okadaic acid on ionomycin-induced LH release. Cultured pituitary cells were incubated with increasing doses of okadaic acid for 24 h. Cells were then washed and incubated with (black bars) or without (dashed bars) ionomycin (1 μM) for 4 h. The medium was collected for LH determination by RIA. Results are mean ± SEM of samples from three experiments ($n = 9$) expressed as percent of the ionomycin response. $*P < 0.05$; $**P < 0.01$ versus ionomycin alone.

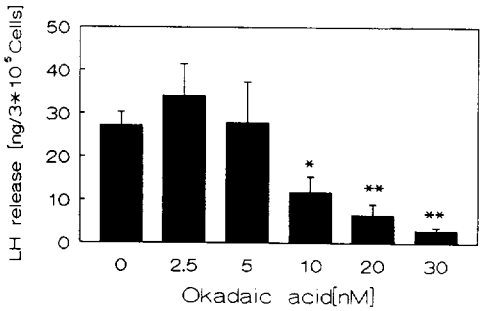

Fig. 7. Effect of okadaic acid on K^+-induced LH release. Cells were pre-incubated with increasing doses of okadaic acid for 24 h. Cells were then washed and transferred to high-K^+ (28 mM)in EBSS buffer containing 2 mM $CaCl_2$ for 4 h. The medium was collected for LH determination by RIA and results are mean ± SEM from triplicate dishes of a representative experiment. Basal release in normal EBSS was 12 ± ng/ml. Similar results were obtained in two other experiments. $*P < 0.05$; $**P < 0.01$.

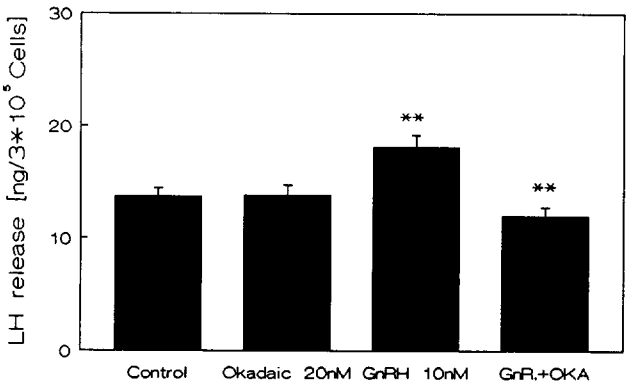

Fig. 9. Effect of okadaic acid on GnRH-induced LH release in Ca^{2+}-free medium. Cells were pre-incubated with okadaic acid (20 nM) for 24 h. Cells were washed and transferred to Ca^{2+} free DMEM containing 250 μM EGTA and GnRH (10 nM) was added for 4 h where indicated. LH was determined in the medium and results are mean ± SEM for three experiments ($n = 12$). $**P < 0.01$.

phatases. Hence, there is an increasing interest in finding the role of protein phosphatases in cell signaling (Cohen, 1989; Fischer et al., 1991, for reviews). Since Ca^{2+} and PKC are implicated in GnRH-induced gonadotropin secretion (Naor and Catt, 1981; Smith and Vale, 1981; Negro-Villar and Lapetina, 1985; Turgeon and Waring, 1986; Davidson et al., 1987; Limor et al., 1987; Strulovici et al., 1987; Johnson et al., 1988; Dan-Cohen and Naor, 1990; Naor, 1990; Chang et al., 1991; Stojilkovic et al., 1991; Naor et al., 1993), it was anticipated that protein phosphorylation is positively correlated with the exocytotic response of GnRH-induced LH release. Indeed TPA and cell permeant diacylglycerol analogs elicit LH release (Naor and Catt, 1981; Smith and Vale, 1981; Negro-Villar and Lapetina, 1985; Turgeon and Waring, 1986; Davidson et al., 1987; Johnson et al., 1988; Chang et al., 1991) and the PKC inhibitor staurosporine inhibited

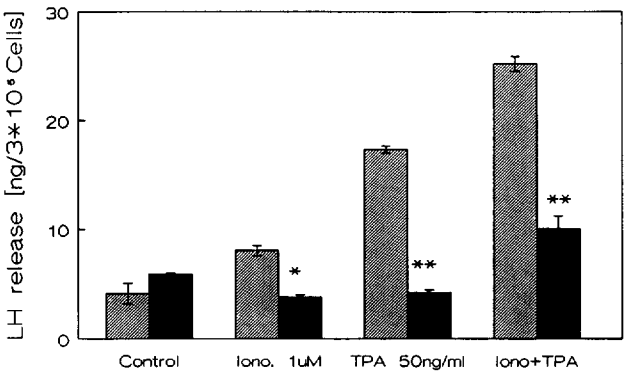

Fig. 8. Effect of calyculin A on ionomycin and TPA-induced LH release. Cells were pre-incubated with (black bars) or without (dashed bars) calyculin A (30 nM) for 30 min and further incubated with ionomycin (1 μM) or TPA (50 ng/ml) or both for 4 h. The medium was collected for LH determination by RIA. Results are mean ± SEM ($n = 4$). Similar results were obtained in two other experiments. $*P < 0.05$; $**P < 0.01$.

GnRH-stimulated LH release (Dan-Cohen and Naor, 1990; Stojilkovic et al., 1991). Down-regulation of endogenous PKC by prolonged treatment with TPA has yielded controversial results (McArdle et al., 1986; Dan-Cohen and Naor, 1990; Stojilkovic et al., 1991). While some investigators found inhibition of GnRH-induced LH release in down-regulated cells (Stojilkovic et al., 1991; Dan-Cohen and Naor, 1990) others found no effect (McArdle et al., 1986).

Therefore the results presented here that the protein phosphatase inhibitors OA and calyculin A inhibit GnRH, TPA and ionomycin-induced LH release are surprising since both drugs should prolong the phosphorylation state of the kinase substrates. We therefore suggest that GnRH-induced LH release is dependent upon a continual cycle of phosphorylation/dephosphorylation of key substrates involved in the process of exocytosis.

Relatively little is known about the phosphoproteins induced by GnRH in cultured pituitary cells. Strulovici et al., 1987 found that GnRH enhanced the phosphorylation of proteins of mol. wt. 42, 34, 11 and 10 kDa and the dephosphorylation of a 15-kDa protein. It is therefore possible that the 15-kDa phosphoprotein is one of the targets of OA and calcyculin A observed here.

Addition of PP-2A or the Ca^{2+}-calmodulin stimulated protein phosphatase, calcineurin, to permeabilized PC12 cells resulted in partial inhibition of Ca^{2+}-dependent norepinephrine secretion (Wagner and Vu, 1990). The results differ in principle from those reported here. On the other hand the Ca^{2+}-dependent dephosphorylation of a 65 kDa protein in the ciliated protozoan *Paramecium tetraurelia* appears to trigger the exocytosis of trichocyst matrices (Gilligan and Satir, 1982). Also OA has been reported to inhibit carbachol-evoked secretion of catecholamines in cultured bovine adrenal medullary cells (Yanagihara et al., 1991). Thus phosphorylation/dephos-

phorylation cycles might play different roles in various cellular systems.

It is possible that activation of PKC by GnRH (Hirota et al., 1985; Naor et al., 1985) indirectly mediates the dephosphorylation event involved in gonadotropin secretion. Such a scheme of events has recently been proposed for the dephosphorylation of c-Jun by PKC (Goode et al., 1992). C-Jun is maintained in its phosphorylated and inactive state by glycogen synthase kinase-3β (GSK-3ß). Phosphorylation of GSK-3β by specific isozymes of PKC inactivates the enzyme, resulting in dephosphorylation and activation of c-Jun (Goode et al., 1992). Our data presented here that OA and calyculin A inhibit TPA-induced LH release support our proposal that PKC might be involved in the dephosphorylation step.

Concerning the site of action of the phosphatase inhibitors on exocytosis, it has been suggested that suppression of Ca^{2+} influx was the site of OA action in carbachol-stimulated adrenal medullary cells (Yanagihara et al., 1991). Indeed in our study OA inhibits high K^+ evoked secretion of LH in support of Ca^{2+} influx as a potential site for the drug action. However, several other lines of evidence presented here support a downstream site of action for OA and calyculin A.

The stimulatory action of the PKC activator TPA was blocked by OA and calyculin A. Also residual LH release elicited by GnRH in the absence of extracellular Ca^{2+} was blocked by OA and the additive response of TPA and ionomycin was inhibited by OA and calyculin A. The collective data support a downstream site of action for the phosphatase inhibitors after Ca^{2+} elevation and PKC activation. Such a site might be the organization of the cytoskeleton during exocytosis. Disassembly of the F-actin network which is required for movement of secretory granules to the plasma membrane might be affected by the phosphorylation state of key proteins. Indeed such a scheme was proposed for the interaction of synapsin I, spectrin (fodrin) and F-actin during neurotransmitter release (Bahler and Greengard, 1987; Benfenati et al., 1992). Thus an analogous system which is sensitive to the phosphorylation/dephosphorylation cycle of cytoskeletal elements might be operating in pituitary cells during GnRH-stimulated gonadotropin secretion.

Acknowledgments

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