**GnRH receptor signaling: cross-talk of Ca\(^{2+}\) and protein kinase C**

Zvi Naor

Department of Biochemistry, Tel Aviv University, Ramat Aviv 69978, Israel

(Correspondence should be addressed to Z Naor)

**Introduction**

Gonadotropin-releasing hormone (GnRH), the first key hormone of the reproductive system, is synthesized in the hypothalamus and stimulates pituitary luteinizing hormone (LH) and follicle-stimulating hormone (FSH) synthesis and release. The neurohormone GnRH binds to a seven transmembrane domain receptor (1-4). The cloned cDNA encodes a 327 amino acid receptor protein which lacks the common cytoplasmic carboxyl terminal domain. The availability of the receptor cDNA will open a new vista for elucidation of receptor structure, ligand-binding sites and development of peptide and nonpeptide agonists and antagonists. The GnRH receptor is expressed exclusively on pituitary gonadotrophs (about 5-10\% of pituitary cells) which consist of 60\% multi-hormonal cells (LH+FSH), 18\% LH- and 22\% FSH-containing cells (5). Occupancy of 20\% of GnRH receptors is sufficient to evoke about 80\% of the biological response (6). GnRH receptor complexes form aggregates and internalization occurs in small vesicles which may transfer GnRH to the lysosome while multivesicular bodies are responsible for receptor recycling (5). GnRH is known to regulate the number of its own receptors (7) and pulsatile administration of GnRH increased GnRH receptor mRNA levels (8).

The extra-pituitary actions of GnRH and its analogs (9) might be mediated by local receptors (e.g. gonadal GnRH binding sites (7)), by desensitization of pituitary receptors followed by decreased serum gonadotropin and gonadal steroids or by both mechanisms. The availability of the receptor cDNA might clarify the above potential sites of action and the physiological relevance of extra-pituitary effects of GnRH and its analogs (see for example 9, 10 and references therein).

**G-proteins and sequential activation of phospholipases**

Following receptor binding, GnRH stimulates a GTP-binding protein (Gq) (11) resulting in enhanced phosphoinositide turnover (5-30 s) (12). Formation of the second messengers inositol-1,4,5-trisphosphate (IP\(_3\)) and early diacylglycerol (DAG) are required for Ca\(^{2+}\) mobilization and protein kinase C (PKC) activation respectively (see 6 for review). After a short lag (~1-2 min), phospholipase A\(_2\) (PLA\(_2\)) and phospholipase D (PLD) are also activated by GnRH in the gonadotroph cell line αT3-1 (13, 14). Activation of PLA\(_2\) and PLD was verified by the production of labeled arachidonic acid (AA, a marker for activated PLA\(_2\)) and labeled phasphatidylethanol and phosphatidic acid (PA) (markers for activated PLD) in prelabeled αT3-1 cells (13, 14). As PA can be converted to DAG by a specific PA-phosphohydrolase, lipid-derived PKC activators are produced by GnRH in a sequential mechanism. Early DAG and Ca\(^{2+}\) (derived via activated phospholipase C (PLC)) might activate Ca\(^{2+}\)-dependent PKC isoforms, while late DAG (derived via PLD) might activate Ca\(^{2+}\)-independent PKC isoforms. On the other hand AA (derived via PLA\(_2\)) is also capable of activating specific PKC isoforms with or without other cofactors (15, 16). Thus, coordinated activation of PKC isoforms might be achieved by sequential activation of PLC, PLA\(_2\) and PLD by GnRH generating lipid-derived second messengers in a well orchestrated, time-dependent fashion.

**Role of calcium in GnRH action**

GnRH stimulates a rapid Ca\(^{2+}\) mobilization from internal IP\(_3\)-sensitive stores followed by external Ca\(^{2+}\) influx via L-type voltage sensitive Ca\(^{2+}\) channels (6, 17 for reviews). In single gonadotrophs GnRH stimulates Ca\(^{2+}\) oscillations and the spiking frequencies are dose-related (17). GnRH also stimulates apamine-sensitive Ca\(^{2+}\)-activated K\(^+\) (SK) channels which give rise to episodic waves of hyperpolarizations which are synchronized with the Ca\(^{2+}\) transients (18). The hyperpolarization waves are also associated with oscillations of action potentials, while external Ca\(^{2+}\) entry might be important for replenishment of the intracellular IP\(_3\)-sensitive Ca\(^{2+}\) pools (17, 18). Unlike neuronal cells which depend on Ca\(^{2+}\) influx, gonadotrophs differ as they resemble exocrine cells which are mainly dependent upon intracellular pools of Ca\(^{2+}\). Indeed, GnRH-induced Ca\(^{2+}\) oscillations were shown to be associated with gonadotropin secretion (19) but the mechanisms responsible for the Ca\(^{2+}\) oscillations are still not clear (17). Nevertheless, we have previously shown that Ca\(^{2+}\)
is necessary but not sufficient to mediate GnRH-induced gonadotropin secretion (20). It seems likely that the rapid first phase of the exocytotic response elicited by GnRH is mediated by IP$_3$-sensitive Ca$^{2+}$ pools (19, 20) while the second “plateau phase” of secretion is mediated by Ca$^{2+}$ mobilization, influx and additional messenger molecules such as protein kinase C (6, 17, 20). More recently, we have shown that Ca$^{2+}$ is also involved in mediating GnRH stimulation of α-subunit mRNA levels in αT3-1 cells (21).

Role of PKC

Protein kinase C plays a key role in signal transduction (see 22–24 for reviews). PKC isoforms are involved in synaptic transmission, memory, learning, cellular growth, differentiation, transformation, metabolism, contraction, exocytosis, regulation of ion channel activity and gene expression. The PKC gene family is classified into conventional PKCs (εPKC: α, βI, βII and γ), novel PKCs (nPKC; δ, ε, η, μ and θ) and atypical PKCs (aPKC: ζ, λ and η) (23). cPKC are tightly coupled to PI turnover and are activated by Ca$^{2+}$, DAG and phosphatidylinerine (PS). nPKCs are Ca$^{2+}$-independent and are activated by DAG and PS while aPKCs are activated by PS and are DAG- and Ca$^{2+}$-independent isoforms (23).

Pituitary cells express PKCa, βII, δ, ε and ζ which represent members of all groups of PKC isoforms (25). Rapid activation of PLC by GnRH (12) might provide Ca$^{2+}$ and early DAG needed for cPKC, while PLD-generated late DAG (14) might be involved in nPKC activation. Arachidonic acid, which is liberated by activated PLA$_2$ (13), will further support selective PKC isoforms activation (15, 16). Thus, sequential activation of various phospholipases by GnRH will provide PKC activators in a time-dependent fashion which might be coordinated with various physiological tasks (e.g. gonadotropin release vs synthesis) elicited by GnRH in a PKC-dependent manner.

We, together with some investigators but not others, found that the criteria for implicating PKC in GnRH-induced gonadotropin secretion and synthesis were fulfilled (see 6 for review). PKC (in particular PKCδ and PKCe) is translocated to the membrane fraction by GnRH; stimulation of LH release by GnRH and TPA is not additive; PKC inhibitors reduced GnRH effect; down-regulation of PKC inhibited GnRH-stimulation of LH release; similar phosphoprotein substrates were described for GnRH and TPA. Nevertheless the role of PKC in GnRH action is still controversial (see 6 for review). More recently we have demonstrated that PKC is involved in GnRH-induced gonadotropin subunits gene expression (21, 26).

Since PKC was implicated in GnRH-induced gonadotropin secretion (6), it was expected that protein phosphorylation would be correlated positively to GnRH-induced gonadotropin release. Thus, the role of persistent protein phosphorylation upon GnRH-induced LH release was studied by the use of selective inhibitors of protein phosphatase type 1 (PP1) and type 2A (PP2A), okadaic acid and calyculin A (27). Both drugs exerted inhibition of GnRH-stimulated LH release with significant inhibition being detected at 10 nM and 30 nM for okadaic acid and calyculin A respectively. Interestingly, stimulation of LH release by the PKC activator TPA or by the Ca$^{2+}$-ionophore, ionomycin, was also abolished by okadaic acid (10-20 nM) or calyculin A (30 nM). The observations indicated that protein dephosphorylation is involved positively in GnRH stimulation of gonadotropin secretion. Moreover, the data suggest that the site of action of PP1 and PP2A during the neurohormone action is downstream to Ca$^{2+}$ and PKC activation (27). Therefore, we conclude that the kinetics and half-life of the phosphoprotein substrates involved in GnRH action play a role in gonadotropin secretion.

We have developed a method of “depletion-insertion” in which PKC-depleted, digitonin-permeabilized pituitary cells are stimulated with purified activated cPKC to reconstitute TPA-induced LH release in normal cells. We found that PKCα and PKCβ, but not PKCγ, mimicked the TPA response (28). Thus PKCα and PKCβ are potential candidates to mediate exocytotic responses. More recent data suggest the involvement of PKCa and PKCe in GnRH action (D Harris, N Reiss & Z Naor, unpublished data).

To follow the fate of PKCα and PKCβ we investigated the effect of GnRH analog upon the mRNA levels of both isotypes. Interestingly, GnRH elevates PKCβ but not PKCα mRNA levels in the αT3-1 gonadotroph-like cell line and the effect is mediated by Ca$^{2+}$ and PKC (29). Thus PKCβ gene expression by GnRH is autoregulated by PKC.

Role of mitogen-activated protein (MAP)-kinase

Signals elicited by receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPCRs) may converge in a set of cytosolic protein kinases, collectively known as the mitogen-activated protein kinase cascade (MAPK) (see 30 for review). RTKs activate MAPK via the adaptor molecule (GRB2) and a guanine nucleotide exchange factor (mSOS), activation of Ras followed by activation of Raf-1, MAPK kinase (MAPKK, MEK) and MAPK. On the other hand, GPCRs activate the MAPK cascade via alternative mechanisms involving PKC or the βγ subunits of G-proteins (30). Since MAPK is translocated to the nucleus, stimulation of MAPK leads to activation of transcription factors initiating cellular responses such as growth and differentiation. It has been shown recently that GnRH stimulates MAPK in pituitary cells and that MAPK is involved
**Figure 1** Proposed cross-talk of Ca\(^{2+}\) and PKC during GnRH-induced gonadotropin release, gonadotropin subunits and PKC\(\beta\) gene expression. Ca\(_{\text{intra}}\) and Ca\(_{\text{extra}}\) are intra- and extracellular pools respectively.

**Figure 2** Proposed model for GnRH receptor activation. G\(_Q\) is activated following the binding of GnRH to its receptor. Thereafter, phospholipase C (PLC-PIP\(_2\)) and phosphatidylcholine specific phospholipase D (PLD-PC) are activated. The activated phospholipases elevate the second messengers DAG and IP\(_3\), which mobilize Ca\(^{2+}\) from internal pools (Ca\(_{\text{intra}}\)) stored in the endoplasmic reticulum (E.R.). The second messengers activate PKC subspecies which are involved in gonadotropin release and biosynthesis and calcium influx. GnRH may cause the stimulation of phospholipase A\(_2\) (PLA\(_2\)) and c-fos expression (not shown) via PKC-dependent activation of MAP kinase (MAPK).
in gene expression of the gonadotropin α-subunit (31–35). We found recently that Ca\(^{2+}\) and PKC mediate the activation of MAPK by GnRH (34, 35). Activation of MAPK by GPCRs provides a sequence of signaling originating at the cell surface and culminating in gene expression in the nucleus. Indeed c-fos is known to be activated by both GnRH and MAPK (17, 30).

**Role of AA**

Activation of phospholipases, in particular PLA\(_2\), results in the release of AA from cellular phospholipids (see 36 for review). The observation that MAPK phosphorylates and activates PLA\(_2\) (30) suggests that release of AA by GnRH is downstream to MAPK activation. GnRH stimulates AA release and formation of several 5-lipoxygenase products of AA such as the leukotrienes LTC\(_4\), LTD\(_4\), LTE\(_4\) as well as 5- and 15-hydroxyeicosatetraenoic acids (HETEs) (37). Moreover, we suggested that AA and some of the lipoxygenase products are involved in GnRH-induced gonadotropin release and gonadotropin subunits gene expression (13, 37). AA and its active lipoxygenase products might act by cross-talk with PKC isoforms (15, 38) or else the leukotrienes might become first messengers in an autocrine/paracrine loop of amplification cycle during GnRH action (37).

**Cross-talk of calcium and PKC**

Examination of the relative roles of Ca\(^{2+}\) and PKC in GnRH action revealed additivity in gonadotropin secretion, suggesting that Ca\(^{2+}\) and PKC act in parallel during exocytosis (26) (Fig. 1). On the other hand, Ca\(^{2+}\) and PKC act sequentially in a non-additive manner during GnRH-induced gonadotropin α-subunit and PKCβ mRNA elevation (21, 29). In addition, either Ca\(^{2+}\) or PKC mediate the rapid phase of GnRH-induced LHβ mRNA elevation but the combined activation of both pathways results in inhibition of LHβ gene expression (26). We suggested that PKC is involved in the rapid phase of GnRH-induced FSHβ mRNA elevation since Ca\(^{2+}\) was found to be inhibitory (26). Thus, differential cross-talk of Ca\(^{2+}\) and PKC is involved in the diverse effects of GnRH upon gonadotropin secretion and synthesis. Different PKC isoforms such as Ca\(^{2+}\)-dependent vs Ca\(^{2+}\)-independent PKCs might be involved in the various functions elicited by GnRH. Alternatively, GnRH responses might be compartmentalized and separated in order to enable the differential cross-talk of Ca\(^{2+}\) and PKC.

Since gonadotrophs are targets for the pituitary adenyl cyclase activating peptide (PACAP), additional cross-talk between GnRH and PACAP might exist (39). Indeed, GnRH inhibits adenyl cyclase activation by PACAP in αT3-1 cells and alters cellular levels of protein kinase A (PKA) subunits (39). Thus, cross-talk might exist not only between signaling molecules but also among ligands acting upon the gonadotrophs.

In summary, GnRH binds to a specific receptor which is coupled to Gq and activates sequentially different phospholipases, initially PLC and later PLD and PLA\(_2\) (Fig. 2). While early DAG and Ca\(^{2+}\) might be involved in rapid activation of selective cPKC, late DAG may activate nPKC. In addition AA might also support selective PKC subspecies activation with or without DAG. Differential cross-talk of Ca\(^{2+}\) and selective PKCs might segregate the signaling to downstream elements which are also activated during the neurohormone action. MAPK is activated in a Ca\(^{2+}\)- and PKC-dependent fashion and the enzyme might be involved in PLA\(_2\) and c-fos activation during gonadotropin subunits gene expression. AA and selective lipoxygenase products also participate in GnRH action, possibly by cross-talk with PKC or by an amplification cycle involving activation of phosphoinositide turnover by leukotrienes (e.g. LTC\(_4\)). A complex temporal and spatial cross-talk of second messengers might mediate the diverse effects elicited by GnRH (e.g. gonadotropin release vs gonadotropin subunits gene expression), the first key hormone of the reproductive cycle.

**Acknowledgements**

The active collaboration of D Ben-Menahem, N Reiss, S Shacham, D Harris, S Lazar, G Yachin, F Przedecki, L Nur-Levi and R Seger is greatly appreciated. The studies were supported by the Eisne Foundation Center-Biology Research Center and Tel Aviv University.

**References**

8. Yasin M, Dalkin AC, Haisenleder DJ, Kerrigan JR & Marshall JC. Gonadotropin-releasing hormone (GnRH) pulse pattern regulates...


29 Shraga-Levine Z, Ben-Menahem D & Naor Z. Activation of protein kinase Cβ gene expression by gonadotropin releasing hormone in αT3-1 cell line: Role of Ca2+ and autorregulation by protein kinase C. *Journal of Biological Chemistry* 1994 269 31028–31033.


Received 29 May 1996
Accepted 1 October 1996