Staurosporine enhances gonadotrophin-releasing hormone-stimulated luteinizing hormone secretion

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In intact sheep gonadotropes, the protein kinase inhibitor, staurosporine, inhibited the stimulatory effect of phorbol 12-myristate 13-acetate (PMA) on luteinizing hormone (LH) secretion. Under the same conditions staurosporine enhanced gonadotrophin-releasing hormone (GnRH)-stimulated LH exocytosis without altering the EC_{50} of GnRH and without affecting basal LH exocytosis. These results suggest that PKC does not play a major role in mediating acute GnRH-stimulated LH exocytosis. Furthermore, they demonstrate that staurosporine enhances GnRH stimulus-secretion coupling. Both extracellular Ca^{2+}-dependent and Ca^{2+}-independent components of GnRH-stimulated LH secretion were enhanced by the drug. Staurosporine had no effect on GnRH stimulation of cAMP and inositol phosphate synthesis. In permeabilized cells staurosporine did not enhance Ca^{2+}- and cAMP-stimulated LH exocytosis. Based on these results we hypothesize that staurosporine inhibits a protein kinase which is activated by GnRH and which negatively modulates GnRH stimulus-secretion coupling.

Luteinizing hormone; Gonadotropin-releasing hormone; Staurosporine; Protein kinase C; Gonadotrope

1. INTRODUCTION

The binding of gonadotropin-releasing hormone (GnRH) to specific receptors on the surface of gonadotropes is followed rapidly by an increase in cytosolic $[Ca^{2+}]_{free}$ and the secretion of luteinizing hormone (LH) (for review see [1]). Ca^{2+} is thought to be the major intracellular second messenger coupling GnRH binding to LH secretion [1]. GnRH also causes (i) rapid translocation of protein kinase C (PKC) from the cytosol to membrane [2,3], and (ii) phosphorylation of cellular proteins which is inhibited by PKC depletion or a PKC inhibitor [4]. Although these results indicate that GnRH causes activation of PKC, studies using gonadotropes depleted of PKC to investigate whether it mediates GnRH-stimulated LH release have produced conflicting results [5–7].

We have used staurosporine, a recently isolated microbial alkaloid [8], to investigate a role for PKC in acute GnRH-stimulated LH secretion. Staurosporine is the most potent PKC inhibitor known though it also inhibits other protein kinases [8,9]. We report here that staurosporine enhances GnRH-stimulated LH exocytosis which argues against a role for PKC and suggests that a protein kinase other than PKC may negatively modulate GnRH stimulus-secretion coupling.

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2. EXPERIMENTAL

Staurosporine, obtained from Boehringer-Mannheim (Mannheim, FRG), was dissolved in dimethylsulphoxide at 1 mM (w/v) and stored at 4°C. Myo-[2-^{3}H]inositol was from Amersham International. Ovine LH (NIADDK-oLH-I-3) and ovine LH antiserum (NIADDK-anti-oLH-1) were kindly provided by the NIDDK. *Staphylococcus aureus* α-toxin was obtained from Dr Sucharit Bhakdi (Institute of Medical Microbiology, Justus Liebig University, Giessen, FRG). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Primary sheep anterior pituitary cell cultures were dispersed and cultured for 48 h in 12-well tissue culture plates as described [10]. In intact cell experiments cells were washed 3–4 times in physiological buffer (buffer I in [10]) and then stimulated in buffer I (containing added agents) for 20 min after which the medium was removed for LH determination by radioimmunoassay [10].

Permeabilized cell experiments were performed as described previously [10]. Briefly, the cells were washed twice with buffer I and then once in Ca^{2+}-free buffer I before being permeabilized by incubation for 20 min at 37°C in intracellular buffer (buffer IC, described in [10]) containing 3 μg/ml α-toxin and 0.5 mM EGTA. Following permeabilization, the cell culture plates were cooled on ice for 10 min before equilibration with ice-cold stimulation buffer for 30 min. Stimulation buffer comprised buffer IC with 10 mM Ca-EGTA buffer (pCa = 7, prepared as described [10]). LH exocytosis was initiated by replacing the stimulation buffer with identical buffer at 37°C. Total cellular LH was measured after solubilizing the cells in Nonidet NP40 (1%, v/v) and LH released is expressed as a percentage of the total cellular LH.

Total inositol phosphate production was measured as described [11]. Briefly, cells were labelled with myo-[2-^{3}H]inositol overnight, washed as above, stimulated in the presence of LiCl (10 mM) for 40 min, and inositol phosphates were chromatographed on Dowex columns. For cAMP measurements, cells cultured in 6-well plates (3 pituitaries/plate) were washed and stimulated in buffer I which was removed after 60 min and replaced with 0.1 M HCl for extraction of cellular cAMP. The extract was neutralized with 100 mM Tris before cAMP determination by RIA (Amersham kit no. TKR 342).

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Table I

Effects of staurosporine on GnRH-stimulated inositol phosphate production

	Total inositol phosphates[a] (% of control)
Control	100
Staurosporine 100 nM	101 ± 25
GnRH 10^{-6} M	264 ± 43
GnRH plus staurosporine	231 ± 39[b]

[a] Mean and SE of 3 experiments
[b] Not significantly different from GnRH alone

Cells prelabelled with myo-[2-^{3}H]inositol overnight were washed and then stimulated for 40 min in the presence of 10 mM LiCl. Cells were preincubated for 30 min with staurosporine or vehicle before stimulation

Table II

Effect of staurosporine on GnRH-stimulated cAMP production

	% LH released[a]	Cellular cAMP[a] (pmol/well)
Control	9.2 ± 0.5	7.2 ± 0.4
Staurosporine 100 nM	7.0 ± 0 3	8.8 ± 0.6
GnRH 10^{-6} M	12.5 ± 0.2	13.6 ± 0 8
GnRH plus staurosporine	16.0 ± 0.8[b]	13.9 ± 0.1[c]

[a] Mean and SE of triplicate determinations
[b] Significantly different from GnRH alone ($P < 0.01$)
[c] Not significantly different from GnRH alone

Cells were preincubated for 30 min with staurosporine and then stimulated for 60 min in the presence of 0.25 mM 3-isobutyl-1-methylxanthine. After stimulation medium was removed for LH determination and the cells were processed for cAMP determination

All data are representative of results from experiments performed at least 3 times except in Table I which combines data from 3 experiments. Data points and error bars represent the mean and range of duplicate determinations (Figs 1–3) or the mean and SE of triplicate (Tables I and II) determinations. Statistical significance was tested, where indicated, using the Student's t-test.

3. RESULTS AND DISCUSSION

In primary cultures of sheep anterior pituitary cells, staurosporine enhanced GnRH-stimulated LH secretion (EC_{50} = 30 nM) as shown in Fig. 1A, a typical experiment. In 8 independent experiments, staurosporine enhanced LH release stimulated by 10^{-6} M GnRH by 104 ± 17% (mean ± SD) without affecting basal release. In contrast, staurosporine (IC_{50} = 10 nM) antagonized the stimulatory effect of phorbol 12-myristate 13-acetate (PMA) on LH secretion indicating that the drug is an effective inhibitor of PKC in gonadotropes (Fig. 1A). This finding argues against a significant stimulatory role for PKC in acute GnRH-stimulated LH secretion. Although the stimulatory effect of staurosporine might suggest an inhibitory role

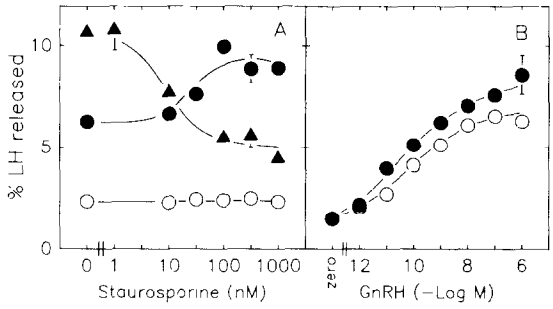

Fig 1 Effects of staurosporine on phorbol-ester- and GnRH-stimulated LH release (A) After preincubation for 30 min with the indicated concentration of staurosporine, cells were stimulated for 20 min in the presence of the same staurosporine concentrations with no addition (○), PMA 100 nM (▲), or GnRH 10^{-6} M (●). (B) After preincubation in the presence (●) or absence (○) of staurosporine (100 nM), cells were stimulated for 20 min with the indicated concentration of GnRH with (●) or wihtout (○) staurosporine.

for PKC in GnRH-stimulated LH secretion this seems unlikely since PKC-activating phorbol esters enhance both basal and GnRH-stimulated LH exocytosis [1,12,13].

Staurosporine had no effect on LH secretion in the absence of GnRH (Fig. 1A) and enhanced secretion at maximal GnRH concentrations without affecting the EC_{50} of GnRH (Fig. 1B). These findings suggest that the drug acts by enhancing GnRH stimulus-secretion coupling and not by stimulating secretion directly or by altering the affinity of the GnRH receptors. This could involve (i) enhancing the GnRH-stimulated generation of second messengers such as Ca^{2+} and cAMP or (ii) increasing the sensitivity of the secretory apparatus to the generated second messengers.

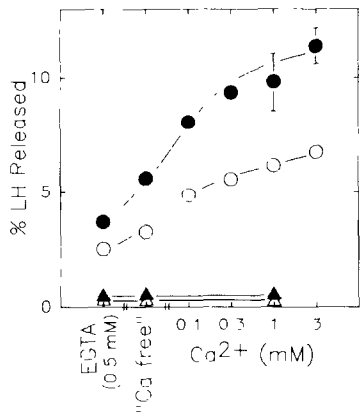

Fig. 2. Effects of staurosporine on GnRH-stimulated LH release at varying extracellular [Ca^{2+}]. Cells were preincubated with (●, ▲) or without (○, △) staurosporine firstly for 25 min in buffer I and then for 5 min in Ca^{2+}-free buffer I before stimulation for 20 min in the presence of the indicated amounts of extracellular Ca^{2+} or EGTA with the following additions: (△) none; (▲) staurosporine 100 nM; (○) GnRH 10^{-6} M; (●) staurosporine plus GnRH. 'Ca free' refers to no added Ca^{2+}

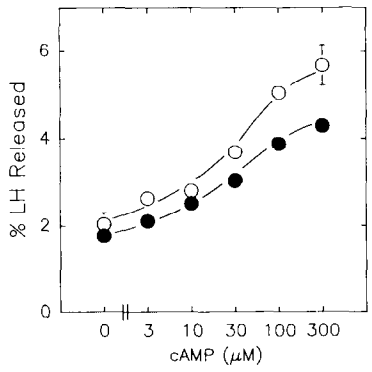

Fig. 3. Effects of staurosporine on cAMP-stimulated LH release. Permeabilized cells were equilibrated at 0°C for 30 min in stimulation buffer (pCa = 7) with staurosporine (●) or vehicle Me_2SO (○) (0.01% v/v) and the indicated cAMP concentrations. Staurosporine was added after 5 min of permeabilization and was present throughout. LH exocytosis was initiated by addition of stimulation buffer at 37°C and LH released after 20 min was measured.

GnRH is known to increase the cytosolic $[Ca^{2+}]_{free}$ by (i) stimulating entry of extracellular Ca^{2+} through plasma membrane Ca^{2+} channels [14,15] and (ii) stimulating the release of intracellular stored Ca^{2+}, most likely through activation of the inositol phospholipid signalling system [1,16]. Since staurosporine enhanced GnRH-stimulated LH release even in the absence of extracellular Ca^{2+}, it appears unlikely that it acts by enhancing Ca^{2+} entry (Fig. 2). PKC has been shown to negatively modulate the inositol phospholipid signalling system in many cell types [17,18]. We therefore examined whether staurosporine had a stimulatory effect on GnRH-stimulated inositol phosphate generation, and found none (Table I). These data do not exclude the possibility that staurosporine might inhibit a cytosolic $[Ca^{2+}]_{free}$-lowering mechanism activated by GnRH, by analogy with other ligands which operate via Ca^{2+}-mobilization [19]. Staurosporine also did not enhance GnRH-stimulated cAMP production under conditions where it enhanced GnRH-stimulated LH secretion (Table II).

α-Toxin-permeabilized cells were used to examine whether staurosporine enhanced the sensitivity of the secretory apparatus to intracellular second messengers. We have previously demonstrated that staurosporine does not enhance Ca^{2+}-stimulated LH exocytosis [20]. Staurosporine did not enhance cAMP-stimulated LH exocytosis but was inhibitory (Fig. 3), as expected from its known inhibitory effects on cAMP-dependent protein kinase in vitro [8,9].

Although the above results have excluded several possible mechanisms for the enhancement of GnRH-stimulated LH release by staurosporine, its mode of action remains elusive. Since staurosporine is an inhibitor of several protein kinases [8,9], it is possible that the drug inhibits an unidentified protein kinase which is a negative modulator of GnRH stimulus-secretion coupling.

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