ORIGINAL ARTICLE

# The effect of valproate (VPA) treatment on inositol phosphates (IPs) accumulation in non-stimulated and GnRH-treated female rat anterior pituitary cells *in vitro*

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**Abstract**

**OBJECTIVE:** Mechanism(s) responsible for VPA-induced effects on reproductive axis activity are not fully recognized. Previously we reported that VPA suppressed only GnRH-stimulated but not the basal LH release from rat anterior pituitary (AP) cells in vitro. Since the inhibitory effect of VPA was exerted only in GnRH-activated cells, potential VPA impact on GnRH-R-coupled IP3/PKC signaling could not be excluded. In this study the effect of VPA on IPs synthesis in non-stimulated and GnRH-treated rat AP cells was examined.

**MATERIAL AND METHODS:** In the first experiment $5 \times 10^5$ cells/ml were incubated for 3h with VPA (10 nM–10 μM), PMA (100 nM), GnRH (100 nM), PMA (100 nM) + VPA (10 nM–10 μM), GnRH (100 nM) + VPA (10 nM–10 μM). In the second experiment cells were preincubated for 24h with 1μCi myo-[$2^3$ H]-inositol, then for 30 min with 10 mM LiCl and finally for 3hr with GnRH (100 nM) VPA (1 μM, 10 μM), GnRH (100 nM) + VPA (1 μM, 10 μM). LH concentration was measured by RIA and intracellular IPs accumulation by ion-exchange chromatography analysis.

**RESULTS:** VPA diminished GnRH-stimulated LH release without affecting PMA-induced LH release at any dose tested. Moreover, VPA-induced increase of IPs accumulation occurred in both non-stimulated and GnRH-treated cells and intensity of cellular response was similar in both groups.

**CONCLUSION:** VPA affects $IP_3$/PKC pathway activity through its up-regulatory effect on IPs synthesis in AP cells. VPA-induced inhibition of GnRH-stimulated LH release from gonadotrope cells appears to be the result of still unrecognized cellular mechanism.

## INTRODUCTION

Valproic acid (VPA), a simple branched, short chain aliphatic fatty acid (2-propylpentanoic acid) is widely used in various seizures, bipolar disorders as well as for migraine prophylaxis (Bialer & Yagen 2007). It is now well established that VPA may cause a number of side effects, including reproductive disturbances what was shown in male and female non-epileptic rats (Roste *et al.* 2002) as well as in humans (Isojarvi *et al.* 2005). Although mechanisms responsible for VPA-induced effects on reproductive axis activity are not fully recognized, existing data suggest both gonadal and hypothalamo-pituitary sites of VPA action. Indeed, VPA was shown to affect testosterone, estradiol and progesterone secretion (Gregoraszczuk *et al.* 2000; Inada *et al.* 2012) as well as to inhibit conversion of testosterone to estradiol in isolated porcine ovarian follicular cells (Tauboll *et al.* 2003). VPA suppressed aromatase expression in cultured rat ovarian follicular cells (Inada *et al.* 2012.), inhibited hCG-induced androstendione secretion and reduced mRNA expression of steroidogenic enzymes to basal levels in primary culture of rat theca-interstitial cells (Fisseha *et al.* 2010). VPA also down-regulated both LH – dependent androstendione synthesis and FSH/IGF-dependent estradiol secretion by bovine theca-interna and granulose cells *in vitro* (Glister *et al.* 2012). Moreover, VPA-induced the decrease of hCG-stimulated testosterone secretion from rat Leydig cells was also reported (Kuhn-Velten *et al.* 1990). It is now well established that VPA directly affects GABA-ergic transmission by increasing GABA turnover in discrete brain structures (Lochner 1999; Johannessen 2000). In prepubertal DBA/2J mice, VPA treatment resulted in disturbances in GnRH secretion and this effect was exerted mainly through VPA impact on GnRH and GABA neurons interactions within the median eminence (Snyder & Badura 1995). Moreover, the disturbances in VPA-evoked GABA-ergic neurons activity were shown to generate puberty delay (Illing *et al.* 2000) as well as to disrupt estrus cycles synchronization in mature female rats (Lankhanpal *et al.* 2007). Recently, VPA was reported to repress GnRHR-1 gene transcription in mouse GnRH neuronal GT1-7 cell line (Gan *et al.* 2012). In our previous study we found that VPA treatment significantly diminished LH and FSH release from GnRH-stimulated male anterior pituitary cells in vitro (Wasilewska-Dziubinska *et al.* 2011). Since the inhibitory effect of VPA has been exerted only in GnRH activated cells, it cannot be excluded that this drug may also affect GnRH-R-coupled $IP_3$/PKC signaling in gonadotrope cells. The data on VPA-induced changes in $IP_3$/PKC pathway activity are still limited, nevertheless in amoeba *Dictyostelium discoideum* prolonged VPA treatment diminished intracellular $IP_3$ level (Williams *et al.* 2002; Eickholt *et al.* 2005; Simshoni *et al.* 2007). Moreover, VPA down-regulated $IP_3$ synthesis in primary rat dorsal root ganglia neurons (Eickholt *et al.* 2005; Simshoni *et al.* 2007). However, an acute stimulation of $IP_3$ accumulation by VPA in mouse cerebral cortex slices was also reported (Dixon & Hokin 1997).

The aim of this study was to compare an effect of VPA treatment on inositol phosphates (IPs) accumulation in non-stimulated and GnRH-treated rat anterior pituitary cells *in vitro*.

## MATERIAL AND METHODS

*Reagents*

Myo-[3H]inositol was purchased from NEN TM (Boston, MA, USA), Sephadex G-25 was obtained from Pharmacia (Uppsala, Sweden) and Dowex resin was obtained from Bio-Rad (Hercules, CA USA). Trypsin, fetal calf serum (FCS) were from BIOMED Vaccine Laboratory (Lublin, Poland). All media and other chemicals were purchased from Sigma (Sigma Aldrich, Germany) and culture dishes from Nunc (Thermo Fisher Scientific, Denmark).

*Isolation and anterior pituitary cells culture*

Four-month-old female random cycling Wistar rats (170–200 g) were kept under standard laboratory conditions on 12/12 h light/dark cycle (lights on at 07.00 a.m). Standard pelleted food (Murigran, Poland) and tap water were available ad libitum. All experiments were approved by the IV$^{th}$ Local Ethical Committee for the care and use of experimental animals.

*Anterior pituitary primary cell culture*

The procedures applied for pituitary tissue dissection and primary cell culture preparation were according to methods described previously (Wasilewska-Dziubińska *et al.* 2011). Finally, $5 \times 10^5$ cells/ml were dispersed on 24-well culture plates, and then pre-incubated for 72 h at 37 °C in DMEM containing 10% FCS and 0.01% mix of penicillin and streptomycin to achieve 80% confluency. For experimental incubations, cells were supplemented with 1 ml of DMEM without FCS. To determine whether VPA can affect PMA induced LH release, cells were incubated for 3h in the presence of 100 nM PMA and increasing doses of VPA (10 nM, 100nM, 1 μM, 10 μM). As a control positive groups, parallel incubations with the above VPA concentration without or in the presence of 100 nM GnRH were maintained. Each assay condition was implemented in triplicate within an experiment, and each experiment was performed two to four times.

*Phosphoinositide analysis*

Phosphoinositide hydrolysis was determined according to method described by Blitek et al (2005). Briefly, $5 \times 10^5$ cells/ml were incubated at 37 °C for 72h in 24-well culture plates in myoinositol-free medium M199 containing 10% FCS and 0.01% mix of penicillin and streptomycin. After reaching a 80% confluency, cells were incubated for 24 h in the fresh serum free

medium M199 in presence of myo-[2³H]inositol (1μCi/ml medium). Next, cells were washed three times with serum free M199 medium, incubated for 30 min with 10 mM LiCl and finally treated for 0.5 and 3h with 100nM GnRH and for 3h with VPA (10 nM–10 μM) or 100nM GnRH + VPA (10 nM–10 μM). After completing incubations, medium was removed and 0.5 ml of ice-cold mixture of methanol and HCl (10:0.1 v/v) was added to each vial. Cells were harvested by scraping, and transferred to glass tubes containing 0.5 ml chloroform. After 30 min incubation on ice, an additional 0.75 ml chloroform and 0.75 ml 0.5 M EDTA were added next all samples were centrifuged at 1000 × g for 5 min. The upper aqueous phase was collected and stored at –20 °C until affinity chromatography analysis.

Inositol phosphates (IPs) were resolved by using anion exchange chromatography on Dowex resin columns (AG 1-X8, formate form) according to Naor *et al.* (1986) and Wallace *et al.* (1993). Before use, columns were equilibred with 10 ml of 5 mM sodium tetraborate, then the samples were applied and 15 ml of distilled water was used to elute unincorporated inositol. Next, columns were sequentially washed with 15 ml of 5 mM sodium tetraborate – 60 mM ammonium formate, 0.1 M formic acid – 0.2 mM ammonium formate, 0.1 M formic acid – 0.4 M ammonium formate and 0.1 ammonium formate. Eluens were collected in 5 ml fractions. These five eluates contained unincorporated inositol, glycerophosphoinositol (GP), inositol monophosphates ($IP_1$), inositol biphosphates ($IP_2$) and inositol triphosphates ($IP_3$), respectively. Two ml aliquots were mixed with 10ml of scintillation liquid and counted in a scintillation counter (LS6000TA, Beckman) and IPs accumulation ($IP_1$+$IP_2$+$IP_3$) is expressed as a percent of basal.

*Radioimnnunoassay*

Rat LH medium concentrations were measured by RIA using antibodies and standards provided by the National Pituitary Agency and Dr. A. F. Parlow (Harbor-UCLA Research and Education Institute c/o Los Angeles Biomedical Research Institute Los Angeles, CA90060). Respective values were expressed in terms of the LH-RP3 reference standard. The intra and inter-assay coefficients of variation were below 9% and 11%, respectively. Changes of LH concentrations were presented as the percentage of the control value which was set at 100% and expressed as mean±S.E.M.

*Statistical analysis*

Statistical analyses of the results were performed with Kruskall-Wallis test followed by Mann-Whitney U test (STATISTICA TM 7.1 PL; StatSoft). Differences in mean values were considered significant if $p \leq 0.05$.

## RESULTS

As shown on Figure 1, VPA significantly inhibited (by 107%, and 110% respectively; $p<0.01$) LH release in GnRH-activated female rat AP cells only at the highest (1 μM and 10 μM) doses tested. In contrast, VPA did not affect PMA-induced LH secretion from the AP cells at any applied dose (Figure 2).

In order to establish GnRH-induced time-dependent pattern of IPs accumulation in the female rat anterior pituitary cells incubations in the presence of 100 nM GnRH were maintained for 30 min, 3h and 5h. As compared to respective non-stimulated control group, IPs accumulation was significantly ($p<0.05$) elevated up to 3h of GnRH stimulation and this increase was parallel to elevated LH release (Figure 3). Therefore, this time

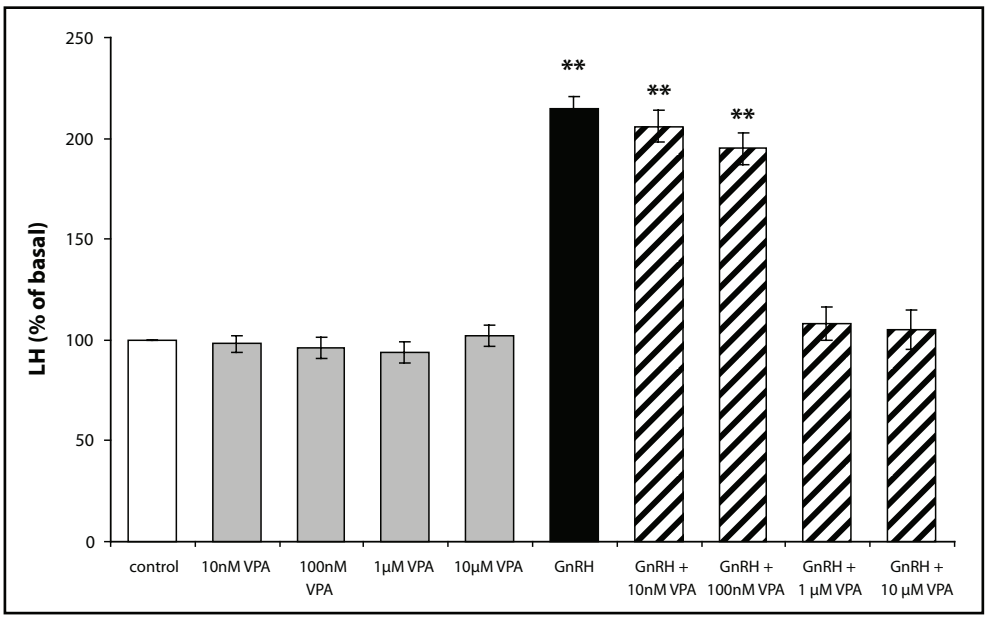


**Fig. 1.** Dose-dependent effect of VPA on basal and GnRH stimulated LH release after 3h treatment. $5 \times 10^5$ AP cells/well were incubated in the presence of increasing (10 nM–10 μM) VPA concentration without and after 100 nM GnRH stimulation. VPA effectively inhibited LH release at higher doses tested (1 μM and 10 μM) but this effect was found only after GnRH receptor stimulation. Control LH concentration was 9.42±0.56 ng/ml. Each bar represents the mean±S.E.M. with percentage of the control value. ** $p<0.01$. control vs 100 nM GnRH, $p<0.01$; 100 nM GnRH *vs* 100 nM GnRH + 1 μM VPA, $p<0.01$; 100 nM GnRH *vs* 100 nM GnRH + 10 μM VPA, $p<0.01$

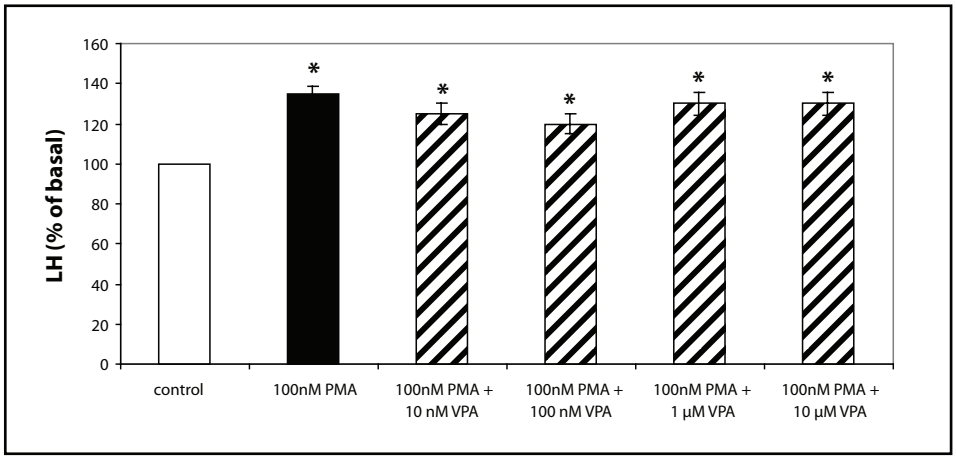


**Fig. 2.** Dose-dependent effect of VPA on PMA-stimulated LH release after 3h treatment. $5 \times 10^5$ AP cells/well were incubated with 100nM PMA in the presence of increasing (10 nM–10 μM) VPA concentration. PMA stimulation induced LH release (135% of control) but this effect was not affected by VPA at any dose tested. Control LH concentration was 9.30±0.61 ng/ml. Each bar represents the mean±S.E.M. with percentage of the control value. *$p$<0.05. 100 nM PMA *vs* control, $p$<0.05.

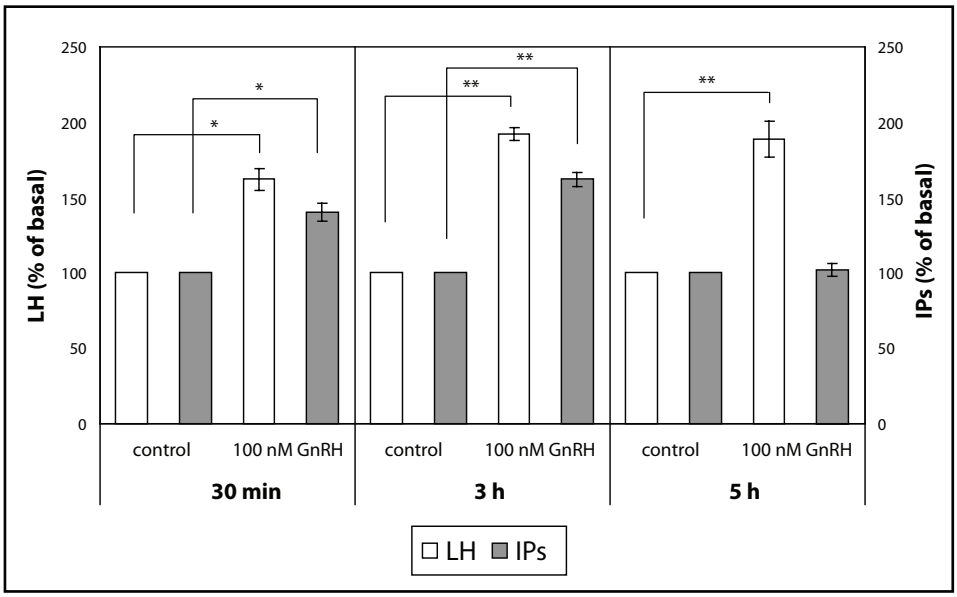


**Fig. 3.** Time-dependent changes of intracellular IPs accumulation and LH release in $5 \times 10^5$ AP cells/well after 100nM GnRH-stimulation. A significant increase of LH release was maintained during the whole (30 min, 3h, 5h) incubation period (162%, $p$<0.05; 192%, $p$<0.01 and 188%, $p$<0.01 of respective basal values). Control LH concentration were 8.74±0.57 ng/ml, 9.09±0.51 ng/ml, 8.03±0.37 ng/ml, respectively. In contrast, IPs accumulation increased after 30 min of GnRH incubation (140%; $p$<0.05) and reached maximum after 3h (162%; $p$<0.01) of respective basal values. * $p$<0.05; ** $p$<0.01.

point was chosen to determine whether VPA could affect IPs formation in GnRH-treated AP cells. As presented on Figure 4, VPA significantly promoted IPs accumulation at both (1μM and 10 μM) applied doses. Indeed, IPs level reached 158% and 161%, respectively, in comparison to the level found in control and taken as 100% in non-treated group cells Obtained results also revealed that VPA-treatment resulted in a comparable IPs accumulation to that found in GnRH-stimulated cells.

## DISCUSSION

Obtained results for the first time revealed that VPA-induced increase of IPs synthesis occurring in both non-stimulated and GnRH-treated anterior pituitary cells and intensity of cellular response was similar in both groups.

The interaction of GnRH with its cognate receptor (GnRHR) in pituitary gonadotropes results in the activation of$G_q/G_{11}$, phospholipase Cβ (PLCβ) what leads to phosphatidylinostol 4,5-biphosphate (PIP2) hydrolysis and finally generates the second messengers $IP_3$ and DAG (Naor 2009). However, an alternative and PLCβ-independent pathway of $IP_3$ formation has been reported in *D. discoideum* as well as in rat liver (Van Dijken *et al.* 1995a) where $IP_3$ was generated due to sequential dephosphorylation of inositol (1,3,4,5,6) pentakisphosphate – [Ins(1,3,4,5,6)$P_5$] by multiple inositol polyphosphate phosphatase enzyme (MIPP) (Van Dijken *et al.* 1995b). Recently, an involvement of prolyl oligopeptidase (PO) in the regulation of $IP_3$ signaling has been suggested (Myöhänen *et al.* 2009). Indeed, an increased intracellular $IP_3$ levels were found in *D. discoideum* after PO gene deletion (Wiliams *et al.* 1999) as well as in astroglioma line U343 along with inhibited enzyme activity (Schulz *et al.* 2002). PO negatively regulates MIPP activity and it has been suggested that cellular inositol phosphate levels are controlled both by inositol recycling and the MIPP-catalized breakdown of higher-order phosphate compounds regulated by PO activity (Ludtmann *et al.* 2011). Studies on recombinant human PO expressed in bacterial vector revealed that

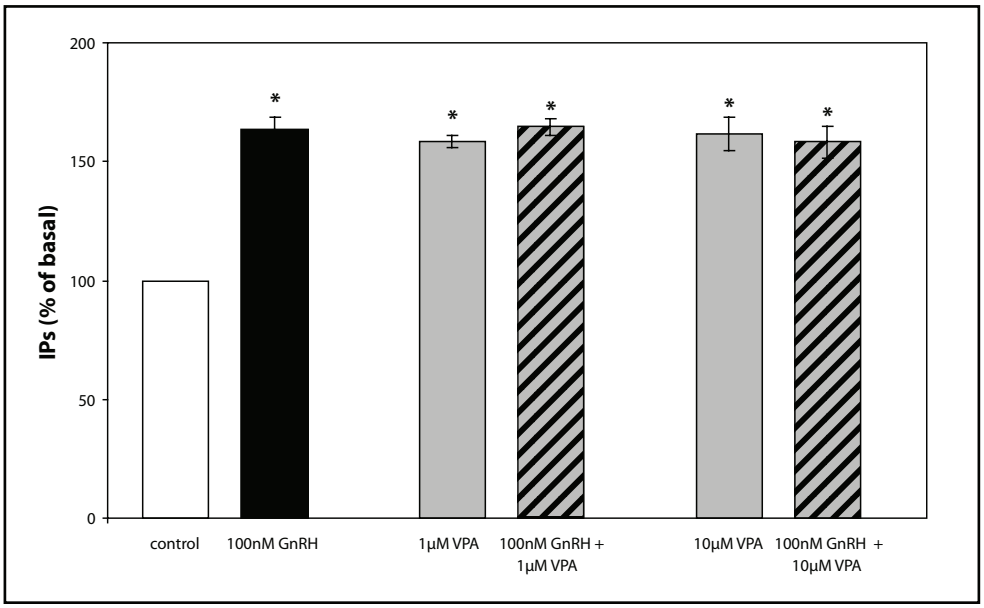


**Fig. 4.** Comparison of VPA and VPA + GnRH effects on IPs accumulation in AP cells. $5 \times 10^5$ cells/well were incubated for 3h with 1μM VPA and 10μM VPA and at both doses tested a significant increase of IPs accumulation was found (158% and 161% of control basal value). 100 nM GnRH-induced IPs intracellular level was not affected by either by 1 μM or 10 μM VPA co-treatment. Each bar represents the mean±S.E.M. with percentage of the control value. * $p<0.05$. control *vs* 100 nM GnRH, $p<0.05$; control *vs* 1 μM VPA, $p<0.05$; control *vs* 10 μM VPA, $p<0.05$.

its activity was directly inhibited by VPA with $K_i$ similar to the therapeutic VPA blood levels. Moreover, the down-regulatory effect resulted from both VPA impact on catalytic activity as well as the binding of substrate to the active site of the enzyme (Cheng *et al.* 2005). Since high density of prolyl oligopeptidase mRNA was found in the anterior lobe of the pituitary (Bellemère *et al.* 2004) PO, as molecular target for VPA, might be, at least in part, responsible for an increased IPs concentration found in our study. Stimulation of gonadotropin release may also occur due to DAG-induced PKC activity in gonadotrope cells (Naor 2009). Although phorbol myristate acetate (PMA), a functional DAG analog and an exogenous PKC activator, stimulated LH release in our study, VPA – in contrast to an inhibitory effect exerted on GnRH activated LH release – did not diminish PMA – induced LH secretion. If so, a post receptor effects of VPA on PKC isoforms synthesis cannot be excluded, what might result in GnRH receptor stimulation.

Several PKC isoforms were found in rat AP (MacEvans *et al.* 1999) and PKC β, δ and ε genes expression remained sensitive for GnRH stimulus. (Shraga-Levine *et al.* 1994; Harris *et al.* 1997). Moreover, by applying a depletion-insertion method, PMA-induced LH secretion was shown to require PKCα and PKCβ isoforms activation (Naor *et al.* 1989). Prolonged VPA treatment weakly decreased specific isoforms activity in human astrocytoma cell line (Kurita 2007). It also exhibited reduction in PKCα and PKCε isoforms activity in rat C6 glioma cells (Chen *et al.* 1994) as well as in rat cortex and hypothalamus (Maniji *et al.* 1996). But acute (up to 4 h) VPA exposure induced an opposite effect since in hippocampal cell line HN33 a transient increase of membrane-associated activity of PKC was reported (Watterson *et al.* 2002). Although VPA effect on PKC isoforms activity in the AP gland still remains unknown, its potential engagement cannot be excluded.

Recently, free fatty acids (FFA) were shown to play a role of signaling molecules *via* interaction with their specific FFA1, FFA2 and FFA3 G-proteins-coupled receptors (Stoddart *et al.* 2008). The expression of mRNA for FFA2 and FFA3 receptors was reported in the rat AP cells (Ishiwata *et al.* 2005). Interestingly, a direct suppressing effect of short-chain fatty acids (SCFAs) on basal and GHRH-induced GH release in goat AP cells *in vitro* was found (Katoh et al.1999). Since VPA also belongs to SCFA, an inhibitory effect exerted on GnRH-induced LH release found in our study might reflect its specific impact on tropic cells releasing activity.

Intracellular effects of VPA are exerted not only via modulation of inositol (Eikholt *et al.* 2005; Wiliams *et al.* 2002) as well as MAPK signaling (Boecker *et al.* 2006) but also through direct VPA impact on genes expression. Indeed, two mechanisms through which VPA can simultaneously affect the expression of multiple genes were suggested: the enhancement of AP-1 binding to DNA (Chen *et al.* 1997; 1999; Arinze *et al.* 2003) and the inhibition of histone deacetylases (HDACs) (Phiel *et al.* 2001; Göttlicher *et al.* 2001; Eyal *et al.* 2004; 2005). Histone acetylation has been shown to be an important regulatory mechanism controlling the transcription of about 2% of transcribed genes (Van Lint *et al.* 1996) among which are LHβ and FSHβ subunit genes (Melamed 2008; Lim *et al.* 2007). Recently, VPA-induced HDAC inhibition has been shown to repress GnRHR-1 gene transcription in mouse GnRH neuronal GT1-7 cell line as early as 2 hours after treatment (Gan *et al.* 2012). Whether VPA-induced diminished LH release in GnRH stimulated-cells found in

our study might, at least in part, result from inhibited HDAC activity requires more detailed research .

In conclusion, obtained data revealed that VPA may affect $IP_3$/PKC pathway activity through its up-regulatory effect exerted on IPs synthesis in AP cells. Therefore, an observed VPA-induced inhibition of GnRH-stimulated LH release from gonadotrope cells appears to be due to another, still unrecognized cellular mechanism.

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