

# Alfa 1 Adrenergic Potentiation of Progesterone Accumulation Stimulated by Vasoactive Intestinal Peptide (VIP) and Pituitary Adenylate Cyclase – Activating Polypeptide (PACAP) in Cultured Rat Granulosa Cells

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## Abstract

**OBJECTIVE:** VIP and PACAP, two structurally related peptides, stimulate cAMP, steroidogenesis and progesterone (PROG) release from cultured rat granulosa cells. VIP and PACAP<sub>38</sub> are known to mimic the effects of  $\beta$ -adrenergic receptor stimulation in the rat pinealocytes causing an increased melatonin synthesis. These effects were markedly potentiated by  $\alpha_1$  adrenergic receptor stimulation.

**METHODS:** We examined the influence of phenylonephrine (PHEN) ( $10^{-4}$  M and  $10^{-5}$  M)- $\alpha_1$  adrenergic receptor agonist and fenoterol (FEN) ( $10^{-5}$  M)- $\beta_2$  adrenergic receptor agonist on PROG accumulation stimulated by VIP ( $10^{-6}$  M) and PACAP ( $10^{-7}$  M) in cultured ovarian granulosa cells of cyclic rats (diestrus) after 2 h and 24 h incubation. The PROG concentrations in supernatants were measured with RIA tests.

**RESULTS:** VIP, PACAP<sub>38</sub>, PHEN and FEN stimulated PROG accumulation after 2 h incubation. The PROG accumulation stimulated both by FEN and VIP, and by FEN and PACAP<sub>38</sub> was not additive. PROG accumulation stimulated by VIP and PACAP<sub>38</sub> was strongly potentiated by PHEN- $\alpha_1$  adrenergic agonist.

**CONCLUSION:** The  $\alpha_1$  adrenergic potentiation of VIP and PACAP<sub>38</sub> stimulatory effects on PROG release from granulosa cells culture was found. VIP, PACAP<sub>38</sub> and  $\beta_2$  adrenergic receptors activation may share the same postreceptor mechanism. There exists simultaneous activation of different receptors – peptidergic and adrenergic ones in cultured granulosa cells of adult cyclic rat.

**Abbreviations and Units**

VIP	- Vasoactive Intestinal Peptide
PACAP	- Pituitary Adenylate Cyclase Activating Polypeptide
PHEN	- Phenylonephrine
FEN	- Fenoterol
NE	- Norepinephrine
FSH	- Follicle Stimulating Hormone
LH	- Luteinizing Hormone
PROG	- Progesterone
mRNA	- messenger Ribonucleic Acid
P450 scc	- cytochrome P450 side chain cleavage enzyme
c AMP	- cyclic Adenosine 3', 5' - Monophosphate
WKY	- Wistar - Kyoto
GRF	- Growth Hormone Releasing Factor
AG	- Aminoglutethimide
PROP	- Propranolol
PRAZ	- Prazosin
ACT	- Actinomycine D
eCG	- equine Chorionic Gonadotropin
hCG	- human Chorionic Gonadotropin
nM	- milimol
M	- Mol
RIA	- Radioimmunoassay
h	- hour

**Introduction**

It has been reported that in the rat, development of the ovarian innervation precedes the onset of folliculogenesis and occurs before follicles acquire responsiveness to gonadotropins, [1]. VIP [2,3] and norepinephrine (NE) [4], the neurotransmitters contained in ovarian nerves are present in the ovary before the gland becomes responsive to gonadotropins and are able to act on early follicles to facilitate the process of molecular differentiation that leads to gonadotropin dependency [5]. In the rat ovary both VIP and NE act via specific receptors coupled to cAMP-generating system, VIP through VIP receptor type 2 [6] VPAC 2 known as PVR 3 or PACAP typ 3 receptor [7] and NE via  $\beta_2$ -adrenergic receptors [8, 9].

Pituitary gonadotropins (FSH, LH) are the most important hormones regulating different ovarian functions. However, the studies on cultured granulosa cells demonstrated the important intra-ovarian regulatory role of several steroidal and nonsteroidal factors [10] including neuropeptides and catecholamines. Previous studies demonstrated that VIP [11] and PACAP [12, 13], members of glucagon/secretin structurally related peptides family, stimulate cAMP, steroidogenesis and progesterone (PROG) accumulation in cultured rat granulosa cells in the presence or absence of FSH. These effects of maximally effective concentrations of VIP and PACAP on progesterone secretion were not additive [13]. The local ovarian synthesis of VIP is also suggested by ability to detect VIP mRNA within rat ovarian tissue [14]. VIP can regulate cytochrome P 450 cholesterol side-chain cleavage (P 450 scc) enzyme gene expression (partially mediated through cAMP) in granulosa cells from estrogen-primed immature rats [15] and the synthesis of P 450 scc enzyme complex [16] responsible for the first reaction in PROG biosynthesis

[17,18]. The results suggest that the stimulatory effect of VIP on ovarian PROG secretion involves regulation of P 450scc gene expression during functional maturation of the prepubertal ovary in the rat [15].

PACAP has been detected in the rat ovary by RIA method [19]. It has been found recently that PACAP is transiently expressed in steroidogenic ovarian cells during periovulatory period in adult cyclic rats [20]. Moreover, PACAP secreted from these cells may induce dose-dependent PROG accumulation suggesting that PACAP could be an auto- or paracrine regulator of periovulatory PROG synthesis in the rat ovary [21]. Recently the reverse-transcription polymerase chain reaction with specific primers to the three cloned PACAP-binding receptors called PAC 1, VPAC 1, VPAC 2 [22] demonstrated both PAC 1 and VPAC 2 mRNA in extracts from preovulatory follicular cells in the rat [23]. Both PAC 1 and VPAC 2 receptors are coupled to adenylate cyclase [7,22,24,25]. PACAP<sub>38</sub>, supposedly interacting with both receptors, and VIP, interacting predominantly with the VPAC 2 receptor, stimulated cAMP production [23].

An important role of catecholamines in regulation of ovarian steroidogenesis in the rat is confirmed by the fact that there is the increased cAMP production and PROG release after stimulation of  $\beta_2$  adrenergic receptors in rat granulosa and luteal cells [8,9,26]. It has been observed that cAMP accumulation in rat pinealocytes by  $\beta$  adrenergic stimulation was potentiated by  $\alpha_1$  adrenergic receptor activation [27]. The interactive site for the  $\alpha_1$  and  $\beta$  adrenergic postreceptor interaction appears distal to the  $\beta$  adrenergic receptor [28]. The effect of  $\beta$  agonists on the cellular cAMP accumulation can be mimicked by VIP [29,30] and PACAP [31] which act upon receptors different from the  $\beta$  adrenoreceptors. Alpha 1 adrenergic potentiation of melatonin biosynthesis stimulated by VIP [31, 32] and PACAP [31, 32] is also well documented [33]. It seems that these interactions may be not restricted only to the pineal gland of the rat but may also occur in ovarian granulosa cells. A partial preliminary report of these findings has appeared recently [34].

**The Aim**

The aim of this study was to evaluate the effect of phenylonephrine (PHEN)  $\alpha_1$  adrenergic agonist and fenoterol (FEN)  $\beta_2$  adrenergic agonist on PROG accumulation stimulated by VIP and PACAP<sub>38</sub> in rat granulosa cells culture.

**Material And Methods**

Adult female Wistar-Kyoto (WKY) rats [220–250] were used throughout this study. They were maintained at 25°C under controlled lighting conditions (lights on at 07.00 h, lights off at 19.00 h), with food and water ad libitum. Vaginal smears were performed to assess the stage of estrous cycle; only animals exhibiting two consecutive 4-day cycles were included in the study.

The ovaries from WKY rats in diestrus were collected under aseptic conditions and were washed 20 min. with culture medium (RPMI 1640+0,1% Antibiotic Antimycotic solution). Ovarian follicles were punctured with a 25 gauge needle. Obtained granulosa cells were separated from adherent tissue by rubbing through a sieve (mesh 50) and digested with 0,1% collagenase at 37°C for 30 min. Dispersed cells were washed three times with culture medium (RPMI 1640+10% FBS+0,5% BSA+0,1% Antibiotic Antimycotic solution) and then counted on hemocytometer. Viability was estimated with trypan blue dye exclusion. Granulosa cells were seeded in culture medium in 24 -well culture plates (at density of  $2 \times 10^5/1$  ml) and cultured for 48 hours in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C. After that period the medium was removed and the cells were cultured in culture medium (RPMI 1640+0,1% Antibiotic Antimycotic solution) supplemented with 25 hydroxy - cholesterol 10 µg/ml as exogenous substrat [35] in the presence of different substances in varying concentration – VIP, PACAP (Sigma) VIP Antagonist ([Ac-Tyr<sup>1</sup>, D-Phe<sup>2</sup>]GRF(1-29) amide) (NEOSYSTEM), Aminoglutethymide 20 µg/ml Phenylonephrine 10<sup>-5</sup> M, Prazosin 10<sup>-4</sup> M, Fenoterol 10<sup>-5</sup> M, Propranolol 10<sup>-4</sup> M, Actinomycine D 10 µg/ml (SIGMA). Cell cultures were maintained for 2 h (short-term experiments) or 24 h (long-term experiments). The method was based on the conditions described previously by [33, 34, 35, 36, 37]. Culture supernatants were then decanted and stored until a hormone analysis. Each assay condition was implemented in triplicate with an experiment, and each experiment was performed two to six times. The supernatant concentrations of PROG were determined by RIA assays using kits (Orion Diagnostica, Finland). For the statistical analysis the unpaired Student's T-test and analysis of variance were used, as appropriate. The data were expressed as the mean ± SEM and the statistical significance was accepted at  $p < 0.05$ .

All experimental procedures were approved by the First Warsaw Ethic Committee for Experiments on Animals (created at the M. Nencki Institute of Experimental Biology, the Polish Academy of Sciences).

## Results

### Short-term experiments

During 2 h incubation VIP and PACAP<sub>38</sub> stimulated PROG accumulation in rat granulosa cells culture in dose - dependent manner

(Fig 1, Tab 1). The stimulatory effects were found at 10<sup>-7</sup> M concentration of VIP and PACAP<sub>38</sub> and at 10<sup>-6</sup> M VIP concentrations of VIP. FEN-β<sub>2</sub> adrenergic agonist and PHEN α<sub>1</sub> adrenergic stimulated PROG accumulation as compared with the control cultures ( $P < 0.05$ ,  $P < 0.05$  respectively) (Fig 2, Tab 2, Fig 3, Tab 3). FEN-β<sub>2</sub> adrenergic agonist and propranolol (PROP) β adrenergic receptor antagonist did not change PROG accumulation stimulated by VIP (Fig 2, Tab 2) and PACAP<sub>38</sub> (Fig 3, Tab 3). The additive effect of PHEN-α<sub>1</sub> adrenergic receptor agonist on PROG accumulation stimulated by VIP (Fig 2, Tab 2) was not observed in the presence of PACAP<sub>38</sub> (Fig 3, Tab 3).

VIP antagonist ([Ac-Tyr<sup>1</sup>, D-Phe<sup>2</sup>]GRF(1-29) amide) had no effect on PROG accumulation stimulated by VIP even in 100 - fold excess. PROG accumulation stimulated by VIP (Fig 2) and by PACAP<sub>38</sub> (Fig 3, Tab 3) was inhibited in the presence of AG.

### Long-term experiments

During 24 h incubation VIP and PACAP<sub>38</sub> stimulated PROG accumulation in dose dependent manner (Fig 4, Tab 4). The stimulatory effects were found at 10<sup>-7</sup> M, 10<sup>-6</sup> M concentration of VIP and 10<sup>-8</sup> M, 10<sup>-7</sup> M of PACAP<sub>38</sub>.

In the control conditions PHEN stimulated PROG accumulation ( $P < 0.0001$ ) and the response was inhibited in the presence of prazosin (PRAZ) α<sub>1</sub> adrenergic receptor antagonist (Fig

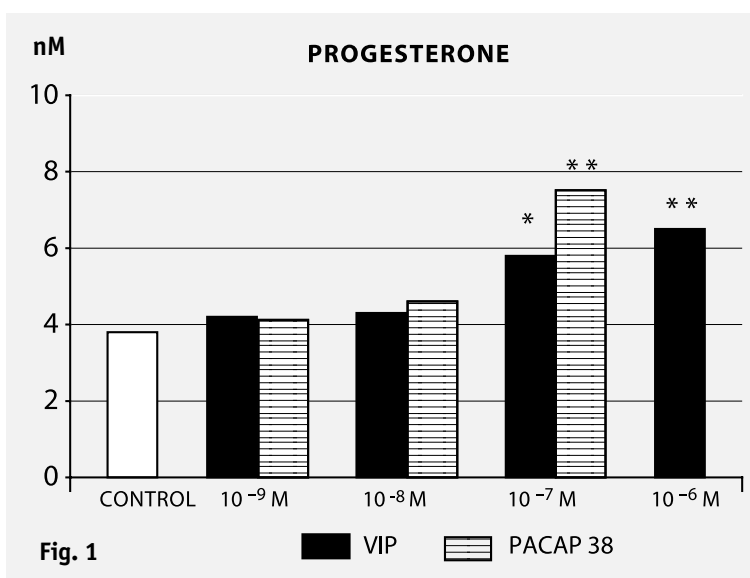


Fig. 1

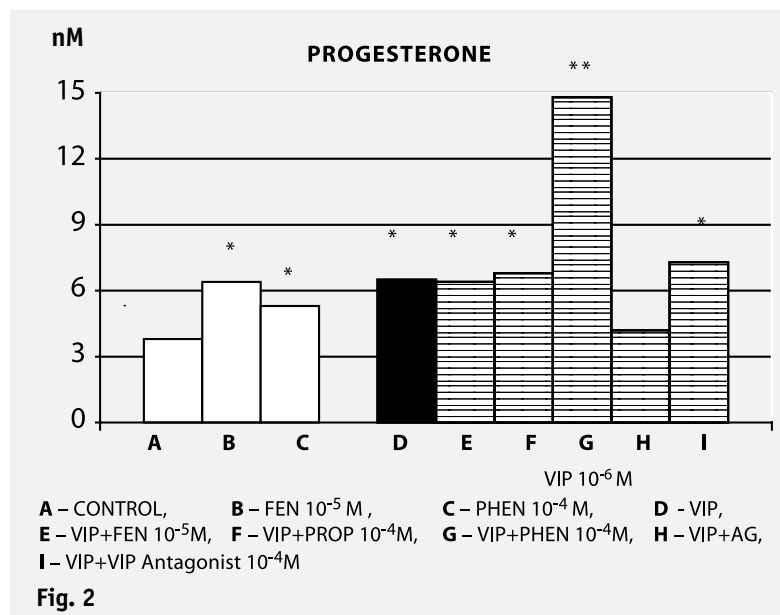
Fig. 1. and Table 1. The effects of VIP and PACAP<sub>38</sub> on PROG accumulation in rat granulosa cells culture (after 2 h incubation). Asterisks indicate significant differences compared to the control \*  $p < 0.05$ , \*\*  $p < 0.01$

PROG nM	CONTROL	VIP (M)				PACAP <sub>38</sub> (M)		
		10 <sup>-9</sup>	10 <sup>-8</sup>	10 <sup>-7</sup>	10 <sup>-6</sup>	10 <sup>-9</sup>	10 <sup>-8</sup>	10 <sup>-7</sup>
MEAN	3.76	4.23	4.26	5.84	6.54	4.12	4.62	7.54
SD	0.52	0.98	0.98	1.12	0.93	0.59	0.97	1.11
n	18	9	12	15	15	12	12	18
SEM	0.18	0.32	0.28	0.29	0.24	0.17	0.28	0.26

CONTROL v.s VIP 10<sup>-6</sup> M  $p < 0.01$

CONTROL v.s VIP 10<sup>-7</sup> M  $p < 0.05$

CONTROL v.s PACAP<sub>38</sub> 10<sup>-7</sup>  $p < 0.01$



**Fig. 2. and Table 2.** The effect of Fenoterol (FEN) 10<sup>-5</sup> M Phenyloephrine (PHEN) 10<sup>-4</sup> M on VIP 10<sup>-6</sup> M stimulated PROG accumulation in rat granulosa cells culture. (after 2 h incubation)

Asterisks indicate significant differences compared to the control

\* p < 0.05, \*\* p < 0.001

PROG nM	Control	FEN	PHEN	VIP	VIP+ PROP	VIP+ FEN	VIP+ PHEN	VIP+ AG	VIP+ Antagonist
MEAN	3.76	6.41	5.26	6.54	6.81	6.40	14.79	4.16	7.31
SD	0.52	1.00	0.92	0.93	0.72	1.02	2.12	0.58	0.85
n	18	12	9	15	9	12	9	12	9
SEM	0.18	0.29	0.30	0.24	0.24	0.29	0.71	0.17	0.28

CONTROL v.s FEN p < 0.05 VIP v.s VIP + AG p < 0.05

CONTROL v.s PHEN p < 0.05 VIP v.s PHEN p < 0.001

CONTROL v.s VIP p < 0.05

5, Tab 5). PRAZ had no effect on PROG accumulation stimulated by VIP. PROG accumulation stimulated by VIP and PACAP<sub>38</sub> was potentiated by PHEN and it was inhibited in the presence of AG (Fig 5, Tab 5).

Actinomycine D (ACT) – an inhibitor of RNA synthesis and AG diminished PROG release in the presence of VIP and PACAP<sub>38</sub> (Fig 5, Tab 5). Stimulated by VIP and PHEN as well as by PACAP<sub>38</sub> and PHEN, PROG accumulation was inhibited by AG (Fig 5, Tab 5).

## Discussion

It is well known that biosynthesis and secretion of ovarian steroid hormones throughout the rat estrus cycle occur in a highly episodic and coordinated fashion that requires precise regulation at cellular level. Our experiments were performed on granulosa cells harvested from adult cyclic rats in diestrus phase of estrus cycle. The 25-hydroxycholesterol in concentration recommended as sufficient to maximal enhancement of progestins secretion [35] was used as an effective exogenous substrate for the study of steroidogenesis [35]. The rate-limiting step in biosynthesis of all steroid hormones is the rate of movement of cholesterol to the side-chain cleavage enzyme complex on the inner mitochondrial membrane [41]. It has been supposed that 25-hydroxycholes-

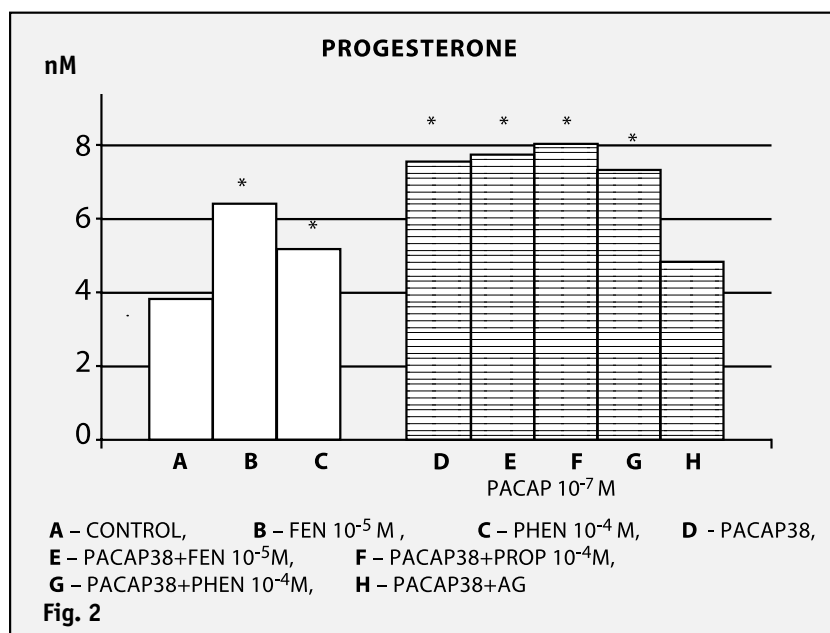
terol may bypass the rate-limiting process of cholesterol movement to the side-chain cleavage enzyme and allowing for measurements of maximal rates of steroidogenesis in vitro [35]. However, other hydroxylated cholesterol derivatives are used to monitor P 450 scc activity in vitro [42]. In our experiments in the presence of 25-hydroxycholesterol VIP and PACAP<sub>38</sub> stimulated PROG accumulation in dose-dependent manner. PACAP<sub>38</sub> has been shown to be more potent than VIP in stimulation of steroidogenesis as it was observed in cultured granulosa cells obtained from estrogen-treated immature rats [12, 13] and eCG/hCG-treated rats [23]. The findings that PACAP<sub>38</sub> was more potent than VIP in the stimulation of PROG accumulation in our experiments and in the stimulation of cAMP production in rat granulosa cells observed by others [13] could indicate the predominant presence of PAC 1 receptors in the rat granulosa cells, as it has been described in other cell types [43]. However, our results can also be reconciled by the concept that PACAP<sub>38</sub> receptors are located on most, if not all granulosa cells [13] and the VIP receptor on a subpopulation of granulosa cells [44]. As it was mentioned in the introduction VIP can regulate expression of cytochrome P 450 scc mRNA in the rat granulosa cells. [15] and P 450 17 alfa-hydroxylase mRNA in the hen granulosa cells [45] and can induce a synthesis and activation of P 450 scc enzyme complex [16]. VIP stimulated PROG synthesis in cultured rat granulosa cells in association with the increased pregnenolone production, the increased rate of conversion of pregnenolone to PROG via 3β-hydroxysteroid dehydrogenase, and the decreased metabolism of PROG via 20α-hydroxysteroid dehydrogenase [11]. Thus PROG accumulation induced by VIP involves selective modulation of key steroidogenic steps concerned with both PROG formation and degradation. However, the cellular mechanism of PACAP<sub>38</sub> stimulatory effect on PROG secretion is not known yet but it seems to be similar to VIP stimulatory effect because there is only one possibility of pregnenolone and PROG synthesis in the granulosa cells and the rate-limiting reaction in PROG synthesis in the ovary is the conversion of cholesterol to pregnenolone. AG – the P 450 scc enzyme inhibitor added to the culture medium inhibited VIP and PACAP<sub>38</sub> PROG accumulation in short-term and long-term incubation in similar manner. This supports the possibility of similar activation by VIP and PACAP<sub>38</sub> P 450 scc enzyme complex

activity in granulosa cells. Inhibition of PROG accumulation stimulated by VIP and PACAP<sub>38</sub> in long-term incubation in the presence of ACT an inhibitor of RNA synthesis was stated. This indicates that accumulation of PROG synthesized from exogenous 25 hydroxycholesterol stimulated by VIP and PACAP in our long-term experiments requires a new RNA synthesis as it was mentioned earlier [17].

The putative VIP antagonist ([Ac-Tyr<sup>1</sup>, D-Phe<sup>2</sup>] GRF(1-29) amide) which inhibits VIP binding to rat and hamster pancreatic membranes [46] failed to affect PROG accumulation stimulated by VIP in ovary granulosa cells. In the similar manner, the same VIP antagonist had no effect on melatonin synthesis stimulated by VIP and PACAP<sub>38</sub> in rat pineal gland [32].

Our observations that PROG production stimulated by activation of PACAP, VIP and  $\beta_2$  adrenergic receptors is not additive suggest that PACAP, VIP and  $\beta_2$  adrenergic receptors may share the same postreceptor mechanism. The  $\alpha_1$  adrenergic potentiation of VIP and PACAP<sub>38</sub> stimulatory effects on PROG release from granulosa cells culture is similar in our experiments to adrenaline and NE augmentation of androgenes synthesis observed in ovarian theca interstitial cells [47]. It has been also observed that in the rat luteal tissue adenylate cyclase was stimulated by PHEN, however, the stimulatory effect of isoprotenerol was the most effective [48]. The mechanism of  $\alpha_1$ -adrenergic potentiation of VIP and PACAP stimulatory effect on PROG accumulation in the granulosa cells requires further investigating in this field.

Synergistic interactions between different receptors in regulating signal transduction mechanisms are known to occur in variety of tissue [49], including the luteal cells [50]. It has been believed that the best example for synergistic interactions between adrenergic receptors and receptors coupled to cAMP is the pineal gland [49]. The  $\alpha_1$  adrenergic potentiation of VIP and PACAP<sub>38</sub> stimulatory effects on PROG release from granulosa cells observed by us is as important as the above mentioned example. It seems that adrenergic and peptidergic agents which can regulate the secretory func-



**Fig 3. and Table 3.** The effect of FEN 10<sup>-5</sup> M, PHEN 10<sup>-4</sup> M on PACAP<sub>38</sub> 10<sup>-7</sup> M stimulated PROG accumulation in rat granulosa cells culture (after 2 h incubation).

Asterisks indicate significant differences compared to the control \* p < 0.05

PROG nM	Control	FEN	PHEN	PACAP <sub>38</sub>	PACAP <sub>38</sub> +FEN	PACAP <sub>38</sub> +PROP	PACAP <sub>38</sub> +PHEN	PACAP <sub>38</sub> +AG
MEAN	3.76	6.41	5.26	7.54	7.73	8.00	7.36	4.15
SD	0.52	1.00	0.92	1.11	0.63	0.78	0.98	0.71
n	18	12	9	18	9	9	12	9
SEM	0.18	0.29	0.30	0.26	0.21	0.26	0.26	0.24
CONTROL v.s FEN	p < 0.05			PACAP <sub>38</sub> v.s	PACAP <sub>38</sub> + FEN N.S			
CONTROL v.s PHEN	p < 0.05			PACAP <sub>38</sub> v.s	PACAP <sub>38</sub> + PROP N.S			
CONTROL v.s PACAP <sub>38</sub>	p < 0.05			PACAP <sub>38</sub> v.s	PACAP <sub>38</sub> + PHEN N.S			
				PACAP <sub>38</sub> v.s	PACAP <sub>38</sub> + AG p < 0.05			

tion of rat granulosa cells, are also interdependently controlled by other multiple local factors.

### In conclusions:

- 1 In similar manner VIP and PACAP<sub>38</sub> may stimulate PROG accumulation in cyclic rat granulosa cells in the presence of 25-hydroxycholesterol in short terms and long terms.
- 2 The  $\alpha_1$  adrenergic potentiation of VIP and PACAP<sub>38</sub> stimulatory effects on PROG release from granulosa cells culture was found.
- 3 VIP, PACAP<sub>38</sub> and  $\beta_2$  adrenergic receptors activation may share the same postreceptor mechanism in short-term stimulation of PROG release from rat granulosa cells.
- 4 There exists simultaneous activation of different receptors – peptidergic and adrenergic ones in adult cyclic rat granulosa cells.

### Acknowledgments

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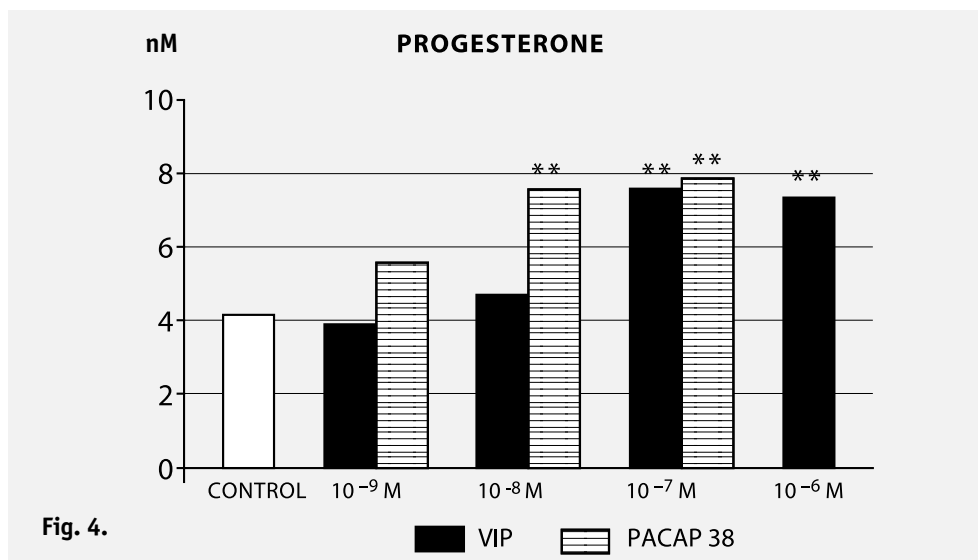


Fig. 4.

■ VIP    ▨ PACAP 38

**Fig. 4.** and **Table 4.** The effects of VIP and PACAP<sub>38</sub> on PROG accumulation in rat granulosa cells culture (after 24 h incubation).

Asterisks indicate significant differences compared to the control \* p < 0.05, \*\* p < 0.01

PROG nM	CONTROL		VIP (M)				PACAP <sub>38</sub> (M)		
	CONTROL	10 <sup>-9</sup>	10 <sup>-8</sup>	10 <sup>-7</sup>	10 <sup>-6</sup>	10 <sup>-9</sup>	10 <sup>-8</sup>	10 <sup>-7</sup>	
MEAN	4.19	3.88	4.75	7.57	7.41	5.60	7.56	7.87	
SD	0.59	0.51	1.05	0.87	0.82	1.07	1.26	0.97	
n	12	6	9	9	12	9	9	12	
SEM	0.17	0.23	0.37	0.30	0.24	0.38	0.44	0.29	

CONTROL v.s VIP 10<sup>-6</sup> M    p < 0.01

CONTROL v.s PACAP<sub>38</sub> 10<sup>-8</sup>    p < 0.01

CONTROL v.s VIP 10<sup>-7</sup> M    p < 0.01

CONTROL v.s PACAP<sub>38</sub> 10<sup>-7</sup>    p < 0.01

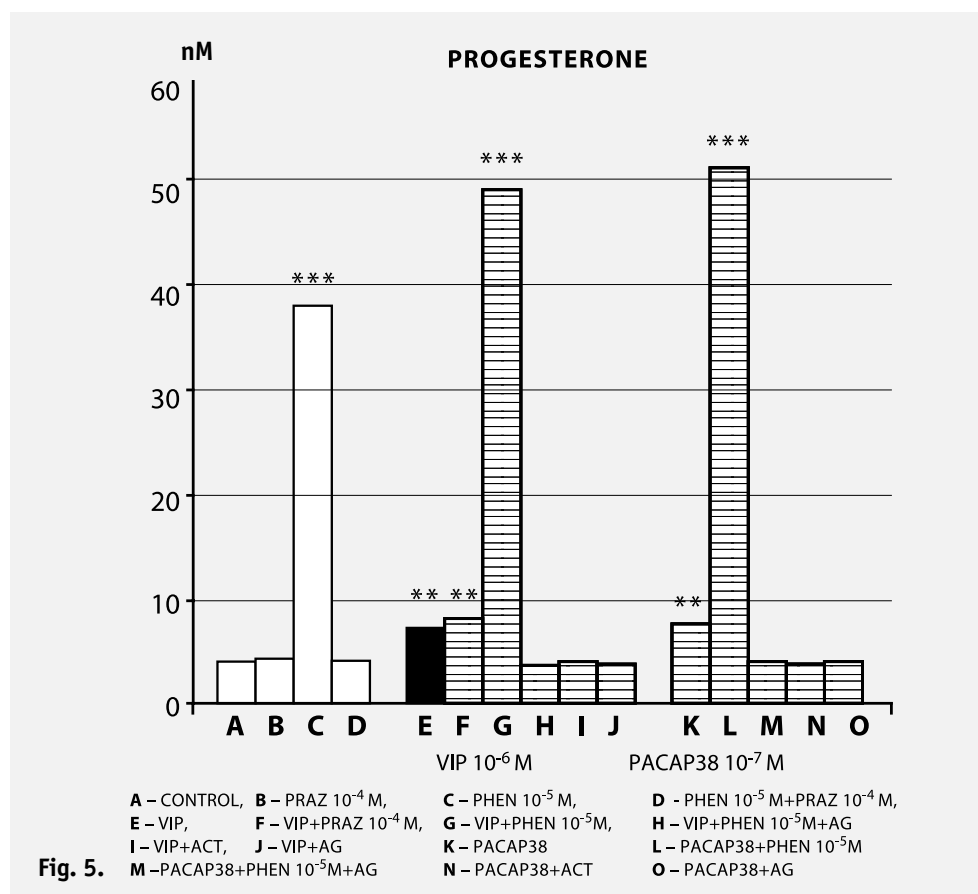


Fig. 5.

A – CONTROL, B – PRAZ 10<sup>-4</sup> M, C – PHEN 10<sup>-5</sup> M, D – PHEN 10<sup>-5</sup> M+PRAZ 10<sup>-4</sup> M,  
 E – VIP, F – VIP+PRAZ 10<sup>-4</sup> M, G – VIP+PHEN 10<sup>-5</sup> M, H – VIP+PHEN 10<sup>-5</sup> M+AG  
 I – VIP+ACT, J – VIP+AG, K – PACAP38, L – PACAP38+PHEN 10<sup>-5</sup> M  
 M – PACAP38+PHEN 10<sup>-5</sup> M+AG, N – PACAP38+ACT, O – PACAP38+AG

## REFERENCES

- Malamed S, Gibney JA, Ojeda SR. Ovarian innervation develops before initiation of folliculogenesis in the rat. *Cell Tissue Res* 1992; **270**:87–93.
- Ahmed CE, Dees WL, Ojeda SR. The immature rat ovary is innervated by vasoactive intestinal peptide (VIP)-containing fibers and responds to VIP with steroid secretion. *Endocrinology* 1986; **118**:1682–1689.
- George FW, Ojeda SR. Vasoactive intestinal peptide enhances aromatase activity in the neonatal rat ovary before development of primary follicles or responsiveness to follicle-stimulating hormone. *Proc Natl Acad Sci USA* 1987; **84**:5803–5807.
- Lawrence Jr IE, Burden HW. The origin of the extrinsic adrenergic innervation to the rat ovary. *Anat Rec* 1980; **196**:51–59.
- Mayerhofer A, Dissen GA, Costa ME, Ojeda SR. A role for neurotransmitters in early follicular development: introduction of functional follicle-stimulating hormone receptors in newly formed follicles of the rat ovary. *Endocrinology* 1997; **138**:3320–3329.
- Usdin TB, Bonner TI, Mezey E. Two receptors for vasoactive intestinal polypeptide with similar specificity and complementary distributions. *Endocrinology* 1994; **135**:2662–2680.
- Lutz EM, Sheward WJ, West KM, Morrow JA, Fink G, Harmar AJ. The VIP<sub>2</sub> receptor: molecular characterisation of a cDNA encoding a novel receptor for vasoactive intestinal peptide. *FEBS Lett* 1993; **334**:3–8.
- Adashi EY, Hsueh AJW. Stimulation of  $\beta_2$ -adrenergic responsiveness by follicle-stimulating hormone in rat granulosa cells *in vitro* and *in vivo*. *Endocrinology* 1981; **108**:2170–2178.
- Aguado LI, Petrovic SL, Ojeda SR. Ovarian  $\beta$ -adrenergic receptors during the onset of pituitary: characterization, distribution, and coupling to steroidogenic responses. *Endocrinology* 1982; **110**:1124–1132.
- Hsueh AJW, Adashi EJ, Jones PBC, Welsh TH Jr. Hormonal regulation of the differentiation of cultures ovarian granulosa cells. *Endocr. Rev* 1984; **5**:76–127.
- Davoren JB, Hsueh AJW. VIP: a novel stimulator of steroidogenesis by cultured rat granulosa cells. *Biol Reprod* 1985; **33**:37–52.
- Zhong Y, Kasson BG. Pituitary adenylate cyclase-activating polypeptide stimulates steroidogenesis and adenosine 3', 5'-monophosphate accumulation in cultured rat granulosa cells. *Endocrinology* 1994; **135**:207–213.
- Heidel JJ, Sneed J, Powell CJ, Davis B, Culler MD. A novel hypothalamic peptide, pituitary adenylate cyclase-activating peptide, regulates the function of rat granulosa cells *in vitro*. *Biol Reprod* 1996; **54**:523–530.
- Gozes I, Tsafirri A. Detection of vasoactive intestinal peptide-

**Fig 5. and Table 5.** The effect of Phenylonephrine  $10^{-5}$  M (PHEN) on VIP  $10^{-6}$  M and PACAP<sub>38</sub>  $10^{-6}$  M stimulated progesterone accumulation in rat granulosa cells culture (after 24 h incubation). Mean  $\pm$  SEM

Asterisks indicate significant differences compared to the control \*\* p < 0.001, \*\*\* p < 0.0001

PROG nM	CONTROL	PRAZ	PHEN	PHEN +PRAZ	VIP	VIP +PRAZ	VIP +PHEN	VIP +PHEN +AG	VIP +ACT	VIP +AG
MEAN	4.19	4.29	37.45	4.29	7.41	8.04	49.08	4.25	4.23	4.19
SD	0.59	0.63	2.91	0.50	0.82	0.69	4.62	0.60	0.81	0.83
n	12	6	9	9	12	6	12	9	9	9
SEM	0.17	0.25	0.97	0.17	0.23	0.28	1.33	0.20	0.27	0.27

PROG nM	PACAP <sub>38</sub>	PACAP <sub>38</sub> +PHEN	PACAP <sub>38</sub> +PHEN +AG	PACAP <sub>38</sub> +ACT	PACAP <sub>38</sub> +AG
MEAN	7.87	51.97	4.83	4.26	4.27
SD	0.92	4.97	0.58	0.77	0.74
n	12	9	9	9	9
SEM	0.26	1.65	0.19	0.25	0.24

CONTROL v.s PRAZ	N.S
CONTROL v.s PHEN	p < 0.0001
PHEN v.s PHEN + PRAZ	p < 0.0001
CONTROL v.s VIP	p < 0.01
VIP v.s VIP + PRAZ	N.S
VIP v.s VIP + PHEN	p < 0.0001
VIP + PHEN v.s VIP + PHEN + AG	p < 0.0001
VIP v.s VIP + ACT	p < 0.01
VIP v.s VIP + AG	p < 0.01
CONTROL v.s PACAP <sub>38</sub>	p < 0.01
PACAP <sub>38</sub> v.s PACAP <sub>38</sub> + PHEN	p < 0.0001
PACAP <sub>38</sub> + PHEN v.s PACAP <sub>38</sub> + PHEN + AG	p < 0.0001
PACAP <sub>38</sub> v.s PACAP <sub>38</sub> + ACT	p < 0.01
PACAP <sub>38</sub> v.s PACAP <sub>38</sub> + AG	p < 0.01

- coding messenger ribonucleic acid in the rat ovaries. *Endocrinology* 1986; **119**:2606–2610.
- 15 Trzeciak WH, Waterman MR, Simpson ER, Ojeda SR. Vasoactive intestinal peptide regulates cholesterol side-chain cleavage cytochrome P450 (P-450 scc) gene expression in granulosa cells from immature rat ovaries. *Mol Endocrinol* 1987; **1**:100–504.
  - 16 Trzeciak WH, Ahmed CE, Simpson ER, Ojeda SR. Vasoactive intestinal peptide induces the synthesis of the cholesterol side-chain cleavage enzyme complex in cultured rat ovarian granulosa cells. *Proc Natl Acad Sci USA* 1986; **83**:7490–7494.
  - 17 Stocco DM, Clark BJ. Regulation of the acute production of steroids in steroidogenic cells. *Endocr Rev* 1996; **17**:221–243.
  - 18 Ronen-Fuhrmann T, Timberg R, King SR, Hales KH, Hales DB, Stocco DM, Orly J. Spatiotemporal expression patterns of steroidogenic acute regulatory protein (StAR) during follicular development in the rat ovary. *Endocrinology* 1998; **139**:303–315.
  - 19 Arimura A, Somogyvari-Uigh A, Miyata A, Mizumo K, Coy DH, Kiada C. Tissue distribution of PACAP as determined by RIA: highly abundant in the rat brain and testis. *Endocrinology* 1991; **129**:2787–2789.
  - 20 Gras S, Hannibal J, Georg B, Fahrenkrug J. Transient periovulatory expression of pituitary adenylate cyclase activating peptide in rat ovarian cells. *Endocrinology* 1996; **137**:4779–4785.
  - 21 Gras S, Hannibal J, Fahrenkrug J. Pituitary adenylate cyclase-activating polypeptide is an auto/paracrine stimulator of acute progesterone accumulation and subsequent luteinization in cultured periovulatory granulosa/lutein cells. *Endocrinology* 1999; **140**:2199–2205.
  - 22 Harmar AJ, Arimura A, Gozes I, Journot L, Laburthe M, Pisegna JR, Rawlings SR, Robberrecht P, Said SI, Sreedharan SP, Wank SA, Waschek JA. International union of pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. *Pharmacol Rev* 1998; **50**:265–270.
  - 23 Gras S, Hedetoft, Pedersen SH, Fahrenkrug J. Pituitary adenylate cyclase-activating peptide stimulates acute progesterone production in rat granulosa/lutein cells via two receptor subtypes. *Biol. Reprod* 2000; **63**:206–212.
  - 24 Arimura A, Shioda S. Pituitary adenylate cyclase activating polypeptide (PACAP) and its receptors: neuroendocrine and endocrine interaction. *Front Neuroendocrinol* 1995; **16**:53–88.
  - 25 Spengler D, Waeber C, Pantaloni C, Holsboer F, Bockaert J, Seeburg PH, Journot L. Differential signal transduction by five splice variants of the PACAP receptor. *Nature* 1993; **365**:170–175.
  - 26 Ratner A, Weiss GK, Sanborn CR. Stimulation by  $\beta_2$ -adrenergic receptors of the production of cyclic AMP and progesterone in rat ovarian tissue. *J Endocrinol* 1980; **87**:123–129.
  - 27 Klein DC, Sugden D, Weller JL. Postsynaptic  $\alpha$ -adrenergic receptors potentiate the  $\beta$ -adrenergic stimulation of pineal serotonin N-acetyltransferase. *Proc Natl Acad Sci USA* 1983; **80**:599–603.
  - 28 Chik CL, Ho AK. Multiple receptor regulation of cyclic nucleotides in rat pinealocytes. *Prog Biophys Mol Biol* 1990; **53**:197–203.
  - 29 Kaneko T, Cheng PY, Oka H, Oda T, Yanaihara N, Yanaihara C. Vasoactive intestinal polypeptide stimulates adenylate cyclase and serotonin N-acetyltransferase activities in rat pineal gland *in vitro*. *Biomed Res* 1980; **1**:84–87.
  - 30 Yuwiler A. Vasoactive intestinal peptide stimulation of pineal serotonin N-acetyltransferase activity general characteristics. *J Neurochem* 1983; **41**:146–153.
  - 31 Chik CL, Ho AK. Pituitary adenylate cyclase-activating polypeptide control of rat pineal cyclic AMP and melatonin but not cyclic GMP. *J Neurochem* 1995; **64**:2111–2117.
  - 32 Yuwiler A, Brammer GL, Bennett BL. Interaction between adrenergic and peptide stimulation in the rat pineal: pituitary adenylate cyclase-activating peptide. *J Neurochem* 1995; **64**:2273–2280.
  - 33 Pfeiffer M, Maronde E, Molina CA, Korf HW, Stehle JH. Inducible cyclic AMP early repressor protein in rat pinealocytes: a highly sensitive natural reporter for regulated gene transcription. *Mol Pharmacol* 1999; **56**:279–289.
  - 34 Wasilewska-Dziubińska E, Borowiec M, Chmielowska M, Baranowska.  $\alpha_1$  adrenergic potentiation of VIP stimulated progesterone accumulation in cultured rat granulosa cells. 5th European Congress of Endocrinology Turin, 9–13 June 2001 Abstract book P–656.
  - 35 Toaff ME, Schleyer H, Strauss III JF. Metabolism of 25-hydroxycholesterol by rat luteal mitochondria and dispersed cells. *Endocrinology* 1982; **111**:1785–1790.
  - 36 Gras S, Ovesen P, Andersen AN, Sorensen S, Fahrenkrug J, Ottesen B. Vasoactive intestinal polypeptide and peptide histidine methionine. Presence in human follicular fluid and effects on DNA synthesis and steroid secretion in cultured human granulosa cells. *Hum Reprod* 1994; **6**:1053–1057.
  - 37 Kotsuji F, Kamitani N, Goto T, Tominaga I. Bovine theca and granulosa cell interactions. Modulate their growth, morphology and function. *Biol Reprod* 1990; **43**:726–732.
  - 38 Leya JM, Rawlins RG, Radwańska E, Beckmann MW. Steroidogenesis of cultured granulosa cells in women at risk for ovarian hyperstimulation syndrome. *Fertil Steril* 1992; **58**:1153–1157.
  - 39 Hughes JFM, Lane TA, Chen TT, Gorospe WC. Effects of cytokines on porcine granulosa cell steroidogenesis. *In vitro Biol Reprod* 1990; **43**:812–817.
  - 40 Kannzaki M, Hattori Horiuchi R, Kojima I. Coordinate actions of FSH and insulin like growth factor-1 on LH receptor expression in rat granulosa cells. *J Endocrinol* 1994; **141**:301–308.
  - 41 Iida S, Papadopoulos V, Hall PF. The influence of exogenous free cholesterol on steroid synthesis in culture of adrenal cells. *Endocrinology* 1989; **124**:2619–2624.
  - 42 Gregoraszczyk L, Piek³o R. Thyroid hormone action in porcine luteal cells Effect of triiodothyronine on mitochondrial cytochrome P 450 -scc activity. *J Physiol Pharmacol* 1998; **49**:467–475.
  - 43 Rawlings SR, Piuz I, Schlegel W, Bockaert J, Journot L. Differential expression of pituitary adenylate cyclase-activating polypeptide/vasoactive intestinal polypeptide receptor subtypes in clonal pituitary somatotrophs and gonadotrophs. *Endocrinology* 1995; **136**:2088–2098.
  - 44 Kasson BG, Meidan R, Davoren JB, Hsueh AJW. Identification of Subpopulations of rat granulosa cells: sedimentation properties and hormonal responsiveness. *Endocrinology* 1985; **117**:1027–1034.
  - 45 Johnson AL, Li Z, Gibney JA, Malamed S. Vasoactive intestinal peptide-induced expression of cytochrome P 450 cholesterol side-chain cleavage and 17  $\alpha$ -hydroxylase enzyme activity in hen granulosa cells. *Biol Reprod* 1994; **51**:327–33.
  - 46 Waelbroeck M, Robberrecht P, Coy DH, Camus J-C, De Neef P, and Christophe J. Interaction of growth hormone releasing factor (GRF) and 14 GRF analogs with vasoactive intestinal peptide (VIP) receptors of rat pancreas. Discovery of (N-Ac-Tyr 1, D-Phe 2)-GRF (1-19) NH<sub>2</sub> as a VIP antagonist. *Endocrinology* 1985; **116**:2643–2649.
  - 47 Dyer ChA, Erickson GF. Norepinephrine amplifies human chorionic gonadotropin-stimulated androgen biosynthesis by ovarian theca-interstitial cells. *Endocrinology* 1985; **116**:1645–1652.
  - 48 Eyster KM, Stouffer RL. Adenylate cyclase in the corpus luteum of the rhesus monkey. II. Sensitivity to nucleotides, gonadotropins, catecholamines and nonhormonal activators. *Endocrinology* 1985; **116**:1552–1558.
  - 49 Mc Grath JC, Brown CM, Wilson VG. Alpha adrenoreceptors: a critical review. *Med Res Rev* 1989; **9**:407–533.
  - 50 Rajkumar K, Chedrese PJ, LY H, Murphy BD. Protein kinase C, an endogenous regulator of hormone-induced cyclic AMP induction in porcine luteal cells. *J Endocrinol* 1991; **130**:273–280.