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Vasoactive intestinal peptide induces the synthesis of the cholesterol side-chain cleavage enzyme complex in cultured rat ovarian granulosa cells

(neuropeptide/regulation/progesterone synthesis/ovarian cells)

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ABSTRACT **Vasoactive intestinal peptide (VIP) has been identified in ovarian nerves and stimulates steroid secretion from immature ovaries. To gain insight into its mechanism of action, the effect of VIP on the synthesis of the cholesterol side-chain cleavage enzyme complex was studied in ovarian granulosa cells from immature estrogen-primed rats. The cells were cultured for 48 hr in serum-free medium; the proteins were labeled with [^{35}S]methionine; and the synthesis of cytochrome P-450, iron–sulfur protein, and NADPH:iron–sulfur protein reductase was evaluated by electrophoretic analysis after immunoisolation with polyclonal antibodies directed against the bovine adrenal enzymes. VIP at concentrations ranging from 0.001 to 1 μM stimulated 3- to 5-fold the synthesis of cytochrome P-450 and iron–sulfur protein. Peptide NH_2-terminal histidine, COOH-terminal isoleucine, which has >50% sequence homology of VIP, stimulated the synthesis of both proteins at ≈50% of VIP effectiveness. Secretin, another member of the glucagon-secretin family of peptides, which has only 30% sequence homology to VIP, was without effect. Similar results were observed with the NADPH:iron–sulfur protein reductase. VIP-induced synthesis of the cholesterol side-chain cleavage enzyme complex was accompanied by a dose-related increase in cAMP accumulation and progestin formation. It is concluded that VIP regulates the synthesis of the ovarian cholesterol side-chain cleavage enzyme complex, which catalyzes the rate-limiting reaction in progesterone biosynthesis, and that the VIP effect is at least partially mediated through cAMP. It is suggested that a stimulatory action of VIP on the synthesis of ovarian progesterone may contribute to regulating the functional development of the ovary.**

It is becoming increasingly evident that the mammalian ovary is regulated by both hormonal and neural inputs (1–3). While the hormonal regulation has been extensively investigated, much less attention has been given to the neural regulatory component (4, 5). Recently, the presence of ovarian peptidergic nerves (6, 7) has been thoroughly documented (8, 9). The occurrence of vasoactive intestinal peptide (VIP)-containing nerves in the immature rat ovary (9) is of particular interest since VIP has been found to stimulate steroid release from immature rat ovaries *in vitro* (9), rat granulosa cells in culture (10), and whole rabbit ovaries *in situ* (11).

The rate-limiting reaction in progesterone synthesis in the ovary is the conversion of cholesterol to pregnenolone. This reaction is catalyzed by the cholesterol side-chain cleavage enzyme complex (SCC), which is comprised of cholesterol side-chain cleavage cytochrome P-450 (P-450$_{SCC}$), iron–sulfur protein, and NADPH:iron–sulfur protein reductase (reductase) (12). The reaction is regulated by follicle-stimulating hormone (FSH), which induces the synthesis of SCC in both bovine (13, 14) and rat (15) granulosa cells in culture, resulting in increased progesterone formation.

The effectiveness of VIP in stimulating progesterone secretion from the ovary (9–11) raises the possibility that the mode of action of VIP involves regulation of SCC synthesis. The present report examines this possibility by utilizing cultured ovarian granulosa cells obtained from immature estrogen-primed rats. With the use of specific antibodies to the components of SCC, induction of the synthesis of these enzymes by VIP was demonstrated. A preliminary report of these findings has appeared (16).

MATERIALS AND METHODS

Radiochemicals. [^{3}H]Progesterone (specific activity, 40–60 Ci/mmol; 1 Ci = 37 GBq), 20α-[^{3}H]hydroxyprogesterone (specific activity, 40–60 Ci/mmol), [^{35}S]methionine (specific activity, 1120 Ci/mmol), ^{14}C-labeled molecular weight protein standards, and Na^{125}I were obtained from New England Nuclear. O^2-monosuccinyladenosine 3′:5′-cyclic monophosphate tyrosyl methyl ester (Sigma) was radiolabeled with ^{125}I as described (17).

Hormones. VIP, peptide NH_2-terminal histidine, COOH-terminal isoleucine (PHI), and secretin were obtained from Peninsula Laboratories (Belmont, CA). Ovine FSH (oFSH-16, AFP-5592C) was donated by the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases (Bethesda, MD).

Antigens and Antibodies. Cytochrome P-450$_{SCC}$, iron–sulfur protein, and reductase, purified to homogeneity from bovine adrenals, were provided by G. David Lambeth (Emory University, Atlanta). Antibodies to these proteins were those described by Kramer *et al.* (18) and were provided by M. R. Waterman (The University of Texas Health Science Center at Dallas). Antibodies to mitochondrial F_1-ATPase from bovine heart were donated by G. A. Breen (The University of Texas at Dallas).

The progesterone antiserum (GDN-377) was provided by G. D. Niswender (Colorado State University, Fort Collins, CO). The 20α-hydroxyprogesterone antiserum was obtained from Endocrine Sciences (Tarzana, CA). Antiserum to cAMP was purchased from Miles Laboratories (Naperville, IL).

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Abbreviations: VIP, vasoactive intestinal peptide; PHI, peptide NH_2-terminal histidine, COOH-terminal isoleucine; FSH, follicle-stimulating hormone; SCC, cholesterol side-chain cleavage enzyme complex; P-450$_{SCC}$, cholesterol side-chain cleavage cytochrome P-450.

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Cell Culture and Radiolabeling of Proteins. Female Sprague–Dawley, 19-day-old rats (Holtzmann, Madison, WI) were implanted s.c. with Silastic capsules containing 100 mg of diethylstilbestrol (19). The animals were killed 4 days thereafter, and ovarian granulosa cells were obtained by puncturing the follicles (20). Three million viable cells were plated onto 60-mm tissue culture dishes and cultured in a humidified atmosphere of 95% air/5% CO_2 at 37°C for 48 hr in a medium comprised of 4 ml of McCoy's 5a medium containing 25 mM glutamine, 10 mM Hepes (pH 7.4) (Irvine Scientific), penicillin (100 international units/ml), streptomycin sulfate (0.1 mg/ml), bacitracin (50 μM), diethylstilbestrol (0.1 μM), transferrin (1 ng/ml), and sodium selenite (1 pg/ml). The medium was supplemented with VIP (1 nM to 1 μM), PHI (1 μM), secretin (1 μM), and/or FSH (30 ng/ml) or dibutyryl-cAMP (1 mM) added at the beginning of the culture and after changing the media at 24 hr. Samples of media were taken for determination of cAMP, progesterone, and 20α-hydroxyprogesterone content and frozen until RIA. Prior to immunoisolation of radiolabeled proteins, the cells were incubated for 2 hr in Ham's F-12 plus Dulbecco's modified Eagle's medium (methionine free), followed by 2 hr of incubation in the same medium containing [^{35}S]methionine (50 μCi/ml). Experiments were terminated by washing the cell layer twice with Gey's balanced salt solution, followed by scraping the cells with a rubber policeman, centrifugation, and washing the cell pellet. The cells were then sonicated and freeze-thawed 3 times in phosphate-buffered saline containing 1% sodium cholate, 0.1% $NaDodSO_4$, and 0.1 mM phenylmethylsulfonyl fluoride.

Immunoisolation and $NaDodSO_4$/PAGE. Newly synthesized, radiolabeled P-450$_{SCC}$, iron–sulfur protein, reductase, and F_1-ATPase were immunoisolated from cell lysates (21) as described (22) and subjected to 0.1% $NaDodSO_4$/PAGE (23) using 10% gels for separating P-450$_{SCC}$, reductase, and F_1-ATPase, and 15% gels for separating iron–sulfur protein. The gels were treated with EN^{3}HANCE (New England Nuclear), dried, and exposed to X-Omat AR films (Kodak). The intensity of the bands on the autoradiograms was evaluated by densitometric scanning. Molecular weights were calculated by least-squares regression analysis using the following ^{14}C-labeled standards: phosphorylase b (M_r, 92,500), bovine serum albumin (M_r, 69,000), ovalbumin (M_r, 46,000), carbonic anhydrase (M_r, 30,000), and cytochrome *c* (M_r, 12,000).

Radioimmunoassays. To measure cAMP, the culture media were boiled for 10 min to inactivate phosphodiesterases and were stored at −20°C prior to the assay. cAMP was determined in duplicate samples after acetylation, as described (24). Progesterone was determined as reported (25). 20α-Hydroxyprogesterone was assayed as recommended by the antiserum manufacturer, except that the buffer and the separation of bound from free hormone were the same as for the progesterone assay (25). All the results are presented as the mean ± SEM of data obtained from at least three separate cultures within each treatment, each performed in duplicate. The significance of differences was determined by the test of Duncan.

RESULTS

Effect of VIP on the Synthesis of SCC. The effect of VIP on the synthesis of SCC, measured as the incorporation of [^{35}S]methionine into the SCC components, is presented in Figs. 1–3. The protein immunoprecipitated with the antibody to P-450$_{SCC}$ had the electrophoretic mobility of purified bovine adrenal P-450$_{SCC}$ (Fig. 1) and a molecular weight of 49,100 ± 300. It was almost completely displaced from the antibody complex by using 5 μg of purified P-450$_{SCC}$ (not shown). Likewise, the protein immunoprecipitated with the

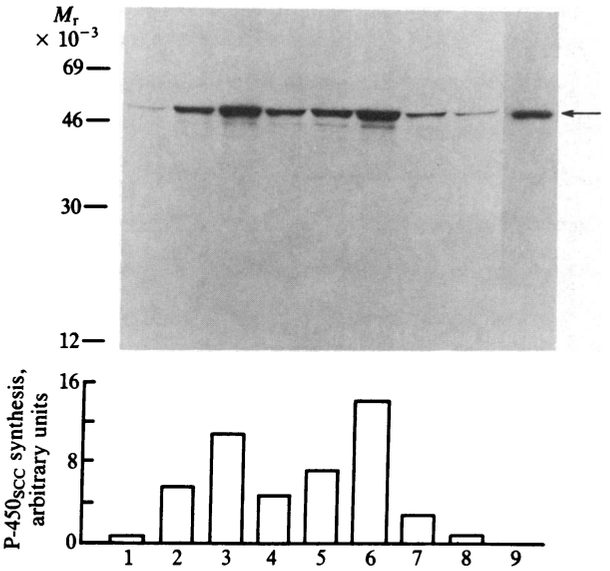

FIG. 1. Electrophoretic analysis of the effect of VIP on P-450$_{SCC}$ synthesis. Granulosa cells were cultured in medium containing FSH (30 ng/ml), dibutyryl-cAMP (1 mM), VIP, PHI, or secretin (1 μM each), or without additions. After 48 hr of culture, the cells were labeled with [^{35}S]methionine (50 μCi/ml) and the radiolabeled cytochrome P-450$_{SCC}$ was immunoisolated from the cell lysates and separated by $NaDodSO_4$/PAGE followed by fluorography. The cells were treated as follows: lane 1, no treatment; lane 2, FSH; lane 3, dibutyryl-cAMP; lane 4, VIP; lane 5, FSH and VIP; lane 6, dibutyryl-cAMP and VIP; lane 7, PHI; lane 8, secretin; lane 9, bovine adrenal P-450$_{SCC}$ coelectrophoresed and stained with Coomassie brilliant blue (arrow). The bars under the autoradiogram represent the areas of the bands obtained from densitometric scanning of the depicted autoradiogram.

antibody to iron–sulfur protein had the electrophoretic mobility of purified bovine adrenal iron–sulfur protein (Fig. 2), a molecular weight of 12,300 ± 300, and was completely displaced from the antibody complex by using 5 μg of purified iron–sulfur protein (not shown). VIP induced the synthesis of P-450$_{SCC}$ (Fig. 1, lane 4), iron–sulfur protein (Fig. 2, lane 4), and reductase (not shown). Densitometric scans of the autoradiograms revealed that VIP stimulated the synthesis of P-450$_{SCC}$ ≈5-fold (Fig. 1, lane 4). The VIP stimulation was ≈20% less than that of FSH (Fig. 1, lane 2), and 60% less than that produced by dibutyryl cAMP (Fig. 1, lane 3). Similar results were obtained when the effect of VIP on the synthesis of iron–sulfur protein was examined (Fig. 2). PHI stimulated the synthesis of P-450$_{SCC}$ and iron–sulfur protein ≈2-fold (Figs. 1 and 2, lanes 7), whereas secretin was ineffective (Figs. 1 and 2, lanes 8). The effects of VIP and FSH on the synthesis of P-450$_{SCC}$ and iron–sulfur protein were nearly additive (Figs. 1 and 2, lanes 5), as were the effects of VIP and dibutyryl-cAMP (Figs. 1 and 2, lanes 6). In this experiment, all these substances were tested at maximally effective concentrations, namely FSH at 30 ng/ml, VIP at 1 μM, and dibutyryl-cAMP at 1 mM. This concentration of FSH was considered to be maximally effective, based on the findings that in our system a concentration of FSH of 50 ng/ml was equally as effective as 30 ng/ml in terms of cAMP accumulation, progestin formation, and induction of SCC components. A similar observation has been made by others in a comparable model system in terms of progestin formation (10).

The synthesis of both P-450$_{SCC}$ and iron–sulfur protein was induced by VIP at concentrations as low as 1 nM. The effect of this dose is compared in Fig. 3 (lane 4) with that of FSH and dibutyryl-cAMP, which were tested in the same experiment. The phosphodiesterase inhibitor isobutylmethylxan-

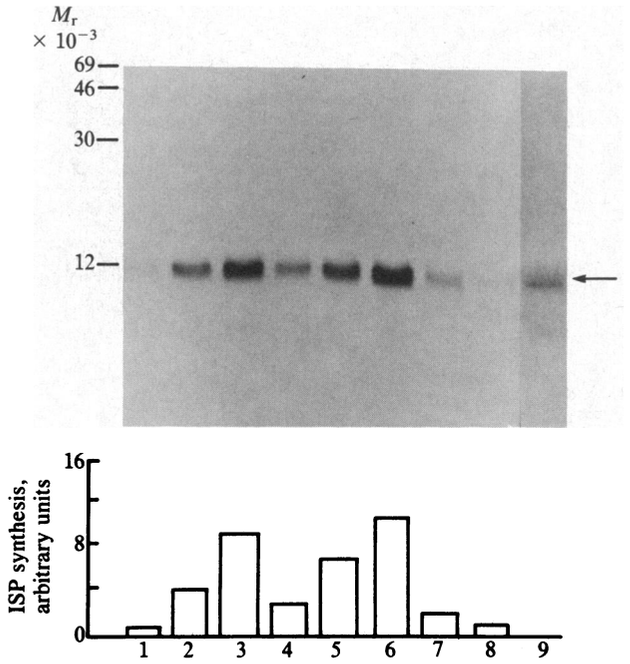

FIG. 2. Electrophoretic analysis of the effect of VIP on iron–sulfur protein (ISP) synthesis. The conditions of cell culture, concentrations of added compounds, separation of radiolabeled iron–sulfur protein by $NaDodSO_4$/PAGE were the same as described in legend to Fig. 1. The cells were treated as in Fig. 1 except that lane 9 shows standard bovine adrenal iron–sulfur protein. The bars under the autoradiogram show the areas of the bands obtained from densitometric scanning of the depicted autoradiogram.

thine (0.1 mM) potentiated the effect of VIP on the synthesis of the two enzymes (not shown).

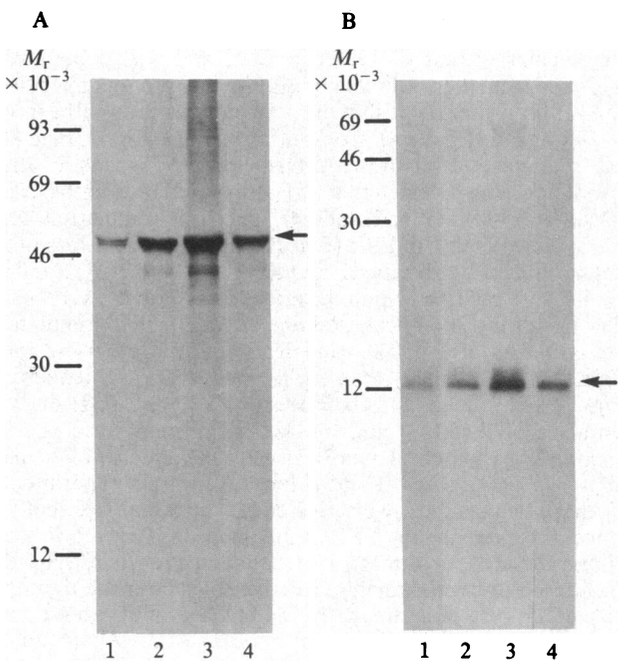

FIG. 3. Effectiveness of VIP at a nanomolar concentration on the synthesis of P-450_{SCC} and iron–sulfur protein. Granulosa cells were cultured for 48 hr, treated with FSH (30 ng/ml), dibutyryl-cAMP (1 mM), or VIP (1 nM), and processed as described in the legend to Fig. 1. The radiolabeled P-450_{SCC} (*A*) and iron–sulfur protein (*B*) were immunoisolated from the cells treated as follows: lanes 1, no treatment; lane 2, FSH; lane 3, dibutyryl-cAMP; lane 4, VIP. Positions of the P-450_{SCC} and iron–sulfur protein standards are indicated by arrows.

A similar pattern of induction of the synthesis of reductase by VIP was observed, whereas mitochondrial F_1-ATPase, whose synthesis is not influenced by FSH (15), was not induced by VIP or the other substances tested (not shown).

Effects of VIP on cAMP Accumulation and Progestin Formation. VIP induced a dose-dependent increase in the accumulation of cAMP in the culture medium (Fig. 4). The effect was slightly less than that caused by FSH at 30 ng/ml, in agreement with the relative degree of induction of the SCC components by the two substances. The VIP effect was observed even at a concentration as low as 1 nM, as was the induction of the SCC enzymes (see Fig. 3, lane 4), and became maximal at 0.1 μM with an ED_{50} of 15 nM (Fig. 4 *Inset*). PHI (1 μM) stimulated cAMP accumulation to ≈60% of the levels seen with 1 μM VIP, whereas secretin was ineffective. The effects of maximally effective doses of VIP plus FSH were nearly additive, paralleling again their degree of induction of the SCC enzymes.

VIP stimulated progesterone formation by the cultured granulosa cells (Fig. 5) in a dose-related manner with an ED_{50} of 3 nM (*Inset*). As seen in the case of the SCC components, the effect of 1 μM VIP on progesterone secretion was less than that seen with FSH at 30 ng/ml or 1 mM dibutyryl-cAMP. In contrast to cAMP, the effects of VIP and FSH on progesterone formation were not completely additive. VIP also stimulated the formation of 20α-hydroxyprogesterone with an effectiveness similar to that shown for progesterone—i.e., lower than that of FSH and dibutyryl-cAMP (data not shown).

DISCUSSION

The foregoing results demonstrate that the peptide neurotransmitter VIP, which occurs in ovarian nerves, induces the synthesis of P-450_{SCC} iron–sulfur protein, and reductase in cultured granulosa cells. These enzymes are components of

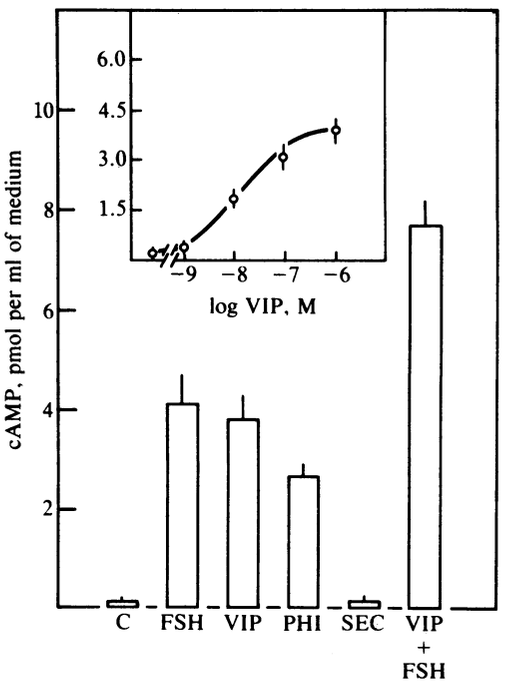

FIG. 4. Effect of VIP on cAMP accumulation in the culture medium of granulosa cells. The cells were cultured under standard conditions (C), or with the addition of FSH (30 ng/ml), and/or 1 μM VIP, 1 μM PHI, 1 μM secretin (SEC). The culture media were collected at 24 hr and duplicate samples were assayed for cAMP. (*Inset*) Dose dependence of VIP-stimulated cAMP accumulation, shown as the mean ± SEM of at least three independent determinations.

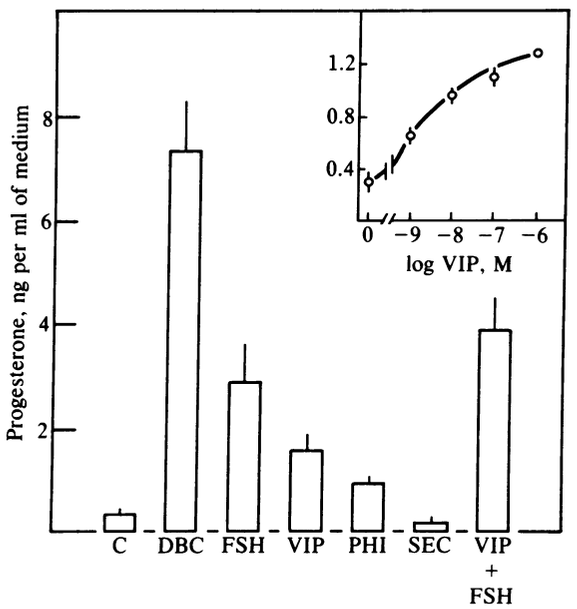

FIG. 5. Effect of VIP on progesterone formation by cultured granulosa cells. The cells were treated as described in the legend to Fig. 4 except that an additional treatment [1 mM dibutyryl-cAMP (DBC)] was introduced. The culture media were collected at 24 hr and duplicate samples were assayed for progesterone. (*Inset*) Dose dependence of VIP-stimulated progesterone formation, shown as the mean ± SEM of at least three independent determinations.

the SCC enzyme complex, whose activity is of prime importance in regulating steroid synthesis in the ovary (26). The induction of the enzymes is specific, coordinated, and high enough to account for the increase in progestin formation observed. Protein specificity of the VIP effect is indicated by its failure to increase the incorporation of [^{35}S]methionine into mitochondrial F_1-ATPase, a constitutive mitochondrial enzyme. The coordinate nature of the induction can be deduced from the fact that the synthesis of the three components of SCC was increased to the same extent. The magnitude of the VIP effect on the induction of the three proteins was less than that of FSH, which has been recently shown to induce the synthesis of SCC in rat granulosa cells (15). VIP stimulated the synthesis of SCC at concentrations as low as 1 nM. In addition to its actions on the synthesis of SCC, VIP stimulated the formation of both cAMP and progesterone in a concentration-dependent fashion.

We have previously reported the presence of VIP in the immature rat ovary at nanomolar concentrations (9), which are well within the range shown here to stimulate both the synthesis of SCC and progesterone formation. These observations suggest that stimulation of SCC synthesis may be a physiological mechanism by which VIPergic nerves (and locally produced VIP) affect steroidogenesis in the developing ovary. The specificity of VIP is demonstrated by the finding that, as in other tissues (27, 28), the stimulatory effect of the peptide was shared by PHI and not by secretin. This, in general, reflects the degree of sequence homology of these peptides with VIP (29).

The stimulation of progesterone production by VIP in granulosa cells appears to be mediated, at least in part, by cAMP (10). As in the case of FSH (13, 15), the VIP action to increase progesterone formation seems to also involve a cAMP-mediated induction of the synthesis of P-450$_{SCC}$ and iron–sulfur protein. This is suggested by the capacity of VIP to increase cAMP formation, the ability of isobutylmethylxanthine to potentiate the VIP effect. Whether the induction of these enzymes by VIP also involves factors other than cAMP remains to be determined. The finding that a greater enzyme induction occurred in the presence of both dibutyryl-cAMP and VIP suggests that this may be the case. The effect of VIP appears to be mostly exerted at the gene-expression level, and not at an enzyme-degradation step, since in recent experiments (unpublished) we have found that VIP increases the level of translatable mRNAs encoding P-450$_{SCC}$ and iron–sulfur protein.

The actions of VIP and FSH, at maximally effective concentrations, on the synthesis of the SCC complex as well as on cAMP accumulation were nearly additive. This is consistent with the recent suggestion that FSH and VIP may act on different subpopulations of granulosa cells (30). On the other hand, the effects of FSH and VIP on progesterone secretion were not completely additive, which might be explained by their stimulatory effect on the conversion of progesterone to 20α-hydroxyprogesterone. Our results show that VIP, like FSH, stimulates 20α-hydroxyprogesterone formation.

It has been reported that VIP stimulates the secretion of progesterone and estradiol from granulosa cells (10) and of testosterone from the immature rat ovary (9). The induction by VIP of the ovarian SCC enzymes demonstrated here provides a mechanism by which VIP may affect the production of these steroids. The SCC complex catalyzes an early and rate-limiting step in steroid biosynthesis—i.e., the conversion of cholesterol to pregnenolone. Stimulation of the synthesis of this enzyme complex by VIP would not only result in increased production of progesterone, but also that of estradiol and testosterone (provided that the availability of the enzymes involved in the formation of these steroids is not rate-limiting). There is evidence, however, that VIP indeed affects additional enzymatic steps. F. W. George and S.R.O. (unpublished data) have found that VIP can increase aromatase activity in the fetal rat ovary even before the ovary acquires responsiveness to FSH. Thus, VIP appears to mimic at least two actions of FSH in the ovary: the induction of SCC synthesis and stimulation of aromatase activity. The question then arises as to the physiological situations during which a VIP involvement would have its greatest impact. Resolution of this issue may derive from the recent observation that FSH and VIP act on different subpopulations of granulosa cells (30).

In conclusion, the present results provide unequivocal evidence that VIP can act at physiological concentrations to induce the synthesis of the SCC, which catalyzes the rate-limiting reaction in ovarian progesterone biosynthesis. While a significant portion of ovarian VIP is contained in nerve fibers of extrinsic origin, the possibility of local synthesis of VIP should also be considered. Regardless of its origin, the similarity of the VIP effect to that of FSH as well as its specificity provide a compelling argument in support of a role for a peptidergic component in the developmental regulation of ovarian steroidogenesis.

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