The Stimulatory Effect of Vasoactive Intestinal Peptides on the Cortisol Production of Guinea Pig *Zona Fasciculata* Cells: an Extra-ACTH Regulatory Model of the Adrenocortical Function

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Abstract

The effect of vasoactive intestinal peptide (VIP) on cortisol production was studied in a primary culture enriched with guinea pig *Zona Fasciculata* (ZF) cells. In ZF cells, VIP stimulates cortisol secretion and enhances the steroidogenic action of ACTH. Compared to ACTH on an equal molar basis, the cortisol-stimulatory effect of VIP is at least 10-fold less potent. As VIP exhibits a wide range of biological actions with widespread distribution in the body, the steroidogenic action of VIP on the adrenal glands is not tissue-specific. There are VIP receptors in ZF cells. With the aid of a VIP receptor antagonist, we found that ACTH and VIP mutually bind each other's receptors with an affinity-ranking order of ACTH > VIP receptor antagonist > VIP. VIP stimulates cortisol production most likely through the cyclic AMP (cAMP) signaling pathway. Both ACTH receptors and the VIP receptors bind VIP receptor antagonist more avidly than VIP, but the bindings do not lead to a consequential effect on cAMP production and cortisol secretion. However, the VIP receptor antagonist counteracted ACTH and VIP to lower both cAMP and cortisol production. In addition, ASIF and BNP-32, which are the proven ACTH receptor antagonists, reduced the cortisol-stimulatory effect of ACTH and VIP. These results suggest that besides ACTH, VIP be an important factor in regulating the cortisol secretion from the adrenal cortex at the site of ACTH receptors. In cases with hypercortisolemia being detected concomitantly with normal or low ACTH levels, we may need to investigate the influential role of VIP.

Key Words: vasoactive intestinal peptide, cortisol, ACTH receptor, adrenal, Guinea pig

Introduction

Vasoactive intestinal peptide (VIP), initially isolated from the porcine duodenum, exhibits a broad range of biological activity (1). Its distribution has been identified at multiple sites throughout the body, including the peripheral and central nervous system specifically associated with VIP neurons (for review, see Ref. 2). VIP has a widespread stimulating effect on the secretory activity of many endocrine cells at VIP receptors, functionally coupled to a VIP-sensitive adenylate cyclase (3). Specifically related to the adrenocortical function, VIP stimulates rat adrenal aldosterone and corticosterone secretion in a perifused capsule-glomerulosa preparation (4). VIP stimulates the rat adrenal *zona glomerulosa* in aldosterone

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secretion with a concomitant increase in both adrenaline and noradrenaline by the adrenal capsular tissue (5). Studies with the human fetal adrenal gland showed that VIP strongly stimulated the chromaffin cells to elaborate a neurite-like extension, suggesting that the effects of VIP on the adrenal cortex could be mediated by the adrenal medulla (6). Nevertheless, VIP directly stimulates a human adrenocortical cell line, NCI-H295, in cortisol secretion (7), and it has been speculated that this stimulatory steroidogenic action may be through the two types of VIP receptors, VIP_1R and VIP_2R, as their expression in the cell was observed using the RT-PCR technique (8). On the other hand, in the dispersed rat adrenocortical cells, VIP enhanced the steroidogenic activities of ACTH by interacting at the ACTH receptor (9). Furthermore, it has been reported that VIP stimulates rat adrenal glucocorticoid secretion through an ACTH receptor-dependent activation of the adenylate cyclase signaling pathway (10). Therefore, there is no consensus about how the stimulation of VIP in relation to the adrenocortical function takes place.

Although ACTH is a primary regulator, the steroidogenic function of the adrenal cortex is clearly modulated by a variety of extra-ACTH substances, possibly through a paracrine or neuronal pathway along the adjacent adrenal medulla. For instance, aldosterone secretion-inhibitory factor (ASIF) and brain natriuretic peptides significantly counteract ACTH's effect on adrenocorticoid production (11). Their mechanism of action involves in a competitive inhibition of ACTH receptor binding and consequential cAMP production (12). Likewise, we have studied VIP in the same primary culture system of guinea pig *zona fasciculata* (ZF) cells, and confirmed the previously known stimulatory action of VIP on cortisol production. Furthermore, our results have determined the ACTH receptors instead of the VIP receptors as the site of VIP action in ZF cells. The underlying mechanism is mediated through the ACTH-dependent cAMP-signaling pathway. These results are in essential agreement with the reported findings in the rat adrenal (9,10).

Materials and Methods

Peptides

A custom-synthesized molecule of guinea pig ACTH peptide, [Phe^2, Nle^4 and Ala^{24}]-ACTH(1-24), is stable for radio-iodination without losing its biological activity. Tested with ZF cells, the peptide possesses a slightly stronger steroidogenic activity than human ACTH(1-24) and ACTH(1-39). Guinea pig vasoactive intestinal peptide differs from human VIP only by four of the 28 amino acids (Leu^5, Thr^9, Met^{19}, and Val^{26}, instead of Val^5, Asn^9, Val^{19}, and Ile^{26} as in human VIP). The VIP antagonist ([Ac-Tyr^1, D-Phe^2]-growth hormone releasing factor 1-29 amide), VIP receptor antagonist ([4Cl-D-Phe^6, Leu^{17}]-human VIP), and VIP_1R (VIP receptor type I) antagonist ([Ac-His^1, D-Phe^2, Lys^{15}, Arg^{16}, Leu^{27}]-VIP(1-7)-GRF (8-27)) were purchased from Phoenix Pharmaceuticals, Inc., Belmont, CA, USA. Guinea pig ACTH and VIP were labeled in our own laboratory with isotopic ^{125}I, using chloramine-T as an oxidant. The ^{125}I-tagged peptides were purified on a Sephadex G-10 column immediately after iodination. The specific radioactivity of the freshly labeled products was calculated by the amount of radioactivity retained by the peptide peak in the column chromatogram, and was above 1,000 mCi/mmol. Aldosterone secretion-inhibitory factor, ASIF(1-39), was purchased from Sigma Chemical Co., St. Louis, MO, USA. Human brain natriuretic peptide, BNP-32, was purchased from Peninsula Laboratories, Inc., Belmont, CA, USA.

Cell Preparation and Culture

The primary culture of a guinea pig adrenocortical preparation enriched with ZF cells has been previously described in detail (13). Cell recovery from tissue dispersion and cell responsiveness to hormone treatment may vary from batch to batch, regardless of the effort made to keep identical conditions for cell preparation and incubation. In general, ZF cells isolated from three or four guinea pigs were dispersed into 32 - 48 wells, and cultured in a 1-ml medium per well. The isolated ZF cells in culture stabilized after three days in a humidified incubator, 37 °C, under 95% air and 5% CO_2. The medium was changed every 2 days prior to the experiment. The steroidogenic activity of the ZF cells reached a plateau on the 5th or 6th day in a continuous culture. Cultured in an ACTH-free medium, cell growth and function declined precipitously once the contaminating fibroblasts overwhelmed the culture on day 7.

Cloned ACTH Receptor cDNA

A guinea pig ACTH receptor (ACTH-R) cDNA of 894 nucleotides (Genbank access number AF104058) was ligated to a pcDNA3.1/V5-His-TOPO vector phage purchased from Invitrogen Corp., Carlsbad, CA, USA. The plasmid-expressing vector was used to transfect COS-7, a strain of monkey kidney mesangial cells, purchased from the Cell Bank of the National Health Research Institute, Taiwan. Unlike ZF cells, initial COS-7 cells lack both ACTH and VIP receptors, as tested by RT-PCR and ligand

Fig. 1. RT-PCR products with RNA isolated from ZF cells (lanes 2, 5 and 8), initial COS-7 cells (lanes 3, 6 and 9), ACTH-R transfected COS-7 cells (lanes 4, 7 and 10). Primer pairs used were 5'-CGAATTCTCTGTGAGGCCAGAGGTCTTAAG-3' and 5'-CTTATCAACGGATACCAGGGATCAGCCATTCTA-3' for ACTH-R (945 bp), 5'-GAAGGCCATTTATACCCTGG-3' and 5'-AAACACCATGTAGTGGACGC-3' for VIP_2R (655 bp), and 5'-ATTCTACCCACGGCAAGTTCAATGG-3' and 5'-AGGGGCGGAGATGATGACCC-3' for GAPDH (224 bp). ZF cells express both ACTH-R and VIP_2R (lanes 2 and 5, respectively). Initial COS-7 cells lack the expression of both receptors (lanes 3 and 6). Transfectant COS-7 cells express only ACTH-R (lane 4) without VIP_2R (lane 7). House-keeping GAPDH gene is expressed in all three lines of cells.

binding. A successfully transformed and stable clone of COS-7, selected with geneticin in culture, confirmed the incorporation of ACTH-R cDNA into the plasmid vector and cell chromosomes, as determined by the RT-PCR technique (Figure 1) and also by an increasing ^{125}I-ACTH binding.

Receptor Binding Test

After four days in culture, the functioning ZF cells were washed with a serum-free medium (modified McCoy's 5A medium, supplied by Life Technologies, Grand Island, NY, USA) containing 0.1% BSA, at 37 °C, for 5-10 min. Then, removing the washing medium, we carried out the binding experiment by incubating the cells for 3 hrs at 4 °C with a cold serum-free medium containing isotopic ACTH(1-24) or VIP ($1 - 8 \times 10^5$ cpm/well) alone or in combination with varied concentrations of unlabeled peptides, i.e., guinea pig ACTH, guinea pig VIP, and VIP receptor antagonist. At the end, after the medium was removed, the cells were gently washed once with phosphate-buffered saline (PBS), and then solubilized in 10% sodium dodecyl sulfate (SDS) in order to count the radioactivity retained by the cells.

A similar experimental procedure was carried out with stable transfectant COS-7 cells to test peptides for their competitive binding of ^{125}I-ACTH.

Studies of Cortisol Production

ZF cells, on the fourth day in culture, were sensitive to the 24-hr treatment of ACTH with a concentration-dependent stimulation, not only in the magnitude of cortisol production but also in the sustaining of responsiveness. Therefore, to test the effect of peptides, we simply added guinea pig ACTH, guinea pig VIP, and/or VIP receptor antagonist into the culture medium for a 24-hr exposure on day 4. The medium was changed daily and collected on three consecutive days, days 4, 5, and 6, for cortisol measurement. In one experiment, we tested the effects of ASIF and BNP-32 on the cortisol-stimulatory actions of ACTH and VIP in a similar protocol but collected only the day-4 medium for cortisol measurement. Cortisol concentrations in the cultured media were determined with a radioimmunoassay technique, using kits purchased from Diagnostic Products Corp., Los Angeles, CA, USA..

Studies of Cyclic-AMP Production

The 4-day-old ZF cells in culture were treated with a serum-free medium containing 0.1% BSA and 0.5 mM isobutyl-1-methyl-xanthine (IBMX, a diesterase inhibitor) at 37 °C for 1 - 2 hr. Then, cells were exposed to the same medium containing ACTH (1×10^{-9} M) alone or in combinations with VIP and VIP receptor antagonist (1×10^{-8} M), and incubation continued for exactly 30 min. At the end, the medium was removed and the plate was placed on ice. The cells were dissolved in cold 10% trichloroacetic acid (TCA), 0.5 ml/well. Each well was rinsed once with 0.5 ml of 10% TCA. The TCA in the combined solution was extracted out four times with an equal volume of ether each time. The TCA-free aqueous layer was lyophilized and reconstituted in an assay buffer for cyclic AMP measurement, using a RIA kit supplied by NEN Life Science Products, Inc., Boston, MA, USA.

Statistical Analysis of Data

Each group consisted of a minimum of 3 wells. The daily cortisol production, receptor binding, and cyclic AMP production of each group were expressed in mean ± S.D. Comparisons between groups were accomplished by t-tests with a significant difference defined at $P < 0.05$. The stimulatory efficacy of peptides on the 3-day cortisol production was also assessed for comparison using one-way analysis of variance (ANOVA) for significant difference, with the critical F-value set at $P < 0.05$.

Results

To test the maximal receptor binding capacity for ACTH or VIP in ZF cells, a fairly large amount of

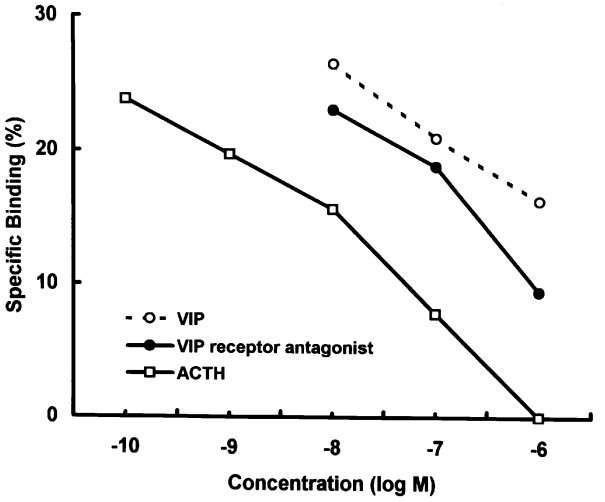

Fig. 2. Competitive binding inhibitions of ^{125}I-ACTH in ZF cells by ACTH, VIP, and VIP receptor antagonist. Cells had been cultured for four days after isolation from guinea pig adrenal glands. An average non-specific binding of 4.1% with ACTH (1×10^{-6} M) was subtracted. Each point represents the mean values of 6 wells with a S.D. less than 5% of the mean.

^{125}I-ACTH or ^{125}I-VIP (nearly 1×10^{6} cpm, equivalent to $2 - 4 \times 10^{-13}$ mole) was added to each well of ZF cells as the maximal binding control. For ACTH, the averaged binding of four control wells was 16.08%, or approximately 5×10^{-14} mole. When 1×10^{-6} M of ACTH, VIP, or VIP receptor antagonist was added to the same radioactive medium, the isotopic binding was inhibited to 5.2%, 13.62%, and 8.64%, respectively. For the test with ^{125}I-VIP, the averaged maximum binding reached only 1.4% (11,300 cpm, or approximately 4.2×10^{-15} mole) and the competitive inhibitions were 0.9%, 1.23%, and 1.09%, with 1×10^{-6} M of ACTH, VIP, or VIP receptor antagonist added, respectively. These results demonstrate that the ZF cells contained a much lower number of VIP receptors, which, surprisingly, bound the ACTH and the VIP receptor antagonist with a higher avidity than the VIP, itself. Thus, the relative affinity of ligand binding to ZF cells ranked in the order of ACTH > VIP receptor antagonist > VIP, tested with either isotopic hormone.

The concentration-dependent inhibition of ^{125}I-ACTH binding in ZF cells by ACTH, VIP, and the VIP receptor antagonist is shown in Figure 2. Since ZF cells are a primary culture, we could not use the conventional receptor-ligand binding approach to estimate the number of receptors in a single cell and differentiate the class of binding receptors. The results show some parallelism among the three peptides in binding the dominant ACTH receptors in the ZF cells, and confirm the finding that the VIP receptor antagonist is stronger than VIP in displacing radioactive ACTH.

Table 1. Competitive Inhibition of ^{125}I-ACTH * Binding in ACTH-R Transfected COS-7 Cells by ACTH, VIP and VIP-Related Peptides (1×10^{-6} M)

	cpm/well†	%
Control	10,126	100
ACTH	4,270	42
VIP	6,881	68
VIP Antagonist	7,001	69
VIP Receptor Antagonist	4,092	40
VIP Type 1 Receptor Antagonist	4,122	41

*A total of 139,100 counts added to each well.
†Mean value of 8 wells with S.D. less than 5% of the mean.

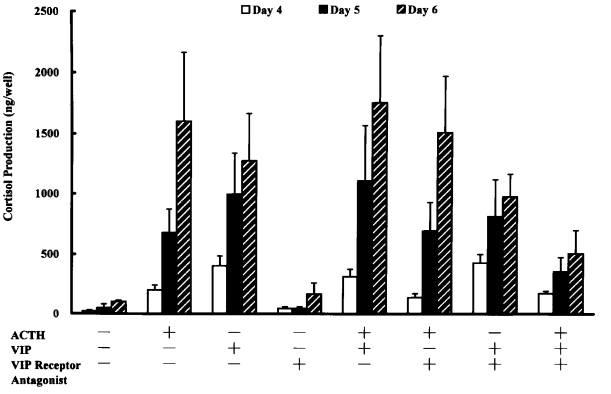

Fig. 3. Effects of ACTH (1×10^{-9} M), VIP (1×10^{-8} M), and VIP receptor antagonist (1×10^{-8} M) on the cortisol production of ZF cells on days 4, 5, and 6 in a continuous primary culture. Each bar represents mean + one S.D. (n = 5). Refer to the text for the significant differences among all the groups in detail.

To further rule out the role of VIP receptors in possible VIP action in ZF cells, the competitive inhibition of ^{125}I-ACTH binding was carried out in ACTH-R transfected COS-7 cells that lacked the VIP receptor. The results in Table 1 indicate that at their maximal concentrations of 1×10^{-6} M, the two VIP receptor antagonists competed equally as well as ACTH in displacing the isotopic ligand binding from ACTH receptors. VIP itself and the VIP antagonist are less effective, – a finding very similar to that of ZF cells. This result implies that the existence of any VIP receptors in ZF cells may not have a significant influence on hormone binding. However, it is an important finding that VIP can bind ACTH receptors and vice versa, as they displace each other.

In repeated measurements for the concentration-dependent stimulation on the cortisol production of ZF cells, VIP is at least 10-fold less potent than ACTH. Therefore, we chose the potency-equivalent concentrations of ACTH (1×10^{-9} M) and VIP ($1 \times$

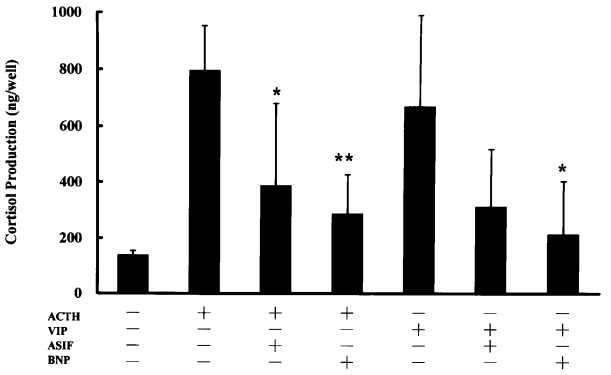

Fig. 4. Effects of ASIF (1×10^{-7} M) and BNP-32 (1×10^{-7} M) on the cortisol production of ZF cells in response to ACTH (1×10^{-8} M) and VIP (1×10^{-7} M) on day 4 in a culture. Each bar represents mean + one S.D. (n = 5). Compared to the control group, ACTH and VIP significantly stimulated cortisol production ($P < 0.001$). **P = 0.0015 and *P < 0.05, as compared to their respective ACTH and VIP groups.

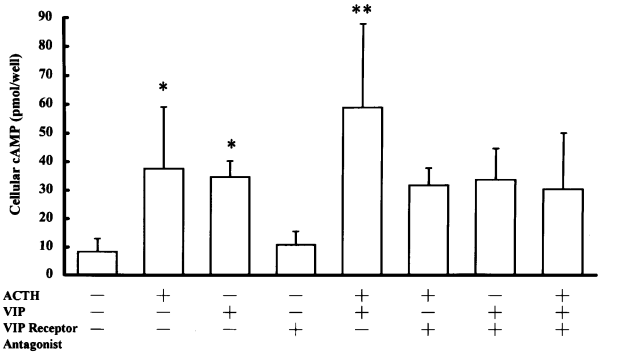

Fig. 5. Effects of ACTH (1×10^{-9} M), VIP (1×10^{-8} M), and VIP receptor antagonist (1×10^{-8} M) on the cyclic AMP production of ZF cells after four days in culture. Each bar represents mean + one S.D. (n = 5). **P < 0.005 and * P < 0.01, as compared to the control groups with or without VIP receptor antagonist.

10^{-8} M) for most experiments. The effects of ACTH, VIP, and VIP receptor antagonist on the cortisol production of ZF cells in a representative experiment are illustrated in Figure 3. Compared to the basal control wells, ACTH and VIP significantly stimulated cortisol production in magnitude during the three days ($P < 0.001$), while the VIP receptor antagonist was inactive. Compared to ACTH alone, VIP enhanced the ACTH effect on cortisol production, although the difference is not statistically significant. The VIP receptor antagonist produced no significant change with ACTH, but significantly lowered VIP activity ($P < 0.05$). The combination of all three peptides resulted in a significant cortisol reduction ($P < 0.05$), as compared to the other groups treated with ACTH or VIP alone, or in the presence of VIP receptor antagonist. The difference between the groups of ACTH plus VIP with and without the VIP receptor antagonist was highly significant ($P < 0.01$).

These results imply that ZF cells contain both ACTH receptors and VIP receptors. ACTH and VIP stimulate cortisol production through both receptors. While ACTH is a powerful stimulator, the effect of VIP depends largely on the availability of receptors. If ACTH has occupied all receptors, the effect of the less potent VIP will no longer be observable. Although VIP stimulates cortisol production through both types of receptor, the post-receptor signal is less effective than that of ACTH. The VIP receptor antagonist binds both types of receptor but is more effective in blocking VIP. Thus, the VIP receptor antagonist is able to distinguish ACTH and VIP in the cellular production of cortisol, probably due to the post-receptor difference between ACTH and VIP. Under a receptor-unsaturated condition, ACTH and VIP may enhance the steroidogenic activity in the adrenocortical cells. The addition of the third molecule, VIP receptor antagonist, somehow distorts the ligand-receptor binding of ACTH and VIP, and sufficiently disturbs the post-receptor events so as to curtail cortisol production drastically.

Figure 4 demonstrates that ACTH receptor antagonists may directly interfere with the cortisol-stimulatory effect of VIP in ZF cells. Both ASIF and BNP-32 significantly diminished the cortisol production in response to ACTH. ASIF also suppressed the cortisol levels stimulated by VIP, but the degree of suppression is not statistically significant. However, the suppression of BNP on VIP's effect on cortisol stimulation, is significant.

The effects of ACTH, VIP and the VIP receptor antagonist on the cellular cyclic AMP (cAMP) production in ZF cells in a representative experiment are illustrated in Figure 5. Again, both ACTH and VIP significantly stimulate the cellular content of cAMP, as compared to the control wells and the wells treated with VIP receptor antagonist alone ($P < 0.01$). VIP receptor antagonist has no effect on the cAMP accumulation of ZF cells in the presence of either ACTH or VIP. The combination of ACTH and VIP gives an additive effect that, however, is somewhat lowered with the addition of a VIP receptor antagonist. Although the overall profiles of cAMP and cortisol production in ZF cells do not match perfectly, the cellular cAMP increased by ACTH and VIP does reflect well with the cortisol-secretion effects of these peptides.

Discussion

Thus, our results provide convincing evidence for the cortisol-stimulatory action of VIP in ZF cells.

VIP shares the same ACTH receptor as the site of action. Furthermore, the signaling pathway through the generation of cellular cAMP seems to mediate its action.

According to the classical model, cortisol secretion is regulated by hormonal interactions among the hypothalamus, the pituitary, and the adrenal glands along the HPA axis. Within a complete HPA regulatory loop, ACTH is the principal stimulator of cortisol biosynthesis and secretion, and cortisol inhibits its own secretion by a negative feedback mechanism through ACTH (14). However, there is increasing evidence suggesting the ACTH-independent regulation of cortisol secretion (15). Many neuronal substances released from the splanchnic nerves and adrenal medulla can directly modulate the adrenocortical function via a peripheral neuroadrenocortical axis (11, 16). The neuroendocrine role of VIP, together with a regulatory action of VIP on the adrenocortical secretion of both aldosterone and cortisol independent of the HPA axis, has been proposed (16, 17). The stimulatory action of VIP on the cortisol secretion examined in the ZF cells provides an additional piece of evidence supporting an extra-ACTH regulatory model of adrenocortical function.

Unlike ACTH, the steroidogenic action of VIP is not tissue specific, as VIP was found to be an important regulatory factor of fetal rat testicular steroidogenesis (18). Besides, the widespread distribution and wide range of biological activity of VIP render it a global neurotransmitter rather than a tissue-specific hormonal regulator. Consequently, the pathophysiological implication of such a well-documented VIP action on adrenocortical steroidogenesis (4-10) has not been fully appreciated.

The tissue specificity of a hormone is mostly decided by the hormone receptors – their availability and high affinity for ligand binding. ACTH is specific to the adrenal cortex, as the ACTH receptor has not been easily found in normal organs other than the adrenal gland (19). Based on its primary structure related to evolution, the tissue distribution in the brain and gut, the functional mechanism through cAMP, and also the similarity in the seven-transmembrane receptors, VIP belongs to the pituitary adenylate cyclase-activating polypeptide (PACAP)/ glucagon superfamily (20). There are at least three big categories of PACAP receptors, distributed in almost any tissue, that bind to VIP (for review, see Ref. 20). There are two VIP receptors (VIP_1R and VIP_2R) with distinctly different amino acid sequences and similar specificity but complementary distributions in the body (21). In the guinea pig adrenal gland, we found the existence of VIP_2R only based on the RT-PCR product and its sequence (unpublished data). However, the significance of the VIP receptor related to the action of VIP in ZF cells has obviously been trivialized by the fact that VIP also actively displaces ligand from ACTH receptor binding. The most interesting finding is the stronger activity of the VIP receptor antagonist compared to VIP or VIP antagonist in the ACTH receptor binding on equal molar concentrations (Table 1). Therefore, the so-called VIP receptor antagonist (22) could actually be a functional ACTH receptor antagonist. Overall, our results confirm the earlier reported results of VIP and ACTH competing for common receptors (9,23) and that the stimulatory effect of VIP on adrenocorticoid secretion is indeed mediated through the ACTH receptor (10). This is particularly true, because the established ACTH receptor antagonists, such as ASIF and human BNP-32, are found to suppress effectively the cortisol-stimulatory action of VIP in ZF cells (Figure 4).

With regard to the ultimate mechanism of VIP action on stimulating cortisol secretion in ZF cells, our results support the notion that VIP induces an increase in cAMP via the same membrane adenylate cyclase pathway as ACTH (11). The adenylate cyclase activation is likely achieved through coupling with a Gs protein. Since the VIP receptor antagonist also binds to the ACTH receptor, but the complex produces no biological activities, a potentially important question is how does the VIP receptor antagonist couple with the G protein? The displacement of VIP or ACTH alone from ACTH receptors, by the VIP receptor antagonist, resulted in a modest reduction of biological activities as compared to individual VIP and ACTH, respectively (Figures 3 and 5). In contrast to the speculation that VIP receptor antagonist is less effective in blocking the combination of VIP and ACTH than either peptide alone, we have found that the same degree of cAMP reduction was associated with significantly more cortisol suppression. This unexpected discrepancy is puzzling and might have resulted simply from a defective experimental design. Otherwise, the signaling mechanism of the VIP receptor antagonist possibly linking to the inositol phosphate (IP) synthesis (24) in ZF cells should also be considered.

Although the physiological significance of VIP's role in the regulation of adrenocorticoid has not been easily recognized (25), the VIP normally present in the adrenal medulla and the sympathetic nerves could serve as a potential stimulator of adrenocortical functioning through a peripheral neuroendocrine pathway. In cases, in which hypercortisolemia persists without excessive ACTH (15), the high levels of VIP could be an influential factor and should been examined. We are especially interested in the extra-ACTH modulation of plasma cortisol by neuropeptides, such as BNP and VIP, in the depressed

patients who have clearly demonstrated the dexamethasone-resistant and ACTH-independent hypercortisolism (26).

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