Vasoactive intestinal peptide (VIP) stimulates cortisol secretion from the H295 human adrenocortical tumour cell line via VPAC1 receptors

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Abstract

Vasoactive intestinal peptide (VIP) shows a wide tissue distribution and exerts numerous physiological actions. VIP was shown in a dose-dependent manner to increase cortisol secretion in the NCI-H295R human adrenocortical carcinoma (H295) cell line (threshold dose 3·3×10−10 M, maximal dose 10−7 M), coupled with a parallel increase in cAMP accumulation. Receptor-specific agonists were employed to determine which of the two known VIP receptor subtypes was involved in cortisol secretion. Treatment with the VPAC1 receptor agonist, [K15, R16, L27]VIP(1–7)/GRF(8–27), produced a dose-dependent increase in H295 cell cortisol secretion (threshold dose 10−11 M, maximal dose 10−7 M) similar to that seen with VIP. Meanwhile, the high-affinity VPAC2 receptor agonist, RO-25–1553, failed to stimulate significantly cortisol or cAMP production from H295 cells. Inhibition of VIP-mediated H295 cell cortisol secretion by PG97–269, a competitive VPAC1-specific antagonist, produced parallel shifts of the dose-response curve and a Schild regression slope of 0·99, indicating competitive inhibition at a single receptor subtype. VIP is known also to interact with the PAC1 receptor, albeit with lower affinity (EC50 of ∼200 nM) than the homologous ligand, PACAP (EC50 of ∼0·5 nM). PACAP stimulated cortisol secretion from H295 cells (EC50 of 0·3 nM), suggesting the presence of functional PAC1 receptors. However, stimulation of cortisol secretion by nanomolar concentrations of VIP (EC50 of 5 nM), coupled with real-time PCR estimation that VPAC1 receptor transcripts appear 1000-fold more abundant than PAC1 transcripts in H295 cells, makes it unlikely that VIP signals via PAC1 receptors. Together, these data suggest that VIP directly stimulates cortisol secretion from H295 cells via activation of the VPAC1 receptor subtype.

Introduction

Vasoactive intestinal peptide (VIP), a 28 amino acid peptide widely distributed in the peripheral and central nervous system, belongs to a family of regulatory peptides that includes secretin, glucagon, pituitary adenylate cyclase-activating polypeptide (PACAP), growth hormone-releasing hormone, gastric inhibitory peptide and parathyroid hormone (Laburthe et al. 2002). Having been isolated originally from the porcine gastrointestinal system (Said & Mutt 1970), where it was found to have vasoactive and secretory properties, actions of VIP have been described in the digestive tract, the brain, the endocrine glands, and the reproductive, immune and cardiovascular systems of man and other mammals. Pathophysiological roles for VIP have been suggested in a variety of tumours and neurodegenerative diseases (Laburthe et al. 2002). VIP has also been shown to play a role in mouse fetal growth (Gressens et al. 1993) and embryonic brain development of rats (Gozes et al. 1999). More recently, VIP has been reported to participate in pain perception (Dickinson & Fleetwood-Walker 1999), the suppression of inflammation (Said 2000) and regulation of the mammalian circadian clock (Harmar et al. 2002, Piggins & Cutler 2003).

The actions of the VIP family of peptides are initiated by interaction with specific receptors belonging to the G protein-coupled receptor superfamily, whose signalling mechanism involves the activation of adenylate cyclase/protein kinase A
and increased cAMP formation (for review, see Laburthe et al. 2002). Due to their closely related amino acid sequences (Miyata et al. 1990), VIP and PACAP share a receptor, the VPAC receptor, for which they display a similar affinity and potency (Shivers et al. 1991, Ishihara et al. 1992). Two VPAC receptor subtypes have been identified and cloned in the human: the VPAC1 (Sreedharan et al. 1993, Couvain et al. 1994) and VPAC2 receptors (Svoboda et al. 1994, Adamou et al. 1995). In addition, VIP can activate the PACAP-specific PAC1 receptor but with 100–1000-fold less potency than PACAP itself (Miyata et al. 1990).

Several putative neurotransmitters, including VIP, have been implicated as regulators of adrenal steroidogenesis (Edwards & Jones 1993). Stimulation of steroid secretion in response to VIP was noted from perfused rat and porcine adrenal glands (Ehrhart-Bornstein et al. 1991, Hinson et al. 1992, 1994). In these systems, the steroidogenic effect was attributed to an indirect action of VIP governed by VIP-stimulated catecholamine release from medullary chromaffin cells, followed by subsequent stimulation of steroidogenesis by the action of catecholamines on adrenocortical cells. The presence of such an indirect mechanism for VIP-stimulated steroidogenesis was supported by the observation that VIP stimulated release of catecholamines from intact adrenal glands in rats and dogs (Wakade et al. 1991, Yamaguchi 1993). In addition, VIP-stimulated aldosterone and corticosterone secretion from rat adrenals was shown to be attenuated by β-adrenoceptor antagonists (Bernet et al. 1994). By autoradiographic detection, Cunningham and Holwarth (1989) showed the presence of VIP-binding sites throughout the rat adrenal cortex. These were concentrated in the subcapsule/zona glomerulosa zone but were present throughout the cortex, suggesting a direct action of VIP on adrenocortical cells. Using in situ hybridisation, Usdin et al. (1994) demonstrated the presence of both VPAC1 and VPAC2 receptors in the rat adrenal gland. Mazzocchi et al. (2002) found that the human adrenal expressed VPAC1 and VPAC2 receptor mRNA in both the zona glomerulosa and adrenal medulla, whereas the zona fasciculata/reticularis did not express detectable levels of VPAC receptor mRNA, though in neither case were these results substantiated at the pharmacological level.

To date, the use of dispersed cell preparations to probe VIP-mediated steroidogenesis has produced conflicting results. Hinson et al. (1992) noted that VIP did not stimulate steroid secretion from dispersed rat adrenocortical cells, whereas Bornstein et al. (1996) reported steroid secretion in response to VIP from dispersed human adrenal cells. They were, however, unable to draw firm conclusions about the possible direct action of VIP because VIP-mediated steroidogenesis was attenuated, though not entirely eliminated, by a β-adrenoceptor antagonist. It was postulated that the steroidogenic effect of VIP was partially mediated by catecholamine release from interspersed chromaffin cells detected within the culture.

In this study, we aim to circumvent the problem of medullary chromaffin cell contamination of dispersed adrenocortical cell preparations by using the established human NCI-H295 (H295) adrenocortical cell line to determine whether VIP can act directly on adrenocortical cells to stimulate steroidogenesis. The H295 cell line was derived from an invasive primary human adrenocortical carcinoma, and it can express appropriate complements of human adrenocortical enzymes and thus is capable of producing glucocorticoids, mineralocorticoids and androgens after suitable treatment regimens (Rainey et al. 1994). The presence of transcripts for both the VPAC1 and VPAC2 receptors has previously been reported in the H295 human adrenocortical carcinoma cell line (Haidan et al. 1998), but the role of specific VIP receptor subtypes in VIP-mediated steroidogenesis was not investigated.

Having demonstrated that VIP is indeed capable of triggering H295 cell steroidogenesis, we use a series of pharmacological tools, including the VPAC1 receptor agonist, [K15, R16, L27]VIP(1−7)/GRF(8−27) (Gourlet et al. 1997b), the long-acting VPAC2 receptor agonist, RO-25–1553 (Gourlet et al. 1997c), and the highly selective and competitive VPAC1 receptor antagonist, PG 97–269 (Gourlet et al. 1997a), to determine whether or not VIP-mediated H295 cell steroidogenesis requires activation of an authentic VIP receptor subtype.

Materials and methods

Cell culture

H295 cells (CRL-2128, ATCC, Rockville, MD, USA) were seeded into 12-well tissue culture plates (5 × 10⁵ cells per well) and maintained in Dulbecco’s modified Eagle’s medium/F12...
containing 2% Ultroser HY (Biosepra, France), 5 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml sodium selenite (as 1% ITS, Invitrogen) at 37 °C with 5% CO₂–95% air. For experiments, the cells were pre-treated in the above medium with forskolin (10⁻⁵ M) for 72 h, replacing the medium every 24 h. The cells showed an increased responsiveness to agonists, including forskolin, after this 72 h pre-treatment (Cobb et al. 1996). Forskolin has been shown to upregulate the expression and activity of CYP17, promoting the production of glucocorticoids over mineralocorticoids (Rainey et al. 1993). After pre-treatment, the cells were washed with Earle’s balanced salt solution (EBSS, Invitrogen), incubated for 1 h in serum-free medium and finally exposed to fresh serum-free medium containing either VIP (Sigma, Poole UK) with or without PG97–269, PACAP (Calbiochem, Nottingham, UK), or the VIP receptor agonists for 4 h at 37 °C. The VIP receptor agonists and antagonist were generous donations from Professor Robberecht (Université Libre de Bruxelles, Brussels, Belgium). After 4 h, the medium was removed and assayed for cortisol and cAMP content. The cell monolayer was washed with EBSS and taken up into 0·3 M NaOH with 0·1% SDS for subsequent protein determination by the Bradford method (Bradford 1976).

Radioimmunoassay

Cortisol and cAMP were measured as described previously (Williams et al. 1989). Briefly, iodinated cortisol (Amersham Pharmacia, Amersham, UK) diluted in 0·1 M citrate buffer, was mixed with the culture medium and anticitisol antibody (Diagnostics Scotland, Edinburgh, UK) for 60 min at 37 °C. The tubes were then centrifuged at 1800 g for 30 min, and the supernatant was decanted. Pelleted antibody-bound counts were measured with a gamma-counter. For cAMP measurement, the experimental medium was incubated overnight at 4 °C with the cAMP antibody and iodinated cAMP. Separation was carried out in a dextran-coated charcoal suspension.

Real-time PCR

H295 cell RNA was extracted with the RNeasy Midi Kit (Qiagen, Crawley, West Sussex, UK) following the manufacturer’s instructions. Samples were DNase treated to eliminate genomic DNA contamination of the final PCR assay. Total RNA concentrations of the samples were determined by measuring absorbance at 260 nm. An amount of 1 µg of total RNA was reverse-transcribed with random hexamers primers and the Taqman reverse transcription system (Applied Biosystems (ABI), Warrington, UK). The resulting cDNA samples were subjected to real-time quantitative PCR, performed on the ABI Prism 7900 sequence detection system with fully validated Assay-on-Demand Primers (ABI). The assay numbers were as follows: Hs00270351_ml (VPAC1) and Hs00153869_ml (PAC1). The thermal cycler conditions were 2 min hold at 50 °C and 10 min hold at 95 °C, followed by 40 cycles of 15 s at 95 °C (denaturation) and 1 min at 60 °C (annealing/extension). Experiments were performed in duplicate for each sample. Analyses were performed using ABI Prism 7900 system software, the fold difference in expression being calculated using the ΔCₜ method with 18S as an internal reference (ABI Prism 7900 sequence detection manual).

Statistical analysis

Results are expressed as means of triplicate wells ± s.d. from a minimum of three independent experiments. Statistical analyses were performed with an unpaired, two-tailed Student’s t-test. Dose-response curves in the presence or absence of antagonists were tested for parallelism. For competitive antagonists, a plot of log (dose ratio-1) versus log[antagonist] should be linear with a gradient of 1 (Rang et al. 1999). Schild regression analysis was performed with Graphpad Prism 3·0 (Graphpad Software Inc, San Diego, CA, USA).

Results

H295 cells treated with VIP (concentration range 10⁻¹¹–10⁻⁶ M) for 4 h produced a dose-dependent increase in cortisol secretion (Fig. 1A). The threshold effect occurred at 3·3 × 10⁻¹⁰ M, reaching a maximum 8·2 (± 1·0)-fold increase over basal at 10⁻⁷ M. The maximal cortisol effect of VIP was equivalent to that produced by 10⁻⁵ M forskolin, a direct activator of adenylate cyclase (Fig. 1A). VIP treatment of H295 cells also
produced a concentration-dependent increase in cAMP accumulation, with the threshold effect occurring at $10^{-11}$ M, reaching a maximum $14.5 \pm 7.5$-fold increase over basal with $10^{-7}$ M VIP. Again the maximal cAMP response was similar to that induced by $10^{-5}$ M forskolin (Fig. 1B). Parallel investigations failed to show any VIP concentration-dependent increase in cGMP levels or phosphoinositide turnover (data not shown).

The biological effects of VIP are mediated by two distinct VIP receptor subtypes, denoted VPAC1 and VPAC2. Receptor subtype-selective agonists and antagonists were therefore employed to determine which of these receptor subtypes is involved in VIP-induced cortisol secretion from the H295 cells. The VPAC1 receptor agonist, \([K^{15}, R^{16}, L^{27}]VIP(1–7)/GRF(8–27)\), produced a concentration-dependent increase in cortisol secretion similar to that seen with VIP (Fig. 2). The EC\textsubscript{50} value for the VIP receptor agonist was 1 nM, in agreement with that seen by Gourlet et al. (1997b). Meanwhile, the long-acting VPAC2 agonist, RO-25–1553 ($10^{-9}$, $10^{-8}$ M), failed to stimulate cortisol levels over basal in H295 cells (Fig. 3). An increase was seen with $10^{-7}$ M RO-25–1553; however, this was found to be non-significant and of a much lesser magnitude than that caused by either $10^{-8}$ M VIP or the VPAC1 receptor agonist (Fig. 2).

For further investigation of whether VIP was working via the VPAC1 receptor, the competitive VPAC1 receptor-specific antagonist, PG97–269, was used. In isolation, PG97–269 had no stimulatory effect on H295 cell cortisol secretion.
However, PG97–269 produced parallel shifts of the log dose-response curve of VIP-stimulated cortisol release and produced a linear Schild regression with a slope of 0·99, not significantly different from unity, consistent with competitive antagonism of VPAC1-mediated cortisol release from H295 cells (Fig. 4B).

Treatment with PACAP38 (10^{-12}–10^{-8} M) increased cortisol secretion from H295 cells in a concentration-dependent manner, suggesting the presence of functional PCAP1 receptors and a possible role in steroidogenesis (Fig. 5). The threshold concentration for PACAP38 was 10^{-11} M, with the maximal cortisol secretion occurring at 10^{-9} M, these being distinct from the demonstrated VIP-mediated cortisol secretion threshold and maxima (Figs 1 and 5). Initial Schild regression analysis of PACAP38-mediated cortisol secretion by the competitive VPAC1 antagonist PG97–269 produced a slope of 0·77, suggesting incomplete competitive antagonism of PACAP38-mediated cortisol secretion by PG97–269 concentrations that inhibit VIP-mediated cortisol secretion. This suggests a probable involvement of a PACAP-specific receptor subtype in PACAP38-signalling in H295 cells, in addition to signalling via VPAC1 (data not shown).

To assess a possible contribution of the PAC1 receptor to the VIP-mediated cortisol response in H295 cells, real-time PCR, employing fully...
validated receptor-specific ‘Assay-on-Demand’ primer sets, was used to estimate the relative abundance of H295 cell VPAC1 and PAC1 receptor transcripts. The average number of PCR cycles (cycle threshold \( C_T \)) required for VPAC1 receptor transcripts to reach the machine-determined fluorescence threshold was found to be 28, compared with a \( C_T \) of 38 for PAC1 receptor transcripts and 16 for the internal 18S RNA control. Since \( C_T \) is proportional to the logarithm of the initial amount of transcript target, and each cycle represents a product doubling, the relative concentration of VPAC1 to PAC1 receptor transcripts is represented by \( 2^{C_{T_{VPAC1}} - C_{T_{PAC1}}} \).

In this case, the determined \( C_{T_{VPAC1}} - C_{T_{PAC1}} \) of 10 represents a 1000-fold higher abundance of VPAC1 receptor transcripts over those for the PAC1 receptor.

**Discussion**

VIP treatment caused a concentration-dependent increase in cortisol secretion from H295 cells. A concomitant increase in cAMP was observed in the absence of change in cGMP levels or phosphoinositide turnover in response to VIP treatment, ruling out the likely involvement of either of these second messenger pathways. It can, therefore, be concluded that VIP regulates steroidogenesis in H295 cells principally via a cAMP-dependent mechanism.

The effects of VIP at the cellular level are mediated principally through interaction with two receptor subtypes, VPAC1 and VPAC2, although it can also bind the PAC1 receptor, albeit with a 100–1000-fold lower affinity than PACAP (Miyata et al. 1990). Previous work has demonstrated the presence of both VPAC1 and VPAC2 receptor transcripts in the H295 cell line (Haidan et al. 1998), but without showing which of these receptor subtypes was directly involved in VIP-mediated steroidogenesis. We have used specific receptor agonists/antagonist to determine whether one or both of these receptors are coupled to cortisol production in the H295 cell line. The VPAC1 receptor agonist, \([K^{15}, R^{16}, L^{27}]VIP(1–7)/GRF(8–27)\), has a low affinity for both the VPAC2 and PACAP receptors and has been shown to have an EC\(_{50}\) of approximately 1 nM for the VPAC1 receptor in humans (Gourlet et al. 1997b). Treatment of H295 cells with the VPAC1 receptor agonist did indeed produce a dose-dependent increase in cortisol secretion, with an EC\(_{50}\) of approximately 1 nM, strongly suggesting the presence of functional VPAC1 receptors in H295 cells. Meanwhile, RO-25–1553 is a highly selective VPAC2 receptor agonist, having been shown to be 3-fold more potent than VIP on the human VPAC2 receptor, while being respectively 600-fold and 10-fold less potent than VIP on the human VPAC1 and PAC1 receptors (Gourlet et al. 1997c).

In this study, \(10^{-9}\) or \(10^{-8}\) M RO-25–1553 failed to stimulate cortisol secretion from H295 cells above basal levels. A non-significant increase in basal cortisol secretion was observed with \(10^{-7}\) M RO-25–1553; however, this effect was much smaller than that seen in response to VIP or the VPAC1 receptor agonist and was probably related to the partial agonist action of high concentrations of RO-25–1553 on human VPAC1.
receptors (Gourlet et al. 1997c). Collectively, these results suggest that VIP-induced steroidogenesis in H295 cells is mediated by activation of VPAC1 receptor subtypes rather than VPAC2 receptor subtypes.

We further sought to investigate whether VIP was working via the VPAC1 receptor, by utilising the known competitive VPAC1 receptor-specific antagonist PG97–269, a chimeric VIP/GRF derivative that has negligible affinity for the VPAC2 or PAC1 receptors (Gourlet et al. 1997a). Schild analysis (Rang et al. 1999) can be used to determine whether pharmacological antagonists impede agonist-mediated receptor activation in a competitive or non-competitive manner. Treatment of H295 cells with PG97–269, in combination with VIP stimulation, produced classic right parallel shifts of VIP dose-response curves with a dose ratio of 0·99. A theoretical Schild slope equal to 1 indicates that the antagonism is competitive and reversible and also that the agonist is acting at a single receptor subtype and that the tissue has no uptake mechanism for the agonist (Rang et al. 1999). Thus, the experimentally determined Schild slope of 0·99 for antagonism of VIP-mediated cortisol secretion by the known competitive VPAC1 receptor-specific antagonist PG97–269 strongly supports the contention that VIP directly stimulates H295 cell steroidogenesis via VPAC1 receptor-mediated activation of the cAMP second messenger signalling pathway, rather than via a VPAC2 receptor.

VIP can also act via the PAC1 receptor (EC50 of 200 nM), whereas PACAP is capable of activating PAC1 receptors with an EC50 of 0·5 nM (Corbitt et al. 2002). The current demonstration that PACAP was able to stimulate cortisol secretion with a similar EC50 of 0·3 nM, implies the presence of a population of functionally coupled PAC1 receptors in the H295 cells. However, the partial competitive inhibition of PACAP-mediated cortisol secretion by the competitive VPAC1 receptor antagonist PG97–269, denoted by a Schild regression result of 0·77, suggests that PACAP may be acting via a combination of both VPAC1 and PAC1 receptors. This latter conclusion is supported by real-time PCR, demonstrating that VPAC1 receptor transcripts appear approximately 1000-fold more abundant than the PAC1 receptor transcripts in H295 cells. Conversely, the small number of PAC1 compared to VPAC1 receptors, coupled with their relatively much lower affinity for VIP (EC50 of 200 nM) (Corbett et al. 2002), makes it unlikely that VIP signals to a significant extent via PAC1 receptors. Together with the stimulation of cortisol secretion from H295 cells by a VPAC1, but not a VPAC2, agonist and the reversible competitive inhibition of VIP-mediated H295 cell cortisol secretion by the VPAC1 receptor-specific antagonist, PG97–269, this leads to the conclusion that VIP-stimulated cortisol secretion from H295 cells is principally mediated by activation of the VPAC1 receptor subtype.

It has previously been suggested that VIP exerts an indirect effect on the adrenal cortex via catecholamine release from medullary chromaffin cells and subsequent activation of β-adrenoceptors on adrenocortical cells, rather than acting directly on adrenocortical VIP receptors. Studies utilising dispersed adrenocortical cell preparations have been uninformative in this regard because contamination with medullary chromaffin cells could not be ruled out. The present study has circumvented this difficulty by showing that VIP directly stimulates cortisol production from the established human NCI-H295R (H295) adrenocortical carcinoma cell line, and that this occurs via activation of the VPAC1 receptor subtype. From the physiological standpoint, it is significant that the presence of both VIP and VPAC1 receptor mRNA has been established in the human adrenal cortex and has been suggested to play a paracrine role in physiological aldosterone release from the zona glomerulosa (Mazzocchi et al. 2002). The present finding of VPAC1 receptor involvement in the promotion of cortisol release in H295 cells is consistent with the multipotent nature of this human adrenocortical cell line and underscores its usefulness as a model system for steroidogenesis in the human adrenal cortex.

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