Actions of vasoactive intestinal peptide on the rat adrenal zona glomerulosa

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Abstract

Previous studies, by this group and others, have shown that vasoactive intestinal peptide (VIP) stimulates aldosterone secretion, and that the actions of VIP on aldosterone secretion by the rat adrenal cortex are blocked by β adrenergic antagonists, suggesting that VIP may act by the local release of catecholamines. The present studies were designed to test this hypothesis further, by measuring catecholamine release by adrenal capsular tissue in response to VIP stimulation.

Using intact capsular tissue it was found that VIP caused a dose-dependent increase in aldosterone secretion, with a concomitant increase in both adrenaline and noradrenaline release. The effects of VIP on aldosterone secretion were inhibited by atenolol, a β_1 adrenergic antagonist, but not by ICI-118,551, a β_2 adrenergic antagonist. Binding studies were carried out to investigate VIP receptors. It was found that adrenal zona glomerulosa tissue from control rats contained specific VIP binding sites ($B_{\max}$ 853 ± 101 fmol/mg protein; K_d 2.26 ± 0.45 nmol/l). VIP binding was not displaced by ACTH, angiotensin II or by either of the β adrenergic antagonists.

The response to VIP in adrenals obtained from rats fed a low sodium diet was also investigated. Previous studies have found that adrenals from animals on a low sodium diet exhibit increased responsiveness to VIP. Specific VIP binding sites were identified, although the concentration or affinity of binding sites in the low sodium group was not significantly different from the controls. In the low sodium group VIP was found to increase catecholamine release to the same extent as in the control group, however, in contrast to the control group, the adrenal response to VIP was not altered by adrenergic antagonists in the low sodium group.

These data provide strong support for the hypothesis that VIP acts by the local release of catecholamines in adrenal zona glomerulosa tissue in normal animals. It does not appear that VIP acts through the same mechanism in animals maintained on a low sodium diet. The mechanism by which VIP stimulates aldosterone in this group remains to be determined.

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Introduction

A range of neuropeptides is present in the rat adrenal cortex (for review see Vinson *et al.* 1994, Toth & Hinson 1995, Nussdorfer 1996, Ehrhart-Bornstein *et al.* 1998). Of these peptides vasoactive intestinal peptide (VIP) has been the most extensively studied. VIP has been shown to be present in nerves supplying the capsule and zona glomerulosa region of the adrenal gland (Hökfelt *et al.* 1981, Holzwarth 1984) and has been shown to exert a significant influence on zona glomerulosa function. Prolonged infusion of VIP stimulates the growth and secretory capacity of the zona glomerulosa of rats and infusion of a VIP antagonist suppresses zona glomerulosa growth and secretory capacity (Rebuffat *et al.* 1994). Experiments *in vitro* have given different results, depending on the tissue preparation used: it has been shown that VIP stimulates

aldosterone secretion by rat adrenal capsular tissue (Cunningham & Holzwarth 1988) and by the intact perfused rat adrenal gland (Hinson *et al.* 1992, 1994). However, experiments investigating the actions of VIP using dispersed zona glomerulosa cells from normal rats failed to demonstrate stimulation of aldosterone secretion (Enyedi *et al.* 1983, Hinson *et al.* 1992).

In 1992 we proposed a hypothesis to account for the differences in responsiveness of the different adrenal preparations (Hinson *et al.* 1992). We proposed that VIP acts on islets of chromaffin cells, known to be present in the zona glomerulosa in the rat (Kovács & Horváth 1973, Palacios & Lafraga 1975, Gallo-Payet *et al.* 1987) and other species (for review see Nussdorfer 1996), to stimulate the release of catecholamines, which then act on adrenal cells to stimulate aldosterone secretion. This hypothesis was supported by the observation that aldosterone secretion in

response to VIP was attenuated in the presence of alprenolol, a β adrenergic antagonist (Hinson *et al.* 1992). Similar observations have subsequently been made by other groups (Mazzocchi *et al.* 1993, Bernet *et al.* 1994), but to date no evidence has been presented to show a relationship between catecholamine release and aldosterone secretion. In addition, the possibility that β adrenergic antagonists might alter VIP binding to its receptor has not been investigated.

The present studies were designed to investigate the actions of VIP and catecholamines on zona glomerulosa function. These studies have employed both normal rats, and rats fed a low sodium diet, as there is evidence that the adrenal response to VIP is altered following dietary sodium depletion (Hinson & Kapas 1995). We have investigated the effects of VIP on catecholamine release by adrenal capsular tissue, and we have used ligand binding studies to investigate the specificity of VIP binding to the zona glomerulosa.

Materials and Methods

Materials

VIP and the VIP receptor antagonist, [4-Cl-D-Phe⁶,Leu¹⁷]-VIP (PL-VIP) were obtained from Bachem (UK) Ltd, Saffron Walden, Essex, UK; ICI 118 551 (($\pm$)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[1-methylethylamino]-2-butanol hydrochloride) was from Semat Technical (UK) Ltd, St Albans, Herts, UK; collagenase (Worthington type I) from Lorne Laboratories Ltd, Reading, Berks, UK. All radiolabels were obtained from Amersham International plc, Amersham, Bucks, UK. All other chemicals were of analytical grade obtained from Sigma Chemical Co., Poole, Dorset, UK or BDH, Dagenham, Essex, UK.

Sodium depletion of animals

Male and female Wistar rats (250–400 g body weight), supplied by A Tuck & Sons, Battlesbridge, Essex, UK and maintained at Queen Mary and Westfield College, were used. Animals were depleted of sodium, as previously described (Fattah *et al.* 1978) by feeding them a diet of wholemeal flour supplemented with 100 nmol calcium carbonate/kg for a minimum period of 21 days, with access to distilled water. Control animals were fed the same diet with the addition of 170 mmol sodium chloride/kg. These experiments were authorised by Home Office project licence number 70/00063.

Preparation of rat adrenal capsules

The rats were stunned and then killed by cervical dislocation. Adrenals were rapidly removed and cleaned of

adhering fat. Capsule fractions (with mainly glomerulosa cells attached) were separated from inner adrenocortical tissue by pressure between glass plates. Capsules were preincubated in Krebs bicarbonate Ringer containing glucose (200 mg/100 ml) (KRBG) for 1 h at 37 °C under an atmosphere of 95% O₂ and 5% CO₂. After preincubation capsules were incubated in fresh KRBG for 1 h under an atmosphere of 95% O₂ and 5% CO₂ in the absence or presence of stimulants $\pm$ inhibitors.

Effect of agonists $\pm$ inhibitors on steroidogenesis

All peptides were dissolved in KRBG to the required concentrations. A range of concentrations of VIP, from 10^{-10} mol/l to 10^{-6} mol/l were used. In other experiments, to test the action of antagonists on VIP-stimulated aldosterone secretion, capsules were incubated with various concentrations of VIP in the presence of 10^{-7} mol/l antagonist for 1 h. After incubation, the capsules were discarded and half the incubation media were placed into clean microfuge tubes and the tubes stored at -20 °C until the media were assayed for aldosterone. Aldosterone was measured in an aliquot of unextracted incubation medium by direct radioimmunoassay (RIA; Kapas *et al.* 1992). A small volume of acetic acid was added to the remaining incubation media (final concentration 10%, v/v) and the media assayed for catecholamines.

Catecholamine assay

The assay of adrenaline and noradrenaline is based on the trihydroxyindole fluorescence method of Brocklehurst & Pollard (1990). The technique involves the oxidation of catecholamines by K₂Fe(CN)₆ and the subsequent generation of the trihydroxyindole fluorophore product by NaOH and ascorbic acid. The oxidation reactions are performed under both acidic and neutral pH conditions to allow measurement of adrenaline and noradrenaline. Briefly, assays were carried out as follows. To one set of 50 μ l samples 500 μ l 0.5 mol/l sodium phosphate buffer (pH 7.0) were added. To another (identical) set of samples 500 μ l 10% (v/v) acetic acid were added. All samples received 50 μ l K₂Fe(CN)₆ and were incubated on ice for 20 min. The reactions were terminated with 1 ml 9 mol/l NaOH containing 0.4% ascorbic acid (w/v), followed by vigorous vortexing. After the addition of 2 ml water, the trihydroxyindole fluorescence product was measured in a spectrofluorimeter (Luminescent fluorimeter LS-50B, Perkin Elmer, Warrington, Lancs, UK) with an excitation wavelength of 412 nm and an emission wavelength of 523 nm.

Receptor binding assay

The binding assay was carried out as previously described (Kapas *et al.* 1996). Rat adrenal capsules were prepared as

described above. After incubation, capsules were homogenised in 150 mmol/l Tris-HCl buffer (pH 7.6) containing 1 µg/ml each of aprotinin and soybean trypsin inhibitor. Homogenates were centrifuged at 800 g for 10 min and the supernatant re-centrifuged at 100 000 g for 1 h. The particulate (membrane) fraction was then resuspended in Tris-HCl buffer containing 100 mmol/l NaCl, 6 mmol/l MgCl₂, 0.1% (w/v) BSA and protease inhibitors (1 µg/ml each of soybean trypsin inhibitor and aprotinin).

Aliquots of membrane suspension (100 µg protein/tube) were incubated with 3-(¹²⁵I-iodotyrosyl)¹⁰VIP (2000 Ci/nmol; final concentration 0.1 nmol/l) with increasing concentrations of unlabelled VIP, PL-VIP, atenolol, ICI 118 551, angiotensin II and adrenocorticotrophic hormone (ACTH) (0.39–50 nmol/l). Non-specific binding was determined by incubating labelled cells with 100-fold excess of unlabelled VIP. Incubations were terminated by the addition of 800 µl ice-cold buffer and the tubes were centrifuged at 10 000 g for 5 min at 4 °C. Supernatants were discarded and the pellets washed twice. After washing, radioactivity bound to the membrane was estimated using a 1272 Clinigamma counter (LKB Wallac, St Albans, Herts, UK). Binding studies were repeated at least three times.

Statistical analysis

Arithmetic means and S.E.M. values were calculated. One-way analysis of variance was used to test whether VIP had a significant effect on basal (control) levels of aldosterone or catecholamine release as appropriate. Student's *t*-tests were used to test whether the above-mentioned responses were affected by the presence of antagonists. Saturation data were analysed by LIGAND (Munson & Rodbard 1980). Student's *t*-tests were also used to test whether the responses of the sodium-depleted group were significantly different to those of the control diet group.

Results

VIP was found to cause a dose-dependent increase in both aldosterone and catecholamine secretion by rat adrenal capsular tissue *in vitro* (Fig. 1). Release of both adrenaline and noradrenaline was observed, with the increase in adrenaline seen in parallel with aldosterone secretion. The minimum concentration of VIP required for significant stimulation of aldosterone and adrenaline was 1 nmol/l, although 10 nmol/l were required for significant noradrenaline secretion. The effects of VIP on aldosterone secretion were inhibited by the β₁ antagonist, atenolol (100 nmol/l), but not by ICI 118 551, a β₂ antagonist (Fig. 2). The effects of VIP were totally specific to the zona glomerulosa: there was no effect of VIP on corticosterone release by inner zone tissue (data not shown).

Figure 1 Effects of increasing concentrations of VIP on aldosterone secretion (Δ), adrenaline (□) and noradrenaline (○) release by rat adrenal capsules. Values are means ± S.E.M. (n=6). ***P*<0.01, ****P*<0.001 (analysis of variance).

Figure 2 Effects of atenolol (100 nmol/l; hatched bars), a β₁ adrenergic antagonist, and ICI 118 551 (100 nmol/l; speckled bars), a β₂ adrenergic antagonist, on the aldosterone response to VIP (100 nmol/l) stimulation. Open bars, absence of inhibitor. Values are means ± S.E.M. (n=6). ****P*<0.001 (analysis of variance).

Ligand binding studies revealed a single population of VIP receptors in both control and the low sodium animals (Fig. 3). The Hill coefficient for the control group was 0.908, and for the low sodium group 0.925. The VIP

Figure 3 Hill plot of VIP binding to adrenal capsular homogenates from (a) control animals and (b) animals maintained on a low sodium diet.

receptors from both groups were found to have a similar K_d : 2.26 ± 0.45 nmol/l in the control group and 1.62 ± 0.32 nmol/l in the low sodium group. While the low sodium group had a higher number of binding sites than the controls (944 ± 153 vs 853 ± 101 fmol/mg protein), this difference was not statistically significant. Displacement studies showed that VIP binding to homogenates of adrenal capsules from the control or low sodium group was not significantly displaced by ACTH, angiotensin II, atenolol or ICI 118 551 (Table 1).

In adrenal capsular tissue obtained from sodium-depleted animals VIP significantly stimulated aldosterone secretion (Fig. 4). The basal level of aldosterone secretion was considerably greater in the sodium-depleted adrenals compared with the controls. In the capsules from sodium-depleted animals VIP also significantly stimulated the release of adrenaline and noradrenaline (Fig. 5). There

were no significant differences between levels of catecholamine release, either basal or stimulated, in the sodium-depleted group compared with the controls. However, in the sodium-depleted adrenal capsules the aldosterone response to VIP stimulation was not attenuated by atenolol (Fig. 4).

Table 1 Effects of various agents (50 μ mol/l) on displacement of 125 I-labelled VIP binding to capsular homogenates from control rats and from rats maintained on a low sodium diet

Agent	125 I-VIP displaced	
	Controls	Low sodium
VIP	$100 \pm 15.0^{***}$	$100 \pm 10.0^{***}$
PL-VIP	$95 \pm 7.0^{***}$	$97 \pm 10.0^{***}$
ACTH	2 ± 0.4	3 ± 0.8
Angiotensin II	1 ± 0.3	3 ± 1.0
Atenolol	5 ± 0.8	4 ± 1.0
ICI 118 551	3 ± 1.0	3 ± 0.6

*** $P < 0.001$ (analysis of variance) compared with control.

Figure 4 The β_1 adrenergic antagonist, atenolol (100 nmol/l; hatched bars), had no effect on the aldosterone response to VIP (100 nmol/l) in adrenals obtained from animals maintained on a low sodium diet. Values are means $\pm$ S.E.M. ($n=6$).

Figure 5 Effect of VIP (100 nmol/l) on (a) adrenaline and (b) noradrenaline release by adrenal capsules from control animals (open bars) and animals maintained on a low sodium diet (hatched bars). Values are means $\pm$ S.E.M. ($n=6$). *** $P<0.001$, compared with appropriate control.

Discussion

The results presented clearly demonstrate that VIP had a dose-dependent effect on the release of catecholamines from adrenal capsular tissue. The release of adrenaline, but not noradrenaline, was found to parallel the increase in aldosterone secretion. The ratio of adrenaline/noradrenaline found in this study is consistent with that previously reported in rat adrenal capsules and in the rat adrenal medulla (Pratt *et al.* 1985). Presumably the catecholamines are derived from the islets of chromaffin cells reported to be present in the zona glomerulosa region of the adrenal cortex (Kovács & Horváth 1973, Palacios & Lafraga 1975, Gallo-Payet *et al.* 1987), as the adrenal capsules were separated from the adrenal medulla in these experiments. It is, however, possible that noradrenaline may be released from nerve terminals remaining in the separated capsular tissue. It is well established that VIP stimulates catecholamine release from adrenal medullary chromaffin cells (Malhotra & Wakade 1987, Wakade *et al.* 1991). In this study it was found that the release of adrenaline in response to VIP displayed the same dose-response characteristics, supporting the contention that local catecholamine release mediates the response to VIP in this tissue.

Most previous studies, investigating the possible role of catecholamines in the aldosterone response to VIP stimulation, have failed to demonstrate release of catecholamines in response to VIP, although we have published some preliminary data (Hinson *et al.* 1992, 1996). Instead, most attention has been focussed on the effects of β adrenergic antagonists, specifically alprenolol and propranolol, non-selective β adrenergic antagonists, and atenolol, a selective β_1 antagonist. These agents have been found to signifi-

cantly inhibit the response of the zona glomerulosa of both rat and human adrenals to VIP stimulation (Mazzocchi *et al.* 1993, Bernet *et al.* 1994, Bornstein *et al.* 1996). In the absence of data on either catecholamine release, or on VIP binding, the mechanism of action of these agents has remained speculative. As previous studies have shown that neuropeptide Y interacts with α adrenoceptors (Martire & Pistrutto 1992), this appeared to be a possible mechanism of the inhibitory action of β adrenergic antagonists on VIP stimulation of aldosterone secretion. The data obtained in the present study, however, clearly demonstrating release of catecholamines in response to VIP, together with the observed specificity of the VIP binding, supports the hypothesis that β adrenergic antagonists inhibit the aldosterone response to VIP by blocking the action of catecholamines released in response to VIP stimulation.

The question as to whether the catecholamines are able to influence aldosterone secretion was not directly addressed by the present study, as there is already much evidence to support the contention that β adrenergic agonists stimulate aldosterone secretion in a variety of different rat adrenal preparations, including adrenal slices (Andreis *et al.* 1995), capsular preparations *in vitro* (Shima *et al.* 1984, Pratt *et al.* 1985, Pratt & McAteer 1989), the isolated perfused adrenal preparation (J P Hinson, unpublished observations) and dispersed zona glomerulosa cell preparations (Horiuchi *et al.* 1987). The question of the physiological role of the VIP-catecholamine system in the regulation of zona glomerulosa function remains unclear.

It has been suggested that the effects of VIP in the adrenal gland are mediated in part via the ACTH receptor (Li *et al.* 1990, Mazzocchi *et al.* 1994). This suggestion was originally made following the observation that VIP displaced binding of labelled ACTH from a subpopulation of

ACTH receptors (Li *et al.* 1990) and was restated on the basis that corticotrophin inhibitory peptide was shown to attenuate the rat adrenal response to VIP stimulation (Mazzocchi *et al.* 1994). Data from the present studies do not support this contention: a single population of VIP binding sites was identified in the zona glomerulosa, and only VIP and its specific antagonist (PL-VIP) were found to displace binding of labelled VIP. Neither ACTH, angiotensin II nor the β adrenergic antagonists were found to displace labelled VIP, suggesting that the zona glomerulosa expresses a single population of VIP receptors. We have not investigated the possibility that ACTH may interact with the specific VIP receptors identified in the adrenal cortex, although this possibility may partly explain the observations of Li and coworkers (1990). This study did not address the question as to which of the VIP receptor subtypes (for review see Harmar *et al.* 1998) are present in the rat zona glomerulosa and mediate the actions of VIP in this tissue, but the binding studies suggest that only one receptor subtype is present.

We have previously observed an increase in adrenal VIP content in animals maintained on a low sodium diet, raising the possibility that locally produced VIP has a role in the adrenal response to perturbed electrolyte balance (Hinson *et al.* 1996). We also reported an increase in the adrenal responsiveness to VIP following dietary sodium depletion, such that aldosterone secretion was significantly stimulated by a lower concentration of VIP in the low sodium group compared with controls (Hinson & Kapas 1995), and proposed that the zona glomerulosa expresses a distinct population of VIP receptors in response to changes in electrolyte balance. The results of the present study do not support this proposal: it was found that adrenals from the low sodium group of animals expressed a population of VIP receptors with the same K_d as controls, and with only a marginally greater B_{max} , which was not significantly different from controls.

One of the most interesting observations made in the present study was that the aldosterone response of adrenals from the low sodium group was not attenuated by the β adrenergic antagonist, atenolol, although catecholamine release was elevated by VIP to the same extent as in the control group. It is not clear why atenolol had no effect in this group, although it is possible that a different subtype of adrenergic receptor is expressed in the adrenals of animals maintained on a low sodium diet. However, the data obtained in the present study suggest that it is not the β_2 subtype of adrenoceptor and other studies have found that activation of α adrenoceptors in the rat adrenal cortex inhibits aldosterone secretion (Lotshaw *et al.* 1991) so this is most unlikely to be the mechanism by which VIP exerts its stimulatory effects in this group. Clearly this requires further study.

In conclusion, these data suggest that VIP stimulates aldosterone secretion indirectly via release of catecholamines. There appear to be different mechanisms medi-

ating the actions of VIP in animals fed a low sodium diet compared with controls, but the nature of these mechanisms is not yet clear. The physiological significance of a VIP–catecholamine system in the regulation of aldosterone secretion also remains unclear.

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