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ADRENAL CORTICAL RESPONSES TO VASOACTIVE INTESTINAL PEPTIDE IN CONSCIOUS HYPOPHYSECTOMIZED CALVES

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SUMMARY

1. Right adrenal and various cardiovascular responses to an intra-aortic infusion of vasoactive intestinal polypeptide (VIP; 4 μg min^{-1} kg^{-1}) have been investigated in the presence and absence of exogenous adrenocorticotrophin, ($ACTH_{1-24}$; 5 ng min^{-1} kg^{-1}, I.V.). The adrenal clamp technique was employed in conscious calves in which the pituitary stalk had been cauterized 3–4 days previously.

2. The I.V. infusion of $ACTH_{1-24}$ increased mean plasma ACTH concentration by between 1000 and 1100 pg ml^{-1} and mean right cortisol output by about 700 ng min^{-1} kg^{-1}. Under these conditions the intra-aortic infusion of VIP produced a further rise in mean adrenal cortisol output, together with a consequential rise in mean arterial plasma cortisol concentration, without affecting the concentration of ACTH in the arterial plasma significantly. In the absence of ACTH the same infusion of VIP had no detectable effect on adrenal cortisol output.

3. In each of the above respects this intra-aortic infusion of VIP closely mimicked the effect of stimulation of the peripheral end of the right splanchnic nerve in these animals, as it also did by causing a substantial fall in adrenal vascular resistance in the absence, but not in the presence, of ACTH.

4. It is concluded that release of this peptide from splanchnic nerve terminals in the adrenal gland most probably accounts, at least in part, for the powerful adrenocortical steroidogenic response to splanchnic nerve stimulation, that occurs in the presence of submaximal doses of ACTH.

INTRODUCTION

It has recently been reported that the sensitivity of the adrenal cortex to the steroidogenic action of adrenocorticotrophin (ACTH) is reduced by about 50% by section of the splanchnic nerves in lambs (Edwards, Jones & Bloom, 1986) and by even more in calves (Edwards & Jones, 1987*b*). Furthermore, experiments in functionally hypophysectomized conscious calves, in which the concentration of ACTH in the plasma can be controlled by I.V. infusions of the exogenous peptide, have provided direct evidence that the cortical response to ACTH is potentiated

strongly by electrical stimulation of the peripheral end of the splanchnic nerve (Edwards & Jones, 1987*a*).

The mechanism whereby splanchnic nerve stimulation causes this enhancement of adrenal cortical activity has yet to be elucidated. The present study was undertaken to investigate the possibility that it depends upon activation of vasoactive intestinal peptide (VIP)-containing neurones, which are known to be present in the cortex (Hökfelt, Lundberg, Schultzberg & Fahrenkrug, 1981; Holzwarth, 1984). Vasoactive intestinal peptide has been shown to stimulate steroid output from adrenal cortical tissue, albeit at higher concentrations than ACTH (Kowal, Horst, Pensky & Alfonzo, 1977; Morera, Cathiard, Laburthe & Saez, 1979; Leboulenger, Leroux, Delarue, Tonon, Charnay, Dubois, Coy & Vaudry, 1983) and is also released from the gland in response to electrical stimulation of the sympathetic innervation (S. R. Bloom, A. V. Edwards & C. T. Jones, unpublished observations). The results show that I.A. infusions of VIP strongly enhance the output of cortisol in the presence of ACTH in the conscious calf and do so sufficiently effectively to account for the steroidogenic potentiation that is observed during splanchnic nerve stimulation.

METHODS

Animals

Pedigree Jersey calves were obtained from local farms shortly after birth and used at ages of 24–43 days (25–32 kg body weight). They were kept in individual pens and maintained on a diet of cow's milk or artificial milk (Easy-mix Volak, Volak Ltd) at a rate of 3–4 l day^{-1}. Food was withheld overnight prior to each operation or experiment.

Experimental procedures

Anaesthesia was induced with chloroform (Chloroform SLR, Fisons) and maintained with halothane (May & Baker; *ca.* 2% in oxygen). Preparatory surgery involved two successive operations at intervals of 3–4 days. On the first occasion the pituitary stalk and the contents of the sella turcica were cauterized as described previously (Edwards, Hansell & Jones, 1986) and narrow-bore polyethylene catheters were inserted into the saphenous arteries so that the tip of one lay in the lower thoracic aorta with the other in the abdominal aorta. These were used subsequently to monitor aortic blood pressure and heart rate and for the collection of arterial blood samples. The catheter with the tip in the thorax was employed for intra-aortic infusions of vasoactive intestinal peptide (VIP) above the level of the adrenal gland. During the second operation the right kidney was removed, the right renal vein was cannulated and an adrenal clamp emplaced (Edwards, Hardy & Malinowska, 1974; Edwards, Furness & Helle, 1980). The right splanchnic nerve was cut immediately below the diaphragm and a Braunula cannula inserted into the jugular vein to provide a conduit for I.V. infusions of ACTH. The animals were maintained by replacement therapy with cortisol (Efcortesol; Glaxo) at a dose of 2·0 mg day^{-1} kg^{-1} and deoxycorticosterone acetate (Sigma) at a dose of 0·2 mg day^{-1} kg^{-1} following cauterization of the pituitary stalk, with an additional dose of 8 mg kg^{-1} on the day of the first operation. These steroids were administered by I.M. injection at 09.00 and 17.00 h and were withheld on the morning of the day on which the adrenal clamp was emplaced and the experiment performed. Following recovery from anaesthesia on the second occasion, arterial plasma glucose was monitored continuously and the animals were given I.V. infusions of glucose (Dextrose Monohydrate; Veterinary Drug Co.) at a dose of 2–3 mg min^{-1} kg^{-1}, if this appeared to be necessary to maintain arterial plasma glucose concentration above 3·0 mmol l^{-1}.

Experiments were carried out 3–4 h after surgery and after full recovery from anaesthesia. Pure synthetic vasoactive intestinal peptide (VIP; Bachem U.K.) was dissolved in an appropriate volume of sterile physiological saline, containing 2% bovine albumin (Sigma) and 1000 K.I.U. ml aprotinin^{-1} (Trasylol; Bayer), for intra-aortic infusion at a dose of 4 μg min^{-1} kg^{-1} in a volume of

1 ml min^{-1}. $ACTH_{1-24}$ (Synacthen; CIBA) was dissolved in saline and infused I.V. at 5 ng min^{-1} kg^{-1} (2·5 ml min^{-1}) for 50 min and the effects of VIP, in the presence of ACTH, were tested by infusing it I.A. for 10 min after ACTH had been infused for 20 min. Assay of ACTH in the infusate emerging from the catheter at the end of the infusion showed that the concentration was 90 ± 10 % of that expected. On each occasion VIP was infused either before and then again during the I.V. infusion of ACTH, or during and then again after ACTH. There was no significant difference between any of the responses of these two groups and the results have therefore been pooled. Heart rate and aortic blood pressure were monitored continuously by means of a Devices M19 recorder. Right adrenal blood flow was estimated gravimetrically and corrected for haematocrit percentage before the output of cortisol from the gland was calculated. Adrenal vascular resistance was estimated by dividing the perfusion pressure (mean aortic blood pressure) by the right adrenal blood flow. Adrenal cortisol output was estimated from the concentration in the adrenal effluent plasma and adrenal plasma flow at the time of collection and expressed as unit weight min^{-1} kg body $weight^{-1}$.

Analytical procedures

Samples of arterial blood were collected at intervals into heparinized tubes containing phenylmethylsulphonyl fluoride (PMSF; final concentration 0·1 mM; Sigma) for haematocrit, glucose, ACTH and cortisol estimations, as were samples of adrenal effluent blood for cortisol estimations. Each was centrifuged at 4 °C as soon as possible and the plasma was then sequestered at −20 °C.

Glucose was measured enzymically by means of a Beckman Mark 2 Glucose Analyzer. ACTH, cortisol and VIP were measured by radioimmunoassay (Jones, Boddy, Robinson & Ratcliffe, 1977; Mitchell & Bloom, 1978). In some instances steroids in the adrenal effluent plasma were extracted with dichloromethane and analysed by high-pressure liquid chromatography (h.p.l.c.) involving separation on a Zorbax-ODS column (25 × 0·4 cm, 5 μm, Dupont Ltd) with 21 % tetrahydrofuran at 1·0 ml min^{-1} and 2000 lbf in^{-2}. Steroids were then detected by measuring absorbance at 240 nm in a Pye–Unicam U.V. detector.

Results are expressed as mean values ± S.E. of mean. Statistical tests were made according to Snedecor & Cochran (1967).

Post-mortem examinations

After each experiment was concluded the animal was killed by the injection of a lethal dose of sodium pentobarbitone and the right adrenal gland together with the adrenal clamp were removed. The positioning of the clamp was then checked and the gland was inspected to ensure that there was no haemorrhage or oedema. The brain was also removed and its base examined to ensure that the pituitary stalk had in fact been destroyed by cautery and without producing intracranial bleeding. Assessment of the success or otherwise of attempted functional hypophysectomy by macroscopic examination was found to correlate well with the changes in plasma ACTH concentration which occurred post-operatively. Animals in which the plasma ACTH concentration had not fallen below 20 pg ml^{-1} were excluded from the series on those grounds alone, as were any in which the adrenal was found to be haemorrhagic.

RESULTS

Cardiovascular responses

The changes in mean aortic blood pressure, heart rate, right adrenal blood flow and right adrenal vascular resistance, which occurred in response to an I.V. infusion of VIP (4 μg min^{-1}) in the presence and absence of exogenous ACTH, given by continuous I.V. infusion (5 ng min^{-1} kg^{-1}), are shown in Fig. 1. VIP caused a closely similar fall in mean aortic blood pressure in both groups. This fall in perfusion pressure was accompanied by a steady fall in mean adrenal blood flow in the presence, but not in the absence, of ACTH. In the animals receiving ACTH the fall in adrenal blood flow, which occurred during the infusion of VIP, could be accounted for entirely by the fall in perfusion pressure and there was no change in mean adrenal vascular

resistance. In contrast, there was no significant fall in right adrenal blood flow in the absence of ACTH, showing that there had been a fall in mean adrenal vascular resistance that roughly matched the fall in mean aortic pressure and also achieved statistical significance ($P < 0{\cdot}01$; Fig. 1).

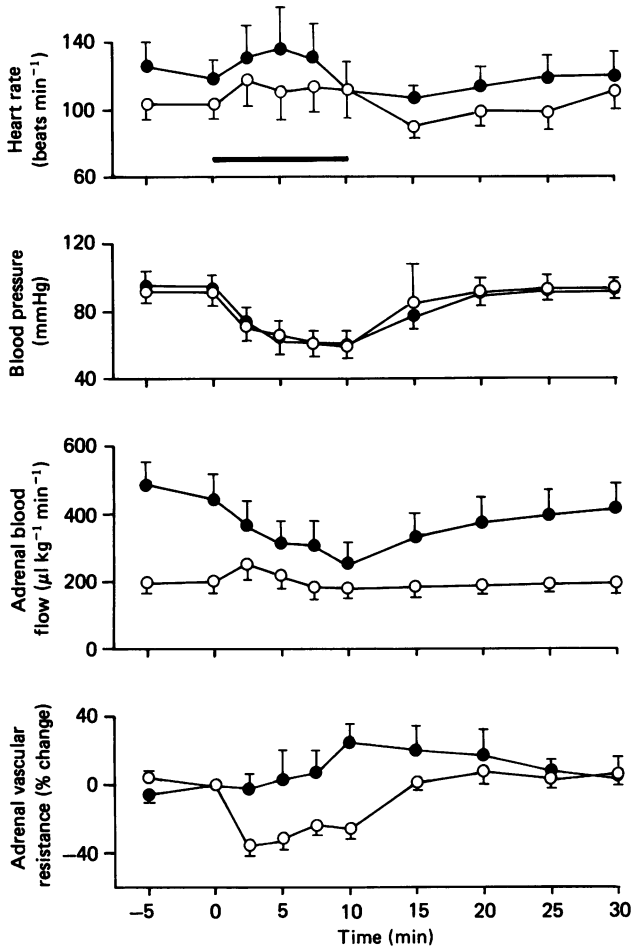

Fig. 1. Comparison of various cardiovascular responses to an intra-aortic infusion of VIP (4 μg min^{-1} for 10 min) in six conscious 3–7-week-old functionally hypophysectomized calves in the presence (●) and absence (○) of exogenous ACTH (5 ng min^{-1} kg^{-1}). The infusion of ACTH was started 20 min before the infusion of VIP and continued for a further 20 min after the VIP infusion was discontinued. Horizontal bar: duration of VIP infusion. Vertical bars: S.E. of each mean value.

Arterial plasma glucose concentration and haematocrit were also monitored routinely but, as no consistent changes in either parameter were recorded, the data are not presented here.

Adrenal cortisol responses

At the point in time at which these experiments were carried out, 3–4 days after the pituitary stalk had been cauterized, mean arterial plasma ACTH concentration

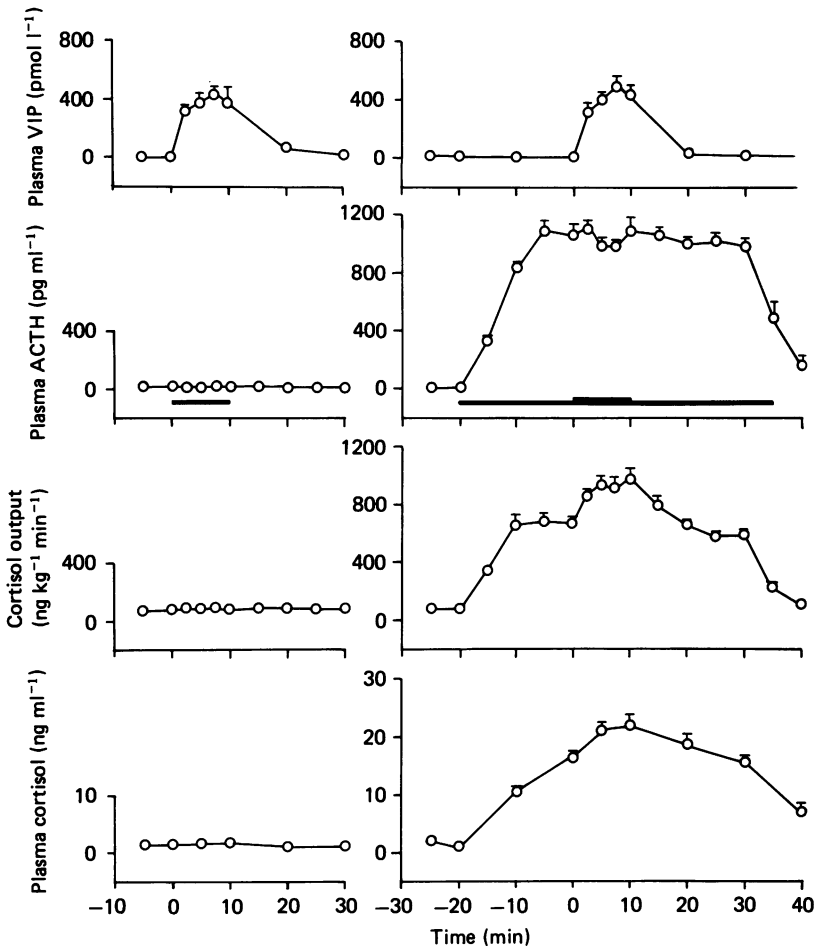

Fig. 2. Comparison of the changes in mean arterial VIP, ACTH and cortisol concentration, together with right adrenal cortisol output, in response to an intra-aortic infusion of VIP (4 μg min^{-1} for 10 min) in six conscious 3–7-week-old functionally hypophysectomized calves in the presence and absence of exogenous ACTH (5 ng min^{-1} kg^{-1}). Time = 0 relates to the time at which the infusion of VIP was started in each case. Open horizontal bar: duration of ACTH infusion. Filled horizontal bars: duration of VIP infusion. Vertical bars: S.E. of each mean value where these exceed the size of the symbol.

had fallen to < 15 pg ml^{-1}, mean right adrenal cortisol output to 77 ± 15 ng min^{-1} kg^{-1} and mean arterial plasma cortisol concentration to 1·3 ± 0·2 ng ml^{-1}. Intravenous infusion of synthetic $ACTH_{1-24}$ produced a rapid rise in its arterial plasma concentration to a plateau of between 1000 and 1100 pg ml^{-1}, which was achieved within 15 min and persisted for the duration of the infusion. Mean plasma ACTH concentration fell rapidly towards the initial value when the infusion was discontinued with a half-life of about 8 min (Fig. 2). The fact that release of endogenous ACTH had been effectively prevented in these animals is attested to by the low (generally undetectable) arterial plasma concentration initially; < 15 pg ml^{-1}, which is directly comparable with a mean value of 514 ± 227 pg ml^{-1} in a group of five

normal conscious intact calves of the same age. The rise in mean plasma ACTH concentration was associated with an increase in mean plasma cortisol output from the right adrenal gland of about 700 ng min^{-1} kg^{-1} over the same period (Fig. 2), together with a rise in mean arterial plasma cortisol concentration of about 17 ng ml^{-1}. The

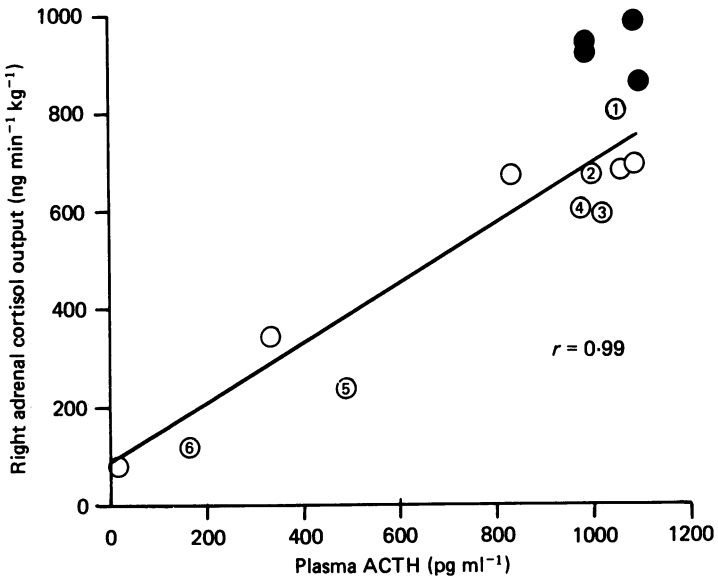

Fig. 3. Relation between mean arterial plasma ACTH concentration and mean right adrenal cortisol output in six, conscious, functionally hypophysectomized, calves given an intra-aortic infusion of VIP (4 μg min^{-1} for 10 min) in the course of a continuous I.V. infusion of exogenous ACTH (5 ng min^{-1} kg^{-1}). ○, prior to the VIP infusion. ●, during VIP infusion. ①–⑥, sequence of samples collected after the infusion of VIP had been discontinued. Regression line calculated by method of least squares applied to the values prior to infusion. See text for further details.

cortisol values measured by radioimmunoassay were 93·8 ± 2·6% (n = 12) of those determined after separation of extracted steroids by h.p.l.c. in a Zorbax-ODS column (see Methods).

Intra-aortic infusion of VIP, above the arterial supply to the right adrenal gland (4 μg min^{-1} for 10 min) resulted in a closely similar rise in the concentration of this peptide in right adrenal venous effluent plasma in both circumstances; to a peak of 435 ± 47 pmol l^{-1} in the absence of exogenous ACTH and 497 ± 62 pmol l^{-1} in its presence (Fig. 2). The average mean rise in the concentration of VIP in right adrenal effluent plasma was also closely similar in both these groups; 416 ± 36 pmol l^{-1} with ACTH and 378 ± 23 pmol l^{-1} without. The adrenal glands of both groups were therefore exposed to a VIPergic stimulus of about the same intensity. In the absence of ACTH this had no detectable effect on mean adrenal cortisol output. However, when the same VIPergic stimulus was imposed in the course of a continuing I.V. infusion of ACTH there was an abrupt and substantial rise in both mean right adrenal cortisol output and mean arterial plasma cortisol concentration, which persisted for the

duration of the VIP infusion. Mean cortisol output rose from 683 ± 39 ng min^{-1} kg^{-1}, just before VIP was infused, to a peak value of 982 ± 71 ng min^{-1} kg^{-1} at the point in time at which that infusion was discontinued ($P < 0{\cdot}01$; Fig. 2), and was associated with a rise in mean arterial plasma cortisol concentration from $16{\cdot}4 \pm 0{\cdot}9$ to $22{\cdot}1 \pm 1{\cdot}6$ ng ml^{-1} over the same period of time ($P < 0{\cdot}02$; Fig. 2). Mean arterial plasma ACTH

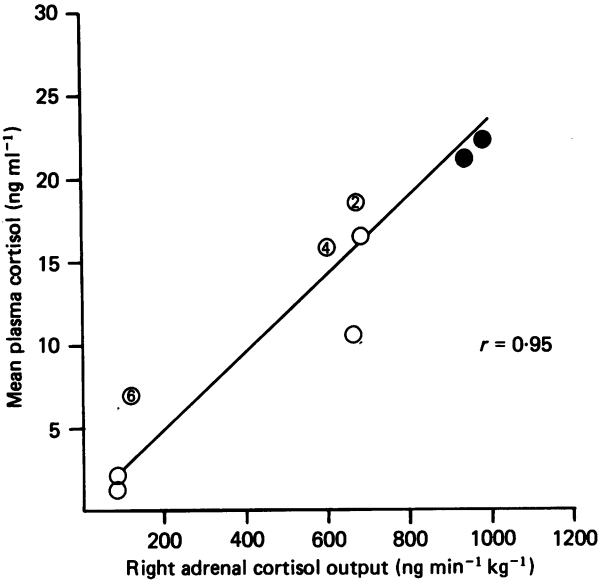

Fig. 4. Relation between mean right adrenal cortisol output and mean arterial plasma cortisol concentration in six, conscious, functionally hypophysectomized calves given an intra-aortic infusion of VIP (4 μg min^{-1} for 10 min) in the course of a continuous I.V. infusion of exogenous ACTH (5 ng min^{-1} kg^{-1}). ○, prior to the VIP infusion. ●, during the VIP infusion. ②–⑥, sequence of samples collected after the infusion of VIP had been discontinued. Regression line calculated by method of least squares applied to all values. See text for further details.

concentration was not significantly affected by the intra-aortic infusion of VIP. Both adrenal cortisol output and mean arterial plasma cortisol levels fell rapidly to the pre-existing level when the infusion of VIP was discontinued at 10 min, and then fell abruptly again to initial levels when the I.V. infusion of ACTH was terminated at 30 min (Fig. 2).

Mean right adrenal cortisol output was found to be linearly related to mean plasma ACTH concentration prior to the intra-aortic infusion of VIP in these experiments ($r = 0{\cdot}99$; Fig. 3), but was greater than could be accounted for in this way during the infusion of VIP. As shown in Fig. 3, cortisol output could be accounted for entirely by the existing plasma ACTH concentration within 10 min after the infusion of VIP, because all the mean values then and thereafter fell below the regression line, without differing significantly from that relation. In contrast, mean plasma cortisol concentration was linearly related to mean adrenal cortisol output throughout ($r = 0{\cdot}95$; Fig. 4).

DISCUSSION

The results of these experiments are directly comparable with those reported by Edwards & Jones (1987*a*), and provide compelling evidence that potentiation of the adrenal cortical steroidogenic response to ACTH, which occurs during stimulation of the splanchnic sympathetic innervation, is due, at least in part, to release of VIP within the adrenal cortex.

This contention is supported by the fact that the potentiating effect of splanchnic nerve stimulation can be mimicked so effectively by an intra-aortic infusion of VIP, which represents a close intra-arterial infusion with respect to the gland. The dose of VIP employed (4 μg min^{-1}) raised the concentration of the peptide in the adrenal effluent plasma to about 400 pmol l^{-1}. This value is roughly double that found in submandibular effluent plasma in the cat during stimulation of the chorda tympani (Bloom & Edwards, 1980). The concentration of VIP in venous effluent plasma is bound to be much lower than that achieved locally, within the tissue, during nerve stimulation, whereas the concentration in the venous effluent plasma is likely to be much higher than that which obtains at receptor sites within the tissue when the peptide is delivered via the vasculature. It is therefore reasonable to suppose that the dose of VIP that was employed in the present experiments represented a VIPergic stimulus to the adrenal cortical cells which was well within the physiological range. As with splanchnic nerve stimulation, VIP was ineffective in the absence of ACTH. Furthermore, VIP reproduced the vascular effect of splanchnic nerve stimulation with respect to the adrenal gland precisely, by causing a substantial and significant fall in adrenal vascular resistance in the absence, but not in the presence, of exogenous ACTH. All these observations are consistent with the reported localization of VIP in the rat adrenal cortex (Holzwarth, 1984) and with its proven steroidogenic activity *in vitro* (Kowal *et al.* 1977; Morera *et al.* 1979; Leboulenger *et al.* 1983). However, it is not known to what extent, if any, other transmitters are involved, and it has yet to be established whether the steroidogenic effect of splanchnic nerve stimulation is exclusively non-adrenergic and non-cholinergic.

The finding that mean arterial plasma cortisol concentration was related linearly to mean adrenal cortisol output throughout these experiments (Fig. 4), just as it was in the previous study involving splanchnic nerve stimulation (Edwards & Jones, 1987*a*), shows that the changes in plasma cortisol concentration faithfully reflected changes in adrenal cortisol production. No doubt, the rate at which cortisol disappeared from the 'cortisol space' increased when the concentration in the plasma rose, but it is reassuring that this did not exceed the expectation of physical concentration dependence because the protocol might conceivably have led to an increase in cortisol metabolism in other ways. If, for instance, the dose of VIP that was infused had affected hepatic or renal blood flow significantly, physiological changes in the rate of cortisol disappearance could be anticipated.

It has been suggested that adrenal cortical responses to ACTH may be both flow and concentration dependent under certain conditions (Urquhart, 1965; Wood, Shinsako & Dallman, 1982). Since splanchnic nerve stimulation increases adrenal blood flow, both in the presence and absence of ACTH, the possibility that an increase in the amount of ACTH reaching the gland, without any change in the concentration

of ACTH in the arterial supply, could not be discounted. Our dubiety that dilatation of the adrenal vasculature could account for the increased output of cortisol is confirmed by the results of the present study because intra-arterial infusion of the putative transmitter (VIP) potentiated the adrenal cortical response to ACTH in the course of a steady fall in mean adrenal blood flow, with no significant change in arterial plasma ACTH concentration. Clearly, if a flow-mediated increase in the supply of ACTH is an important mechanism for VIP action, as it may be for adrenal responses to haemorrhage (Engeland, Lilly & Gann, 1985), this would require an increased flow of blood to the cortex in the face of a fall in total adrenal blood flow.

We are therefore led to the conclusion that release of VIP, from nerve terminals in the adrenal cortex, contributes to the adrenal cortical steroidogenic response which has, hitherto, been widely supposed to depend entirely upon release of ACTH from the pituitary gland. The results of our several studies (Edwards *et al.* 1986; Edwards & Jones, 1987*a*, *b*; S. R. Bloom, A. V. Edwards & C. T. Jones, unpublished observations, and the present paper) seem to show that VIP produces this effect by a direct action on the secretory cells. As mentioned previously, experiments *in vitro* have established that VIP is capable of stimulating steroidogenesis in adrenal cortical cells (Kowal *et al.* 1977; Morera *et al.* 1979; Leboulenger *et al.* 1983) as it does in the ovary (Frederichs, Lundquist, Mathur, Ashton & Landgrebe, 1983; Davoren & Hsueh, 1985; Ahmed, Dees & Ojeda, 1986), another steroidogenic organ in which nerve endings containing VIP-like immunoreactivity have been identified (Ahmed *et al.* 1986). In this situation the effect has been attributed to increased synthesis of the ovarian cholesterol side-chain cleavage enzyme complex, which represents the rate-limiting reaction in progesterone synthesis, and is mediated, at least in part, via cyclic AMP (Trzeciak, Ahmed, Simpson & Ojeda, 1986). It has yet to be established whether the same or a similar mechanism is implicated in the adrenal cortex but it has been shown that it stimulates the production of cyclic AMP in adrenal cells *in vitro* (Morera *et al.* 1979), as it does in other cells. An intracellular effect on cyclic AMP would provide a mechanism for VIP potentiation of the action of ACTH. ACTH acts via Ca^{2+}- and cyclic AMP-dependent pathways, the latter having the ability to potentiate the former (Sayers, Beall & Seelig, 1972; Sala, Hayashi, Catt & Dufau, 1979; Cheitlin, Buckley & Ramachandran, 1985).

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