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ADRENAL CORTICAL AND MEDULLARY RESPONSES TO ACETYLCHOLINE AND VASOACTIVE INTESTINAL PEPTIDE IN CONSCIOUS CALVES

By A. V. EDWARDS and C. T. JONES

From the Physiological Laboratory, University of Cambridge, Downing Street, Cambridge CB2 3EG and the Laboratory of Cellular and Developmental Physiology, Institute for Molecular Medicine, University of Oxford, Headley Way, Oxford OX3 9DS

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SUMMARY

1. Adrenal responses to intra-aortic infusions of acetylcholine and vasoactive intestinal peptide (VIP) have been investigated in functionally hypophysectomized calves given exogenous adrenocorticotrophic hormone (ACTH, 2 ng min^{-1} kg^{-1} I.V.).

2. Infusions of VIP at a dose of 0·13 μg min^{-1} kg^{-1} caused a small, but significant increase in adrenaline and noradrenaline output which was, however, far below the level recorded previously in response to acetylcholine (0·7 μg min^{-1} kg^{-1}). In contrast, these doses of the two agonists produced closely similar rises in adrenal cortisol output.

3. The steroidogenic effects of acetylcholine and VIP were found to be strictly additive and no evidence of potentiation was obtained in relation to either cortical or medullary responses or in the case of any of the cardiovascular responses which were monitored.

4. Intra-aortic infusions of VIP, at a dose which produced a substantial increase in adrenal steroidogenesis (0·065 μg min^{-1} kg^{-1}), had no effect on the output of catecholamines, enkephalin-like immunoreactivity or corticotrophin-releasing factor, either in the presence or absence of acetylcholine.

5. It is concluded that VIP is unlikely to modulate adrenal medullary responses to muscarinic stimulation in this species as it has been claimed to do in the rat and does not potentiate adrenal steroidogenesis in response to acetylcholine as it does to ACTH.

INTRODUCTION

The output of cortisol from the adrenal gland in response to adrenocorticotrophic hormone (ACTH) is potentiated by intra-aortic infusions of small amounts of both acetylcholine and of vasoactive intestinal peptide (VIP) in the conscious calf (Bloom, Edwards & Jones, 1987; Jones, Edwards & Bloom, 1991). The response to acetylcholine is attributable to activation of muscarinic receptors (Jones & Edwards, 1992). Release of VIP is thought to be implicated in the potentiation of the steroidogenic response to ACTH during splanchnic nerve stimulation, both in the conscious calf (Bloom *et al.* 1987) and the perfused adrenal gland of the pig,

when the nerve supply is preserved (Erhart-Bornstein, Bornstein, Scherbaum, Pfeiffer & Holst, 1991). In the rat, both VIPergic and muscarinic receptors have been implicated in the control of catecholamine secretion from the adrenal medulla (Wakade & Wakade, 1983; Malhotra & Wakade, 1987*a*, *b*; Wakade, Blank, Malhotra, Pourcho & Wakade, 1991). The possibility that VIP plays an important part in the control of catecholamine secretion under physiological conditions is strengthened by the finding that it is most important in mediating secretion during splanchnic nerve stimulation at low frequency (1 Hz; Wakade, 1988). This can be maintained for many hours in the isolated perfused rat adrenal gland preparation, unlike the response to high frequency stimulation (10 Hz), which fatigues relatively rapidly. Pretreatment with atropine and hexamethonium reduced the secretion of catecholamines in response to stimulation at 10 Hz by more than 80 %, whereas that which occurred in response to stimulation at 1 Hz was reduced by only about 35 % in the presence of these blocking agents.

Both muscarinic and VIPergic activation mobilize Ca^{2+} from intracellular stores following the generation of inositol 1,4,5-trisphosphate (IP_3), providing a possible pathway for mutual synergy, whereas nicotinic stimulation of chromaffin cells depends upon Ca^{2+} entry across cell membranes (Malhotra, Wakade & Wakade, 1988). VIP has been found to stimulate catecholamine biosynthesis in isolated bovine adrenal chromaffin cells (Houchi, Oka, Misbahuddin, Morita & Nakanashi, 1987; Waymire, Craviso, Lichteig, Johnston, Baldwin & Zigmond, 1991) and elevates the levels of enkephalin precursors (Wilson, 1987) but nothing is known of interactions between these two agonists in the adrenal glands of conscious animals. Accordingly, the present study was undertaken to assess the extent to which they interact in the potentiation of adrenal steroidogenesis and of various medullary responses.

Some of these results have been presented previously in a preliminary form (Jones & Edwards, 1993).

METHODS

Animals

Pedigree Jersey calves were obtained from local farms shortly after birth and used at ages ranging between 21 and 42 days (24–38 kg body weight). They were kept in individual pens and maintained on a diet of cow's milk or artificial milk (Easy-mix Volac, Volac 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, Loughborough, Leicestershire) and maintained with halothane (May & Baker, Dagenham, *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 polytetrafluoroethylene (Teflon) catheters were inserted into the saphenous arteries so that the tips lay in the lower thoracic aorta. These were used subsequently to monitor aortic blood pressure and heart rate, for collection of arterial blood samples and for intra-aortic infusions of acetylcholine and/or 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, Greenford, Middlesex) at a dose of 2·0 mg day^{-1} kg^{-1} and deoxycortisone acetate (Sigma, Poole, Dorset) at a dose of 0·2 mg day^{-1} kg^{-1} following cauterization of the pituitary stalk, with an additional dose of 8·0 mg kg^{-1} cortisol on the day of the first operation. These steroids were administered by I.M. injection at 09.00 h 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., Bury St Edmunds, Suffolk) 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, during which time the animals had made a full recovery from anaesthesia. Acetylcholine (acetylcholine chloride; Sigma) was made up as a stock solution 1 mg ml^{-1} in 0·25 M sodium dihydrogen orthophosphate which was then diluted with an appropriate volume of sterile physiological saline for infusion at a dose of 4·5 nmol min^{-1} kg^{-1} (1 ml min^{-1}) for 10 min. ACTH(1–24) (Synacthen; Ciba, Horsham, W. Suffolk) was dissolved in saline and infused I.V. at 2 ng min^{-1} kg^{-1} (2·5 ml min^{-1}) for 50 min and the effects of acetylcholine (0·7 or 0·35 μg min^{-1} kg^{-1}) and VIP (0·13 or 0·065 μg min^{-1} kg^{-1} were tested by infusing them, either singly or together intra-aortically for 10 min during the I.V. ACTH infusion. 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. Aortic blood pressure was monitored continuously by means of a Devices M19 recorder. Right adrenal blood flow was estimated gravimetrically and corrected for haematocrit 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 before, during and after the 10 min experimental infusion period into heparinized tubes containing phenylmethylsulphonyl fluoride (PMSF; final concentration 0·1 mM; Sigma Chemical Co, St Louis, MO, USA) for haematocrit, ACTH, and cortisol estimations. Samples of adrenal venous effluent blood were collected in the same way for cortisol, [Met^5]enkephalin and corticotrophin-releasing factor (CRF) estimations and into tubes containing 2–3 mg EDTA for catecholamine estimations. Volumes of about 8 ml were collected over periods of 1–2 min depending on the rate of adrenal blood flow at the time. Each was then centrifuged at 4 °C as soon as possible and the plasma stored at −20 or −70 °C.

Adrenaline and noradrenaline, were measured by high performance liquid chromatography (HPLC) with electrochemical detection (Arkinstall & Jones, 1985). ACTH and cortisol were measured by radioimmunoassay (Jones, Boddy, Robinson & Ratcliffe, 1977). In some instances steroids in the adrenal effluent plasma were extracted with dichloromethane and analysed by HPLC involving separation on a Zorbax-ODS column (25 × 0·4 cm, 5 μm, DuPont UK Ltd, Stevenage, Herts.) with 21 % tetrahydrofuran at 1·0 ml min^{-1} and 13800 kPa. Steroids were then detected by measuring absorbance at 240 nm in a Pye–Unicam UV detector. Corticotrophin releasing factor (CRF) was determined by radioimmunoassay, essentially as described by Vale *et al.* (1983). The antibody used cross-reacted with bovine, ovine and human CRF. It showed no cross-reactivity with ACTH, β-lipotropin, pro-opiomelanocortin, β-melanocyte-stimulating hormone, β-endorphin, [Met^5]enkephalin, vasoactive intestinal peptide, neuropeptide Y, sauvagine, vasopressin or oxytocin. [Met^5]enkephalin was measured by a specific radioimmunoassay as described previously (Edwards *et al.* 1986; Edwards & Jones, 1987). Briefly, untreated plasma was assayed in order to determine the content of the free peptide, and other samples of plasma were assayed after proteolytic digestion to liberate all the [Met^5]enkephalin from any high molecular weight precursor molecules that were present; these values are referred to as total [Met^5]enkephalin.

Results are expressed as mean values $\pm$ S.E.M. They were analysed statistically by means of Student's *t* test.

Postmortem examinations

After each experiment was concluded the animal was killed by the injection of a lethal dose of sodium pentobarbitone (Sagatal; May & Baker) 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.

Animals in which the plasma ACTH concentration had not fallen below 10–15 pg ml^{-1} (the detection limit of the assay) or cortisol output below 200 ng min^{-1} kg^{-1}, were excluded from the series on those grounds alone, as were any in which the adrenal gland was found to be haemorrhagic; oedema was not encountered.

RESULTS

Adrenal cortical responses

Adrenal cortical responses to acetylcholine and VIP were investigated by infusing them intra-aortically, either together or alone, at doses previously shown to potentiate steroidogenesis (0·7 and 0·13 μg min^{-1} kg^{-1} respectively for 10 min; Bloom *et al.* 1987; Jones *et al.* 1991) in six conscious calves. Both agonists produced

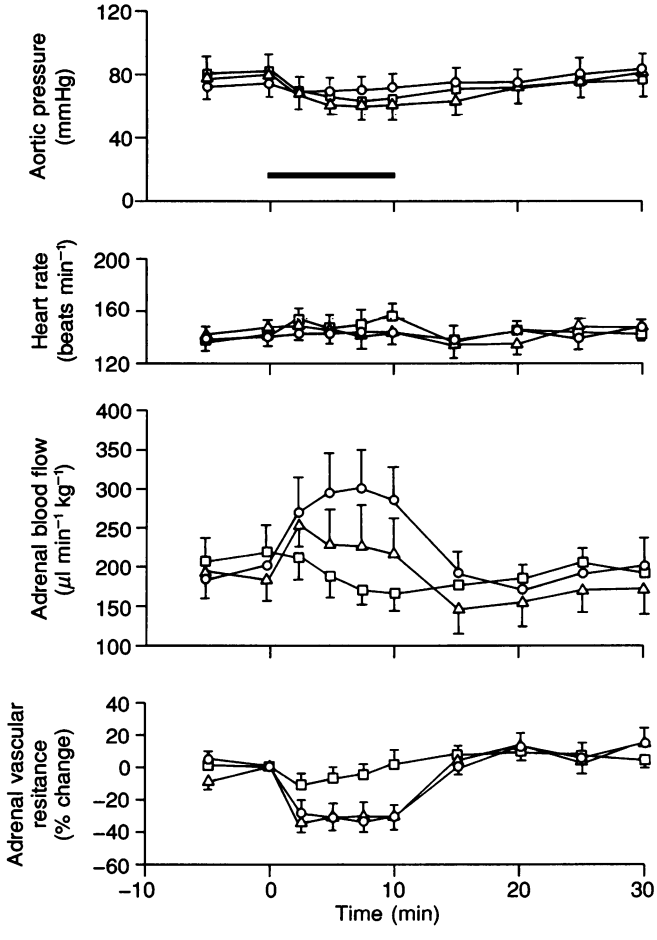

Fig. 1. Changes in mean aortic blood pressure, heart rate, right adrenal blood flow and vascular resistance in 6 conscious functionally hypophysectomized calves receiving exogenous ACTH (2 ng min^{-1} kg^{-1} I.V.), given intra-aortic infusions of acetylcholine (○; 0·7 μg min^{-1} kg^{-1}) or VIP (□; 0·13 μg min^{-1} kg^{-1}) or both agonists together (△). Horizontal bar, duration of intra-aortic infusions. Vertical bars represent S.E.M.

a small but statistically significant fall in mean aortic blood pressure. Thus, the mean average pressure during the infusion of acetylcholine was 69·0 ± 0·5 mmHg compared with a mean average of 75·0 ± 1·5 mmHg before and after ($P < 0·01$). The corresponding values during and before and after the VIP infusion were 65·3 ± 2·2

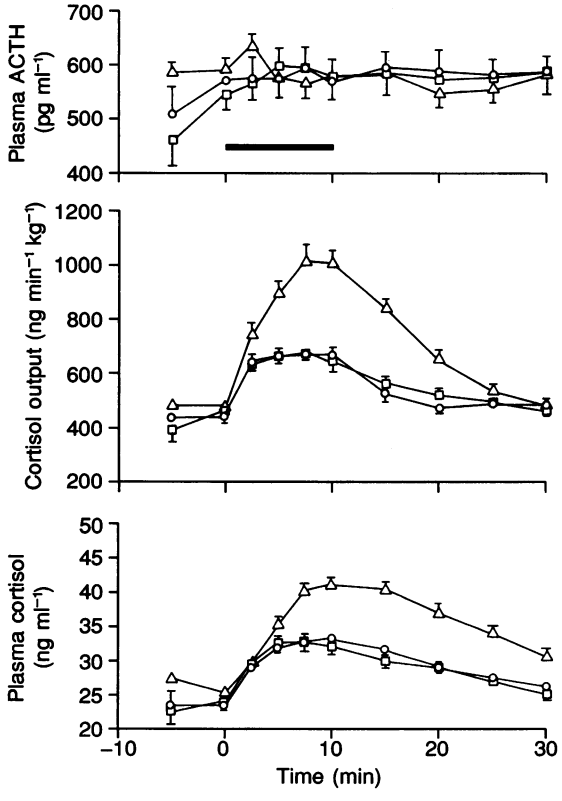

Fig. 2. Changes in mean plasma ACTH and cortisol concentration and right adrenal cortisol output in 6 conscious functionally hypophysectomized calves receiving exogenous ACTH (2 ng min^{-1} kg^{-1} I.V.), given intra-aortic infusions of acetylcholine (○; 0·7 μg min^{-1} kg^{-1}) or VIP (□; 0·13 μg min^{-1} kg^{-1}) or both agonists together (△). Horizontal bar, duration of intra-aortic infusions. Vertical bars, represent S.E.M.

and 75·0 ± 1·5 mmHg ($P < 0·01$) and in respect of the combined infusion 61·0 ± 1·9 and 73·8 ± 2·7 mmHg ($P < 0·01$). This hypotensive effect was associated with mild bradycardia and a variable rise in right adrenal blood flow (Fig. 1). Estimation of right adrenal vascular resistance from the flow and perfusion pressure showed that it was reduced more effectively by acetylcholine than by VIP (Fig. 1) and there was no evidence of any potentiation of this vascular response when the two agonists were given together.

Neither agonist produced any change in the concentration of ACTH in the arterial plasma but both produced a substantial increase in adrenal cortisol output, the extent of which happened to be closely similar (Fig. 2). Mean cortisol output rose from 442 ± 23 ng min^{-1} kg^{-1} at time = 0 to a mean peak output of

676 ± 11 ng min^{-1} kg^{-1} at 7·5 min during the infusion of acetylcholine, and from 461 ± 20 to a peak of 667 ± 18 ng min^{-1} kg^{-1} at 7·5 min during the infusion of VIP; i.e. by 234 and 206 ng min^{-1} kg^{-1} respectively. If the effects of the two agonists were purely additive when they were given together one would therefore expect

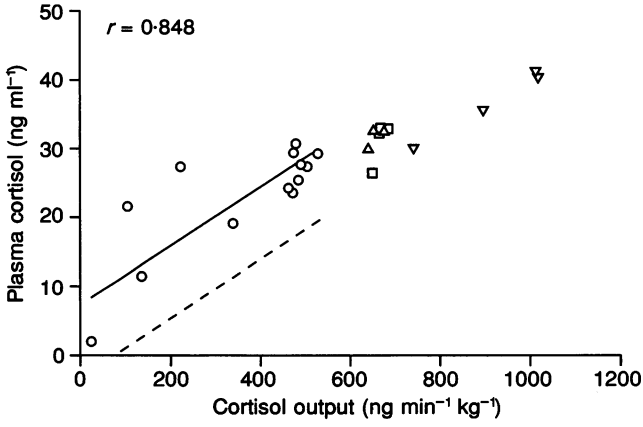

Fig. 3. Relation between right adrenal cortisol output and plasma cortisol concentration in 6 conscious functionally hypophysectomized calves receiving exogenous ACTH (2 ng min^{-1} kg^{-1} I.V.), given intra-aortic infusions of acetylcholine (0·7 μg min^{-1} kg^{-1}) or VIP (0·13 μg min^{-1} kg^{-1}) or both agonists together. ○, values before and after the intra-aortic infusions. □, values during acetylcholine infusions. △, values during VIP infusions. ▽, values during combined acetylcholine and VIP infusions. Regression line calculated by the method of least squares applied to values before and after the intra-aortic infusions. The dashed line represents regression + 2 S.D.

the output to rise by about 440 ng min^{-1} kg^{-1}. It actually rose from 481 ± 10 to a mean peak of 1012 ± 62 ng min^{-1} kg^{-1} at 7·5 min, i.e. by about 530 ng min^{-1} kg^{-1} or some 20 % greater than the prediction based on simple addition (Fig. 2). These changes were associated with rises in the concentration of cortisol in the peripheral plasma (Fig. 2). At the end of the infusion of acetylcholine (10 min) mean arterial plasma cortisol concentration had risen from 23·5 ± 0·6 to 33·3 ± 0·7 ng ml^{-1} and at the end of the VIP infusion from 24·2 ± 1·3 to 32·2 ± 1·2 ng ml^{-1}, i.e. by 9·8 and 8·0 ng ml^{-1} respectively. At the end of the combined infusion mean plasma cortisol concentration had risen from 25·3 ± 0·5 to 41·0 ± 1·2 ng ml^{-1}, i.e. by 15·7 ng ml^{-1} or some 12 % less than that to be expected from a simple additive effect (17·8 ng ml^{-1}).

The extent to which the rise in mean arterial plasma cortisol concentration reflected the rise in adrenal cortisol output, unaffected by any change in disappearance rate which either agonist might produce was assessed by plotting the two mean variables against one another (Fig. 3). In the absence of acetylcholine and VIP there is a linear relation (Edwards & Jones, 1987). This finding was confirmed ($r = 0·848$) and all the mean values during each of the three infusions was found to fall within 2 S.D. of this relation showing that the changes in plasma cortisol concentration could indeed be accounted for by the changes in adrenal cortisol output.

Right adrenal cortisol output was linearly related to the rate at which ACTH was presented to the gland (estimated from the mean arterial plasma ACTH concentration and mean right adrenal plasma flow) for all samples collected before and after the infusions of acetylcholine and/or VIP ($r = 0{\cdot}981$; Fig. 4). As reported

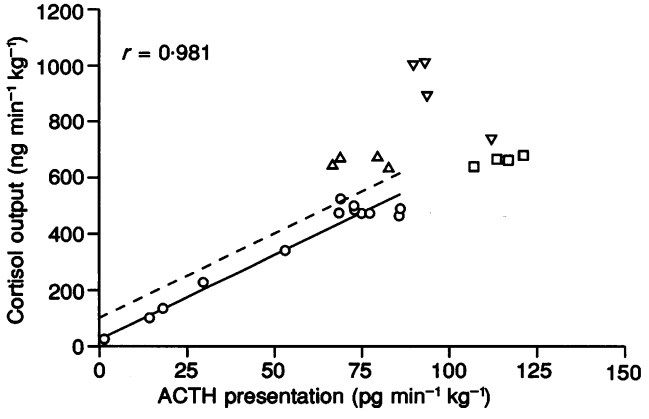

Fig. 4. Relation between right adrenal ACTH presentation rate and cortisol output in 6 conscious functionally hypophysectomized calves receiving exogenous ACTH (2 ng min^{-1} kg^{-1} I.V.), given intra-aortic infusions of acetylcholine (0·7 μg min^{-1} kg^{-1}) or VIP (0·13 μg min^{-1} kg^{-1}) or both agonists together. ○, values before and after the intra-aortic infusions. □, values during acetylcholine infusions. △, values during VIP infusions. ▽, values during combined acetylcholine and VIP infusions. Regression line calculated by the method of least squares applied to values before and after the intra-aortic infusions. The dashed line represents regression + 2 S.D.

previously (Jones *et al.* 1991), the same relation was preserved during acetylcholine infusions. However, mean right adrenal cortisol output was consistently higher than that predicted from the rate of ACTH presentation during intra-aortic infusions of VIP, with or without acetylcholine (Fig. 4). All but one of the values lay more than 2 S.D.s above the regression relating cortisol output to ACTH presentation, indicating that VIP exerted a significant additional steroidogenic effect of it own, as has also been reported previously (Edwards, 1989).

The peak outputs of cortisol from the right adrenal gland which were attained during the combined intra-aortic infusions of acetylcholine and VIP exceeded maxima obtained previously in response to ACTH alone (Edwards, Hardy & Malinowska, 1975) or indeed those obtained in any previous study employing this model. It therefore seemed possible that mutual potentiation might have been precluded by the fact that a maximal response was obtained when the effects just happened to be additive. The possibility of such a coincidence was investigated in a further group of six animals in which the doses of the agonists were each reduced by half.

This protocol produced closely similar patterns of cardiovascular responses to those recorded using the higher doses, with reduced extent as expected (data not shown). Acetylcholine produced a rise in mean adrenal cortisol output from

258 ± 24 ng min^{-1} kg^{-1} at time = 0 to a mean peak value of 393 ± 54 at the end of the infusion (10 min); i.e. an increment of 135. VIP caused a rise in mean cortisol output of from 257 ± 36 to 401 ± 48 ng min^{-1} kg^{-1}, i.e. an increment of 144. Thus the increment to be expected when both agonists were given together if their effects

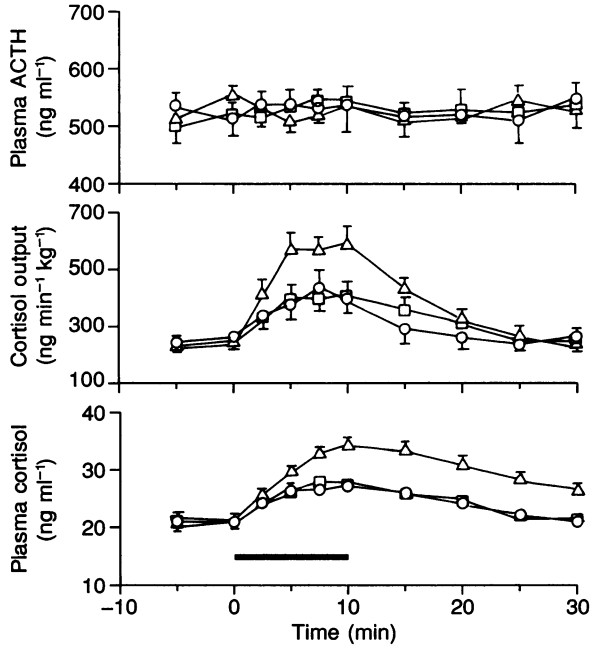

Fig. 5. Changes in mean plasma ACTH and cortisol concentration and right adrenal cortisol output in 6 conscious functionally hypophysectomized calves receiving exogenous ACTH (2 ng min^{-1} kg^{-1} I.V.), given intra-aortic infusions of acetylcholine (○; 0·35 μg min^{-1} kg^{-1}) or VIP (□; 0·065 μg min^{-1} kg^{-1}) or both agonists together (△). Horizontal bar, duration of intra-aortic infusions. Vertical bars represent S.E.M.

were purely additive would be 279 ng min^{-1} kg^{-1}. In fact mean adrenal cortisol output rose during the combined infusions by an increment of 332 ± 34 ng min^{-1} kg^{-1}; which was within 20% of that predicted. The mean incremental cortisol output during the combined infusions (332 ± 34) was not significantly higher than the mean of the summed increments during the two single infusions in each animal (259 ± 47 ng min^{-1} kg^{-1}; $P > 0{\cdot}2$). Mean plasma cortisol concentration rose by 5·2 ng ml^{-1} during the acetylcholine infusion and by 6·5 ng ml^{-1} during the VIP infusion. During the combined infusion mean plasma cortisol concentration rose by 11·4 ng ml^{-1}, which was within 3% and *less* than that expected by simple addition.

Adrenal medullary responses

Intra-aortic infusions of VIP (0·065 μg min^{-1} kg^{-1}) had no detectable effect on the outputs of either noradrenaline or adrenaline from the adrenal gland and failed to potentiate the output of either amine in response to acetylcholine (0·35 μg min^{-1} kg^{-1}; Fig. 6). Similarly, this dose of VIP failed to stimulate the

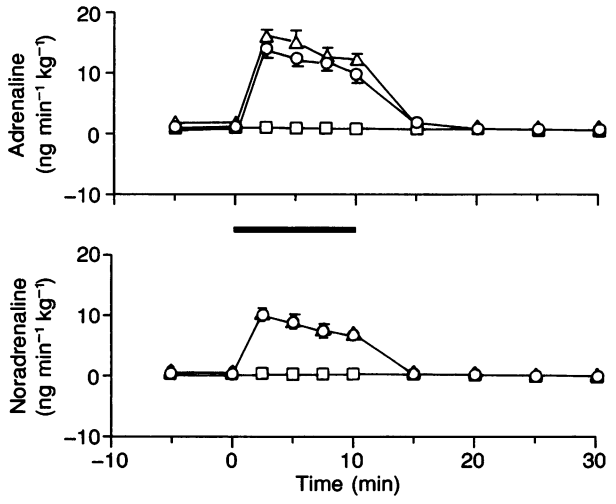

Fig. 6. Changes in mean catecholamine output from the denervated right adrenal gland in 6 conscious functionally hypophysectomized calves receiving exogenous ACTH (2 ng min^{-1} kg^{-1} I.V.), given intra-aortic infusions of acetylcholine (○; 0·35 μg min^{-1} kg^{-1}) or VIP (□; 0·065 μg min^{-1} kg^{-1}) or both agonists together (△). Horizontal bar, duration of intra-aortic infusions. Vertical bars represent S.E.M.

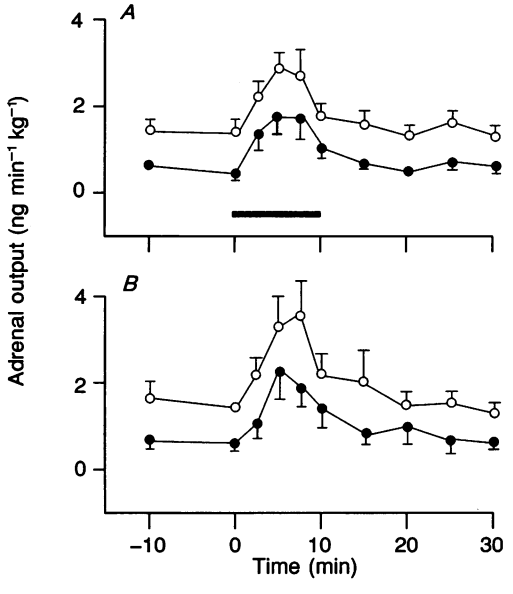

Fig. 7. Changes in the output of adrenaline (○) and noradrenaline (●) from the denervated right adrenal gland of 6 conscious functionally hypophysectomized calves, given intra-aortic infusions of VIP (0·13 μg min^{-1} kg^{-1}). *A*, receiving exogenous ACTH (2 ng min^{-1} kg^{-1} i.v.). *B*, without ACTH. Horizontal bar, duration of intra-aortic infusions. Vertical bars, represent S.E.M.

release of either free or total enkephalin or of CRF from the gland, or to affect the release of these peptides in response to acetylcholine (data not shown). At the higher dose of VIP (0·13 $\mu g\ min^{-1}\ kg^{-1}$) there was a small but perceptible release of both adrenaline and noradrenaline from the denervated adrenal gland, which was not affected by plasma ACTH concentration (Fig. 7).

DISCUSSION

The results of these experiments confirm previous findings that both acetylcholine and VIP potentiate the adrenal steroidogenic response to ACTH in the conscious calf, without affecting plasma ACTH concentration (Bloom *et al.* 1987; Jones *et al.* 1991), and extend them, by showing that both responses are dose dependent over the ranges tested (0·35–0·70 $\mu g\ min^{-1}\ kg^{-1}$, acetylcholine and 0·065–0·130 $\mu g\ min^{-1}\ kg^{-1}$ for VIP). In animals in which attempted functional hypophysectomy was found to be unsuccessful both agonists produced a pronounced rise in plasma ACTH concentration, underlining the necessity for employing hypophysectomized animals, with controlled ACTH status, in any study of the direct effects of the agonists on the adrenal gland. The response to acetylcholine is abolished by atropine and so attributable to activation of muscarinic receptors (Jones *et al.* 1991), as it has also been shown to be in bovine adrenal cortical cells *in vitro* (Hadjian, Ventre & Chambaz, 1981). In other tissues, including the cerebral cortex of the rat (Raiteri, Marchi & Paudice, 1987), vesicular ganglia of the cat (Kavatani, Rutigliano & De Groat, 1985) and isolated guinea-pig adrenal medullary cells (Misbahuddin, Houchi, Nakanishi, Morita & Oka, 1986) VIP strongly potentiates muscarinic activation. The best characterized of such mechanisms are the secretory and vasodilator response to acetylcholine in the submandibular gland of the cat (Lundberg, Änggård, Fahrenkrug, Hökfelt & Mutt, 1980), in which it is supposed that VIP enhances the binding of muscarinic ligands (Lundberg, Hedlund & Bartfai, 1982).

No evidence was obtained in the present experiments to suggest potentiation between acetylcholine and VIP in relation to any cardiovascular parameter or adrenal steroidogenesis. Rather the responses appeared to be strictly additive. This finding, regarding steroidogenesis, is in accord with that by Leboulenger and colleagues in the inter-renal tissue of the frog (Leboulenger, Benyamina, Delarue, Netchitaïlo, Saint-Pierre & Vaudry, 1988). These authors attribute the finding that the effects are strictly additive to the fact that activation of VIPergic and muscarinic receptors in the adrenal cortex of the frog stimulate adrenal steroidogenesis by two entirely separate mechanisms (Leboulenger, Charnay, Dubois, Rossier & Vaudry, 1984; Delarue *et al.* 1986) and that may well turn out to be the explanation in the adrenal cortex of the calf. The precise pathway by which VIP induces steroidogenesis in the adrenal cortex in mammals has yet to be established. It might act in a cAMP-dependent fashion as it does in certain other tissues (Amiranoff & Rosselin, 1982) or by increasing the breakdown of phosphatidylinositol, as it does in the superior cervical ganglion of the rat (Audigier, Barberis & Jard, 1986). Acetylcholine has been shown to stimulate cortisol secretion in bovine adrenal fasciculata-reticularis cells *in vitro*, via m3 muscarinic receptors linked to a polyphosphoinositide-specific phospholipase-C (Walker, Strachan, Lightly, Williams & Bird, 1990) and increase intracellular Ca^{2+},

at least partly by releasing it from an intracellular pool (Walker, Strachan, Nicol, Williams & Bird, 1991).

Adrenal medullary responses to intra-arterial infusions of acetylcholine at the top of this dose range are largely annulled by atropine (Jones *et al.* 1991). They are therefore presumably also mediated via muscarinic receptors, recently found to be of an M4 subtype in bovine adrenal medullary cells *in vitro* (Fernando, Abdallah, Evinger, Foray & Elfakahany, 1991). VIP was also found to release detectable amounts of adrenaline and noradrenaline when infused intra-aortically at a dose of 0·13 μg min^{-1} kg^{-1}. However, it had no potentiating effect on catecholamine output when given together with acetylcholine at a slightly lower dose (0·065 μg min^{-1} kg^{-1}), chosen to minimize vascular effects while still exerting a pronounced steroidogenic response on the cortex. Others have reported that it potentiates catecholamine secretion *in vitro* both in the presence and the absence of acetylcholine (Misbahuddin *et al.* 1986; Houchi *et al.* 1987; Waymire *et al.* 1991) although the precise mechanism is controversial. It also stimulates proenkephalin A mRNA expression and increases levels of enkephalin-containing peptides in bovine adrenal chromaffin cells *in vitro* (Wilson, 1987; Wan & Livett, 1989) whereas it had no detectable effect on adrenal enkephalin output in the present experiments. The most likely explanation for these differences would appear to be differences in the concentration at which the peptide was presented to the gland. From a previous study in which the concentration of VIP in the adrenal venous effluent plasma was measured during such intra-arterial infusions of the peptide in conscious calves (Bloom *et al.* 1987), one can predict that the maximum possible concentration in the adrenal extracellular fluid would have been about 200 pmol l^{-1}. This happens to coincide with the levels found in submandibular effluent blood in cats during electrical stimulation of the chorda tympani (Bloom & Edwards, 1980) showing that it is well within the physiological range. The *in vitro* studies referred to above employed much higher concentrations of the peptide ranging from 10 nM to 20 μM. However the actual concentration which is achieved at the receptor sites when VIP is released from nerve terminals is, of course, unknown.

It is concluded that muscarinic adrenal medullary responses are unlikely to be potentiated by VIP *in vivo* and that the adrenal cortical steroidogenic effects of acetylcholine and VIP are strictly additive in this species.

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