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EFFECTS OF SYNTHETIC ADRENOCORTICOTROPHIN ON ADRENAL MEDULLARY RESPONSES TO SPLANCHNIC NERVE STIMULATION IN CONSCIOUS CALVES

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SUMMARY

1. Right medullary and various cardiovascular responses to stimulation of the peripheral end of the splanchnic nerve have been investigated in the presence and absence of exogenous adrenocorticotrophin, $ACTH_{1-24}$, (5 ng min^{-1} kg^{-1}). The adrenal-clamp technique was employed in conscious calves, after the pituitary stalk had been cauterized and they had recovered from anaesthesia.

2. The intravenous infusion of $ACTH_{1-24}$ increased the plasma ACTH concentration by about 1100 pg ml^{-1} and right adrenal venous output of cortisol by about 400 ng min^{-1} kg body $weight^{-1}$. Stimulation of the splanchnic nerve at 4 Hz for 10 min had no effect on either arterial plasma ACTH concentration or the adrenal output of cortisol.

3. Closely similar amounts of both adrenaline and noradrenaline were released in response to nerve stimulation in the presence and absence of exogenous ACTH. In contrast, the fall in adrenal vascular resistance of about 40%, which normally occurred in response to splanchnic nerve stimulation, was completely abolished by ACTH.

4. The adrenal produced relatively large quantities of met-enkephalin-containing peptides. During splanchnic nerve stimulation the output of these increased 20–100-fold, at which time free met^5-enkephalin accounted for only 10–20% of total. During ACTH infusion the output of free met^5-enkephalin was reduced at rest and during nerve stimulation, but that of total met-enkephalin-containing peptides was unaffected. These results indicate that ACTH or an adrenal steroid may alter the processing of proenkephalin in the adrenal medulla acutely but not total opiate secretion. Alternatively, the presence of ACTH could act by influencing the population of chromaffin cells activated by splanchnic nerve stimulation.

INTRODUCTION

Justification for the association of the adrenal medulla and cortex is to be found in the long-term control of phenyl-ethanolamine-*N*-methyl transferase activity, and hence adrenaline output by cortisol (Wurtman & Axelrod, 1966). The anatomical

basis for this is a proposed intraglandular portal system between cortical sinusoids and medullary capillaries (Coupland, 1975; Henderson & Daniel, 1978). However, in the rat, which seems to be the only species in which the adrenal microvasculature has been investigated thoroughly, there appears to be no direct connexion between the capillary beds and the cortical sinusoids which empty into peripheral branches of the adrenal vein (Kikuta & Murakami, 1982). This suggests that direct cortical effects on the medulla are transmitted in some other way and it may well be relevant that the adrenal cortical epithelium is fenestrated (Motta, Muto & Fujita, 1979). Interaction between the medulla and cortex of the adrenal gland might also be reciprocal. Thus, although the changes in adrenal blood flow which occur in response to splanchnic nerve activity (Edwards, Furness & Helle, 1980), could be mediated entirely *via* the innervation to the gland (Mikhail, 1961), potentially vasodilator peptides, such as the enkephalins are released from the medulla and are capable of activating the pituitary–adrenal axis (De Souza & Van Loon, 1982). Since ACTH itself exerts a potent vasodilator effect on the adrenal vasculature (Edwards, Hardy & Malinowska, 1974) this represents an alternative effector mechanism. Angiotensin II provides an example of an agonist which is capable of affecting both parts of the gland; increasing the release of adrenaline from the medulla (Feldberg & Lewis, 1964; Reit, 1972) in addition to promoting the secretion of aldosterone from the cortex.

Observations which have suggested that factors additional to adrenocorticotrophin (ACTH) influence adrenal cortical secretion might be explained, at least in part, by intra-glandular modulation. These include both diurnal and acute changes in the sensitivity to ACTH (Engeland, Byrnes, Presnell & Gann, 1981; Kaneko, Kaneko, Shinsako & Dallman, 1981). Moreover, neural or other mechanisms may be involved in controlling both the short- and long-term responses to ACTH (Dallman, Engeland & McBride, 1977; Krieger, 1979; Kaneko *et al.* 1981; Engeland *et al.* 1981; Wilkinson, Shinsako & Dallman, 1982). A potential candidate for such a role, possibly acting entirely within the gland, is met-enkephalin, an analogue of which has been shown to reduce the response to ACTH of rat adrenal cortical cells *in vitro* (Guaza, 1984).

The existence of such regulatory mechanisms has been investigated in the present study by attempting to discover whether splanchnic nerve stimulation, and hence adrenal medullary activity, exerts short-term effects on the response of the cortex to ACTH. Undiluted adrenal effluent blood can be collected from conscious calves, without damaging the innervation, by means of an 'adrenal clamp' (Edwards *et al.* 1974). The peripheral end of the splanchnic nerve in these animals can be stimulated below behavioural threshold (Bloom & Edwards, 1980), thus avoiding anaesthesia which modifies the adrenal medullary responses to stimulation via the innervation substantially (Edwards *et al.* 1980) and could also affect cortical responses. This technique has been employed in the present experiments to establish whether there is evidence of direct interaction between the cortex and medulla when the adrenal gland is subjected to simultaneous stimulation via the splanchnic nerve and with infusions of ACTH. The pituitary stalk had been destroyed by thermocautery in each animal in order to prevent variations in the release of endogenous ACTH.

METHODS

Animals

Pedigree Jersey calves were obtained from local farms shortly after birth and used at ages of 22–42 days (28–45 kg body weight). They were kept in individual pens and maintained on a diet of artificial milk (Easy-mix Volac, Volac Ltd.) at a rate of 3–4 l day^{-1}. Food was withheld overnight prior to each experiment.

Experimental procedures

Anaesthesia was induced with chloroform (Chloroform SLR, Fisons) and maintained with halothane (May & Baker; *ca.* 2% in oxygen). Preparatory surgery involved the insertion of narrow-bore polyethylene catheters into the saphenous arteries so that the tips lay in the abdominal aorta. These were used subsequently to monitor aortic blood pressure and heart rate and for the collection of arterial samples. A braunula cannula was inserted into a jugular vein to provide a conduit for I.V. infusions of ACTH. Following the removal of the right kidney and cannulation of the right renal vein an adrenal clamp was emplaced as described previously (Edwards *et al.* 1974, 1980). The right splanchnic nerve was cut immediately below the diaphragm and the peripheral end enclosed in a fluid electrode designed to minimize the spread of stimulus to surrounding tissues. The ventral surface of the basisphenoid bone was then exposed via the parapharyngeal approach and a dental drill employed to bore a hole, 3–4 mm diameter, through it in the mid-line and visualize the ventral aspect of the pituitary lying in the sella turcica. A flexible thermocautery electrode, insulated except at the tip, was then inserted through the basisphenoid and the substance of the gland until the tip was positioned just below the pars tuberalis. This was then thoroughly cauterized, as were the contents of the sella during withdrawal of the electrode. Haemorrhage from the surrounding sinuses was controlled by hyperventilation and tilting the operating table so as to raise the head and reduce the intracranial venous pressure. Finally, the sella turcica and the hole in the basisphenoid were packed with absorbable gelatin sponge (Sterispon; Allen & Handburys) and the wound closed.

Experiments were carried out 3–4 h after surgery and when the animals had recovered from anaesthesia. A standard 20–30 V square-wave electrical stimulus (pulse width 0·5 ms) was employed at a frequency of 4 Hz for 10 min. In animals given $ACTH_{1-24}$ (Synacthen, CIBA), the peptide was dissolved in an appropriate volume of saline and infused I.V. at a dose of 5 ng min^{-1} kg^{-1} (2·5 ml min^{-1}) for 50 min and the splanchnic nerve was stimulated for 10 min after the ACTH had been infused for 20 min. Assay of ACTH in the infusate emerging from the catheter at the end of the period of infusion showed that the concentration was 90 ± 10% of that expected. 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 catecholamines, cortisol and enkephalins from the gland were calculated. Adrenal vascular resistance was calculated by dividing the perfusion pressure (mean aortic blood pressure) by the right adrenal blood flow. The outputs of catecholamines, cortisol and enkephalins from the gland were expressed as unit weight min^{-1} kg body $weight^{-1}$, having been estimated from the concentration of each in the adrenal effluent plasma and adrenal plasma flow at the time of collection.

Analytical procedures

Samples of arterial blood were collected at intervals into heparinized tubes containing phenylmethylsulphonyl fluoride (final plasma concentration 0·1 mM; Sigma Chemical Co.) for haematocrit, glucose, ACTH and cortisol estimations. Right adrenal venous effluent blood samples were collected at the same intervals into heparinized tubes containing 2–3 mg EDTA for catecholamine estimations and phenylmethylsulphonyl fluoride for cortisol, ACTH and enkephalin estimations. Each was centrifuged at 4 °C as soon as possible and plasma stored at −20 or −70 °C.

Glucose was measured enzymatically by means of a Beckman Mark 2 Glucose analyser. Adrenaline and noradrenaline were estimated by a modification of von Euler & Floding's trihydroxyindole method (von Euler & Floding, 1955) as described previously (Bloom, Edwards, Hardy, Malinowska & Silver, 1975). 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 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 detected by measuring absorbance at 240 nm in a Pye-Unicam U.V. detector.

Met- and leu-enkephalin were measured by specific radioimmunoassay essentially as described by Gros, Pradelles, Rouget, Bepoldin, Dray, Fournie-Zaluwski, Roques, Pollard, Llorens-Cortes & Schwartz (1978). The antibody against met^5-enkephalin gave 50% binding at 6 pg of peptide $tube^{-1}$, its cross-reaction with related peptides was: 3·5% leu^5-enkephalin, 2% met-enkephalyl-arg-phe, 2% met-enkephalyl-arg-gly-leu, < 0·1% each α- and β-endorphin and dynorphin. The antibody against leu^5-enkephalin gave 50% binding at 3 pg $tube^{-1}$ and showed 4% cross-reactivity with met^5-enkephalin for which the results reported below were corrected. Prior to assay 0·1% 2-mercaptoethanol was added to adrenal effluent plasma which was then heated to 95 °C for 30 min. Plasma contained met-enkephalin in the range 60–7000 pg ml^{-1} and leu-enkephalin in the range 6–600 pg ml^{-1}. The recovery of standard met^5- and leu^5-enkephalin added to plasma was 85–92%; no correction was made for this. Intra- and inter-assay coefficients of variation were about 9% and 12% respectively.

Met-enkephalin-containing peptides were measured either on samples of untreated perfusate or after proteolytic digestion to liberate met^5-enkephalin from any released precursor essentially as described by Lewis, Stern, Kimura, Rossier, Stein & Udenfriend (1980) and by Chaminade, Foutz & Rossier (1984). A 60 or 120 min incubation at 37 °C with 60 μg ml^{-1} of trypsin treated with L-(tosylamido 2-phenyl)ethyl chloromethyl ketone (TPCK-trypsin, Worthington Biochemicals) followed by 60 min incubation at 37 °C with 0·1 μg ml^{-1} of carboxypeptidase B (Boehringer Corp.). This procedure liberated 90–94% of the met^5-enkephalin peptide from pro-enkephalin peptide B, E and F. Moreover the recovery of met^5-enkephalin taken through the digestion procedure was 93·6 ± 2·8% (6).

Enkephalin-containing samples were subjected to gel-filtration chromatography and h.p.l.c. Gel filtration was carried out on a Sephadex G100 (100 × 2·5 cm, Superfine; Pharmacia) eluted at 5 ml h^{-1} with 1 M-acetic acid at 4 °C. The 5 ml fractions were freeze-dried after collection and resuspended in 10% acetic acid containing 0·1% 2-mercaptoethanol. Separation by h.p.l.c. was carried out on a Bondapak C8 reversed phase column (25 × 0·4 cm; Waters Associates) eluted at 1 ml min^{-1} and *ca.* 1500 lbf in^{-2} with a 0–50% linear gradient of acetonitrile (Fisons Ltd.) in 0·25 M-triethylammonium formate, pH 3·3. Fractions of 1 ml were collected and freeze-dried. Enkephalin peptides used for validation of assay, digestion and separation procedures were obtained from Peninsula Laboratories.

Results are expressed as means ± S.E. of mean, statistical analyses 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 clamp were removed. The positioning of the clamp was then checked and the gland 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 been destroyed by cautery without producing intracranial bleeding. Assessment of the success or otherwise of attempted functional hypophysectomy by macroscopic examination was found to correlate well with changes in plasma ACTH concentration which occurred post-operatively. Animals in which plasma ACTH concentration had not fallen below 150 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

Stimulation of the peripheral end of the right splanchnic nerve at 4 Hz for 10 min produced an abrupt rise in mean aortic blood pressure in conscious hypophysectomized calves, which reached a peak at 2·5 min and declined thereafter in spite of continued stimulation. This rise in blood pressure was accompanied by a fall in mean heart rate, which was abolished by atropine and can therefore be attributed to reflex

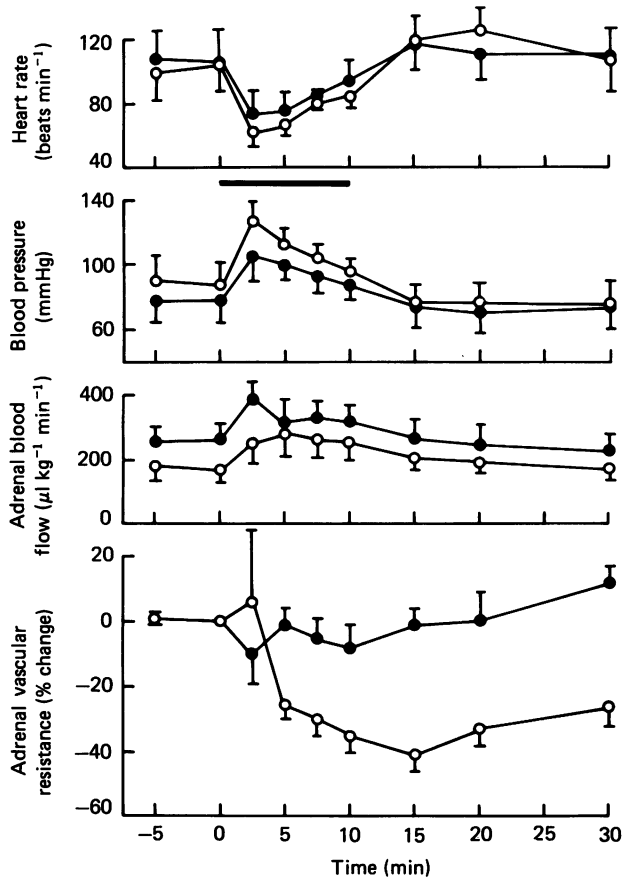

Fig. 1. Comparison of various cardiovascular responses to stimulation of the peripheral end of the right splanchnic nerve at 4 Hz for 10 min in conscious 3–6-week-old hypophysectomized calves in the presence (●, $n = 4$) and absence (○, $n = 4$) of exogenous ACTH (5 ng min^{-1} kg^{-1}). Horizontal bar: duration of stimulation. Vertical bars: S.E. of each mean value.

parasympathetic cardiac inhibition. Closely similar changes in both mean heart rate and aortic blood pressure occurred in the presence of exogenous ACTH (5 ng min^{-1} kg^{-1}, Fig. 1). There was a small rise in blood flow through the right adrenal gland in response to stimulation in both groups. During infusions of ACTH the time course of this rise in adrenal blood flow corresponded to that of the hypertensive response and, as there was no significant change in adrenal vascular resistance, it could be attributed entirely to the rise in perfusion pressure. However, in the absence of exogenous ACTH the rise in mean adrenal blood flow followed a different time course from the rise in aortic blood pressure and there was a delayed, but substantial, prolonged and statistically significant fall in adrenal vascular resistance, which reached a nadir of $-41 \pm 5\%$ 5 min after stimulation had been discontinued ($P < 0{\cdot}01$, Fig. 1).

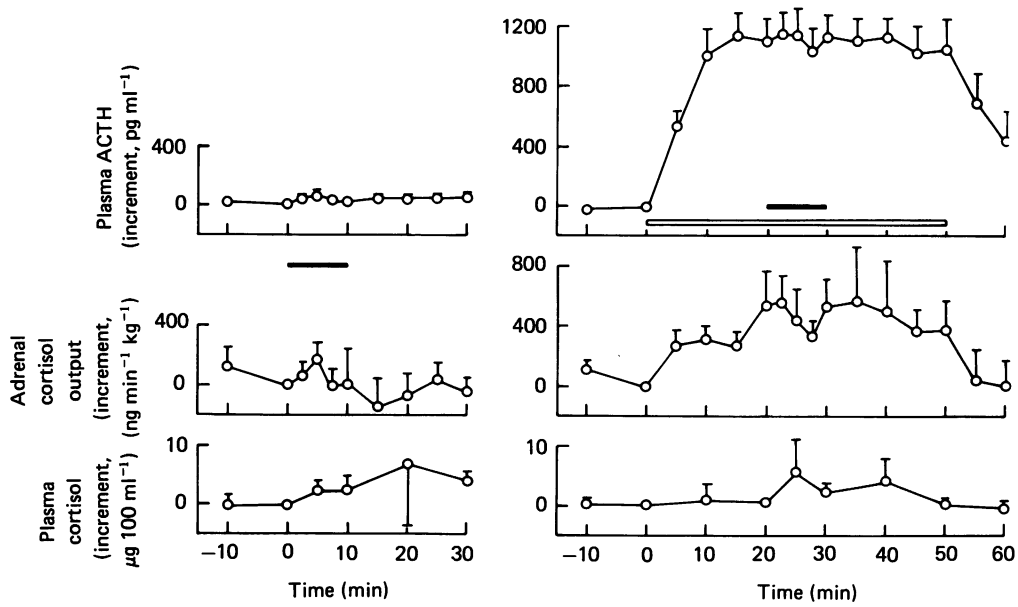

Fig. 2. Comparison of the changes in mean arterial plasma ACTH, cortisol and right adrenal cortisol output, in response to stimulation of the peripheral end of the right splanchnic nerve (4 Hz for 10 min) in conscious 3–6-week-old hypophysectomized calves in the presence ($n = 4$) and absence ($n = 4$) of exogenous ACTH (5 ng min^{-1} kg^{-1}). Open horizontal bar: duration of ACTH infusion. Filled horizontal bars: duration of stimulation. Absolute values at time = 0 in the absence of exogenous ACTH: plasma ACTH 42 ± 14 pg ml^{-1}, plasma cortisol $10{\cdot}6 \pm 2{\cdot}3$ μg 100 ml^{-1}, cortisol output 1117 ± 206 ng min^{-1} kg^{-1}. Before infusions of ACTH: plasma ACTH 72 ± 31 pg ml^{-1}, plasma cortisol $8{\cdot}5 \pm 1{\cdot}8$ μg 100 ml^{-1}, cortisol output 945 ± 176 ng min^{-1} kg^{-1}.

Haematocrit and arterial plasma glucose concentration were also monitored during these experiments and the expected increase in both occurred in response to splanchnic nerve stimulation. Neither response was significantly affected by the intravenous infusion of exogenous ACTH.

Adrenal cortical responses

Intravenous infusion of exogenous $ACTH_{1-24}$ produced a rapid rise in concentration to a plateau that was achieved in 15 min and persisted for the duration of the infusion. Mean plasma ACTH concentration rose by about 1100 pg ml^{-1} from an initial value of 72 ± 31 pg ml^{-1} and returned rapidly towards the initial value when the infusion was stopped, with a half-life of about 7·5 min (Fig. 2). The initial ACTH concentration in the hypophysectomized calves was much lower than the mean value of 514 ± 227 pg ml^{-1} in intact calves. The rise in plasma ACTH concentration during $ACTH_{1-24}$ infusion was associated with an increase in cortisol output from the right adrenal gland of about 400 ng min^{-1} kg^{-1} at 20 min. At this time the right splanchnic nerve was stimulated (4 Hz for 10 min), which had no effect on the enhanced output of cortisol (Fig. 2). The ACTH-induced rise in the output of cortisol from the adrenal gland failed to produce a significant increase in the concentration

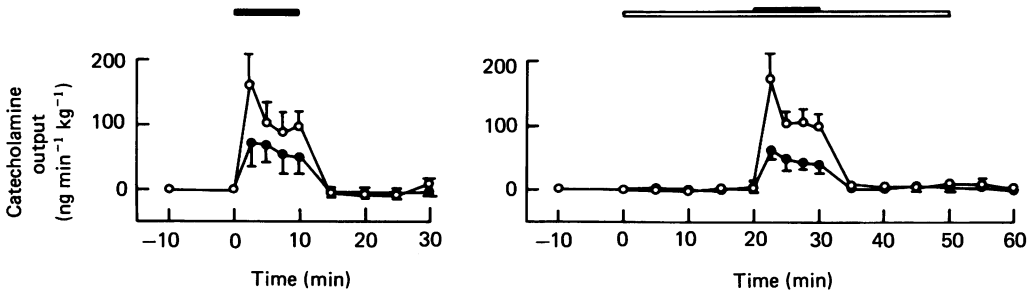

Fig. 3. Comparison of the changes in the mean outputs of adrenaline (○) and noradrenaline (●) from the right adrenal gland in response to stimulation of the peripheral end of the right splanchnic nerve (4 Hz for 10 min) in conscious 3–6-week-old hypophysectomized calves in the presence ($n = 4$) and absence ($n = 4$) of exogenous ACTH (5 ng min^{-1} kg^{-1}). Open horizontal bar: duration of ACTH infusion. Filled horizontal bars: duration of stimulation. Vertical bars: S.E. of each mean value. Absolute values immediately before stimulation in the absence of exogenous ACTH: adrenaline 18 ± 13 ng min^{-1} kg^{-1}, noradrenaline 8 ± 7 ng min^{-1} kg^{-1}. In the presence of ACTH: adrenaline 14 ± 14 ng min^{-1} kg^{-1}, noradrenaline 7 ± 5 ng min^{-1} kg^{-1}.

of steroid in peripheral plasma, presumably because it was already high ($8{\cdot}5 \pm 1{\cdot}8$ μg 100 ml^{-1}, Fig. 2). The steroid output, as measured by cortisol radioimmunoassay, was $91{\cdot}4 \pm 2{\cdot}6$% of that estimated by h.p.l.c. and spectrophotometric detection (see Methods).

Stimulation of the peripheral end of the right splanchnic nerve produced no significant change in mean plasma ACTH or cortisol concentration whether or not exogenous ACTH was infused (Fig. 2). Thus these experiments yielded no evidence that adrenal medullary activity influences steroid output from the cortex under these particular experimental conditions.

Adrenal medullary responses

Unlike cortisol, the outputs of both adrenaline and noradrenaline from the adrenal gland were trivial prior to stimulation of the splanchnic nerve. In the absence of ACTH the initial values were 18 ± 13 ng min^{-1} kg^{-1} (adrenaline) and 8 ± 7 ng min^{-1} kg^{-1} (noradrenaline); in the presence of ACTH the corresponding values were 14 ± 14 and 7 ± 5 ng min^{-1} kg^{-1}. Splanchnic nerve stimulation produced a rapid increase in the output of both. The responses were biphasic with the outputs rising to a peak at 2·5 min and then falling to a plateau of about 110 ng min^{-1} kg^{-1}, which was maintained thereafter until stimulation was discontinued, when output fell rapidly to the resting range (Fig. 3). The incremental values during stimulation were closely similar in the two groups, thus the outputs of adrenaline at 2·5 min were 160 ± 50 ng min^{-1} kg^{-1} in the absence of ACTH and 170 ± 40 in the presence of ACTH; the corresponding values for noradrenaline were 73 ± 37 and 63 ± 13 ng min^{-1} kg^{-1}. Similar values were obtained previously in normal conscious calves of the same age in response to stimulation of the peripheral end of the right splanchnic nerve (Edwards *et al.* 1980).

The mean output of met-enkephalin-like immunoreactivity from the denervated

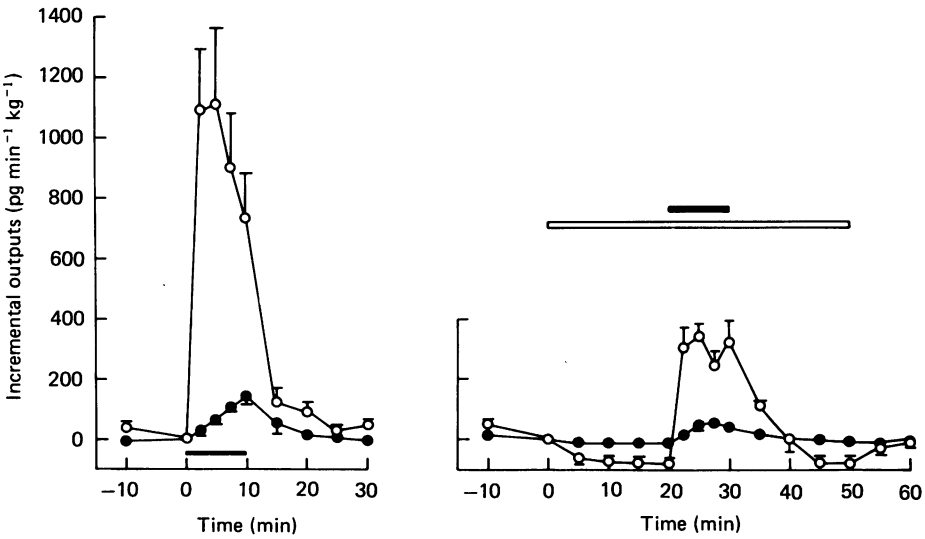

Fig. 4. Comparison of the changes in the mean outputs of met- (○) and leu-enkephalin (●) in response to stimulation of the peripheral end of the right splanchnic nerve (4 Hz for 10 min) in conscious 3–6-week-old hypophysectomized calves in the presence ($n = 4$) and absence ($n = 4$) of exogenous ACTH. Open horizontal bar: duration of ACTH infusion. Filled horizontal bars: duration of stimulation. Vertical bars: S.E. of each mean value. Absolute values immediately before stimulation in the absence of ACTH: met-enkephalin 124 ± 20 pg min^{-1} kg^{-1}, leu-enkephalin 21 ± 4 pg min^{-1} kg^{-1}. In the presence of ACTH: met-enkephalin 67 ± 10 pg min^{-1} kg^{-1}, leu-enkephalin 7 ± 2 pg min^{-1} kg^{-1}.

adrenal gland prior to stimulation (150 ± 30 pg min^{-1} kg^{-1}) was substantially and significantly greater than that of leu-enkephalin-like immunoreactivity (16 ± 4 pg min^{-1} kg^{-1}, $P < 0{\cdot}01$). The output of both was reduced by intravenous infusion of $ACTH_{1-24}$; after 20 min that of met-enkephalin-like immunoreactivity had fallen to 67 ± 10 whilst output of leu-enkephalin-like immunoreactivity had fallen to 7 ± 2 pg min^{-1} kg^{-1} ($P < 0{\cdot}05$). Stimulation of the splanchnic nerve increased the output of both met- and leu-enkephalin-like immunoreactivity and this effect was significantly inhibited by infusion of $ACTH_{1-24}$ (Fig. 4). In the absence of exogenous ACTH nerve stimulation elicited an abrupt rise in output of met-enkephalin-like immunoreactivity to a peak incremental value of 1110 ± 250 pg min^{-1} kg^{-1} at 5 min, whereas the output of leu-enkephalin-like immunoreactivity rose more slowly, and by substantially less to a mean incremental peak of 146 ± 28 pg min^{-1} kg^{-1} at 10 min. During infusion of $ACTH_{1-24}$ the pattern of release was similar but the amounts released were significantly smaller and the corresponding peak incremental values were 340 ± 40 pg min^{-1} kg^{-1} (met-enkephalin; $P < 0{\cdot}05$) and 62 ± 6 pg min^{-1} kg^{-1} (leu-enkephalin; $P < 0{\cdot}05$). In both groups the mean outputs of the two peptides returned rapidly to the initial range when stimulation was discontinued (Fig. 4). Furthermore, the outputs of both had returned to normal, in the group given $ACTH_{1-24}$, within 10 min when the infusion was terminated.

Separation of adrenal effluent by gel filtration demonstrated that the immuno-assayable met-enkephalin-like peptide fraction was heterogeneous although most of

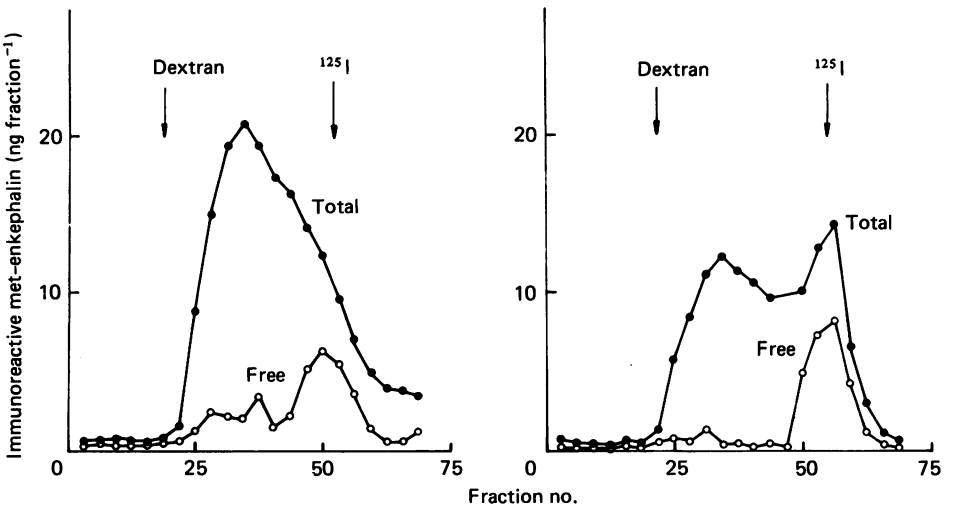

Fig. 5. Separation of met-enkephalin-containing peptides in adrenal effluent during stimulation of the splanchnic nerve at 4 Hz. Heat-treated, freeze-dried plasma was dissolved in 1 ml of 10 % acetic acid containing 0·1 % 2-mercaptoethanol and separated on a Sephadex G-100 column (100 × 2·5 cm, superfine) by elution at 5 ml h^{-1} with 1 M-acetic acid containing 0·1 % 2-mercaptoethanol; 5 ml fractions were collected and freeze-dried. The samples were adrenal effluent separated and either assayed without prior proteolytic digestion (○), or assayed after incubation for 2 h with 60 μg ml^{-1} of TPCK-trypsin followed by 1 h with 0·1 μg ml^{-1} of carboxypeptidase B both at 37 °C (●). The arrows indicate elution position of dextran and ^{125}I; met^5-enkephalin eluted close to ^{125}I. Fractions were assayed by radioimmunoassay for met-enkephalin-containing peptides using the antibody raised against met^5-enkephalin (see Methods). Examples from two sets of adrenal effluent samples are shown.

the assayable material coincided with the met^5-enkephalin peak. Proteolytic digestion of eluate peptides for 2 h caused a large increase in the peak co-incident with met^5-enkephalin and of peaks eluting earlier with $M_r > 20\,000$ (Fig. 5).

H.p.l.c. separation of the peak of peptide that is predominantly assayed in adrenal effluent plasma when proteolytic digestion is not employed, showed that it co-eluted with met^5-enkephalin (Fig. 6). It was estimated that 85·4 ± 5·1 % (8) of the enkephalin peptide assayed in untreated adrenal effluent was met^5-enkephalin. This fraction (42–57 on Sephadex G100) was essentially similar after proteolytic digestion (Fig. 6).

The concentration of total met-enkephalin-containing peptides measured after proteolytic digestion was always at least 4-fold higher than that of 'free' peptide. If the proteolytic digestion of met-enkephalin-containing peptides provides a fraction which on assay reflects total release of such peptides from the adrenal, it is possible to compare output of met^5-enkephalin with that of the proenkephalin family. Such an analysis is provided in Fig. 7 where output by assay from untreated plasma (free) is compared with the values obtained after prior digestion with trypsin and carboxypeptidase B. Before infusion of $ACTH_{1-24}$ adrenal output of met-enkephalin-containing peptides was 0·43 ± 0·10 ng min^{-1} kg body $weight^{-1}$ with met^5-

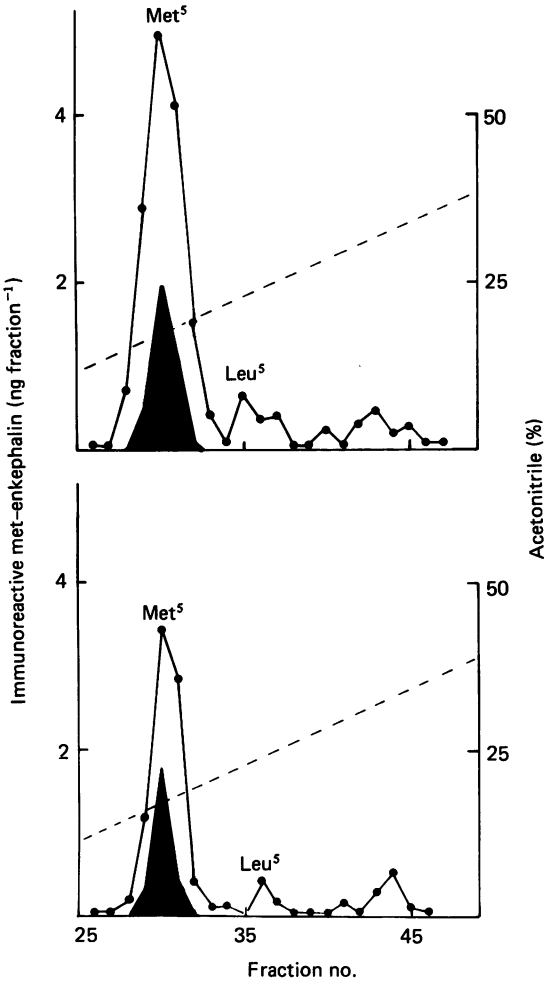

Fig. 6. Separation by h.p.l.c. of enkephalin peptides from fractions 47–55 in adrenal effluent. Freeze-dried samples from fractions 47–55 after Sephadex G-100 chromatography (Fig. 5) were separated on a Bondapak C8 reversed phase column (25 × 0·4 cm) eluted with 0·25 M-triethylammonium formate (pH 3·3) and a linear 0–50% gradient (---) of acetonitrile at 1 ml min^{-1} and 1500 lbf in^{-2}. Fractions of 1 ml were collected and freeze-dried. Met-enkephalin-containing peptides were measured by radioimmunoassay using an antibody against met⁵-enkephalin (see Methods). The upper profile shows peptides isolated by gel-filtration after proteolytic digestion, the lower being without such digestion (see Fig. 5). The shaded areas relate to the elution of 3 ng of standard met⁵-enkephalin.

enkephalin accounting for only 40% of this (Fig. 7). During splanchnic nerve stimulation the output of total peptide increased almost 10-fold to $3{\cdot}90 \pm 1{\cdot}01$ ng min^{-1} kg^{-1} ($< 0{\cdot}01$) and met⁵-enkephalin then accounted for about 20% of the total met-enkephalin-containing peptide released by the adrenal ($P < 0{\cdot}05$). Infusion of $ACTH_{1-24}$ had no significant effect on the output of the met-enkephalin peptides (Fig. 7), but as the production of met⁵-enkephalin was depressed it now represented

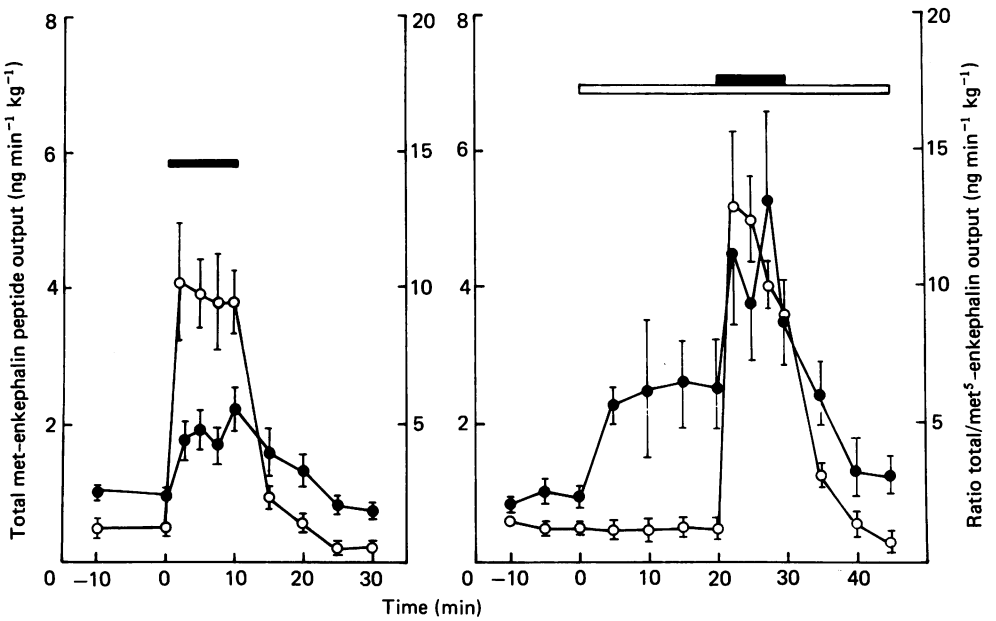

Fig. 7. Output of met-enkephalin-containing peptides from the calf adrenal (ng min^{-1} kg body $weight^{-1}$) in response to the infusion of $ACTH_{1-24}$ at 5 ng min^{-1} kg^{-1} (▭) and to stimulation of the splanchnic nerve at 20 – 30 V and 4 Hz (▬). Met-enkephalin-containing peptides (○) were measured by radioimmunoassay with an antibody against met^5-enkephalin, after prior digestion with trypsin and carboxypeptidase B (see Methods). The ratio of this fraction of total met-enkephalin to that measured as met^5-enkephalin (i.e. without prior proteolytic digestion, see Fig. 4) is also plotted (●).

< 10% of the total met-enkephalin fraction. Splanchnic nerve stimulation in the presence of exogenously supplied ACTH caused a similar increase in the output of the met-enkephalin-containing peptides to that in its absence. However, the smaller increase in production of met^5-enkephalin under these conditions meant that it now represented < 10% of the fraction of enkephalin-containing peptides (Fig. 7).

DISCUSSION

Whereas there is strong evidence that there are alterations over periods of hours or days in the adrenal cortical response to ACTH that cannot be explained by ACTH alone (Krieger. 1979; Kaneko *et al.* 1981; Engeland *et al.* 1981; Wilkinson *et al.* 1982), it has yet to be established whether changes in cortisol output that occur over much shorter periods can be caused by mechanisms other than those involving variations in plasma ACTH. Corticosteroid responses to mild haemorrhage have been reported where there is little or no change in plasma ACTH concentration (Engeland, Lilly & Gann, 1985). The present experiments provide no evidence to support the contention that adrenal medullary activity affects the release of glucocorticoids from the adrenal cortex. The dose of ACTH employed (5 ng min^{-1} kg^{-1}) was expected to produce a submaximal increase in cortisol output of about 300 ng min^{-1} kg^{-1}, as had

been found previously (Edwards, Hardy & Malinowska, 1975). In the event mean cortisol output was increased by about 400 ng min^{-1} kg^{-1}. However, both the initial cortisol output and the concentration of the steroid in peripheral plasma were greater than normal, which may be a consequence of the preceding anaesthesia and surgery. The plasma ACTH concentration fell quite rapidly after cauterizing the pituitary stalk, to lower values than found in intact calves. However, extremely high levels would have been present during surgery, and studies in dogs have shown that the steroidogenic effects of sustained high plasma levels of ACTH can persist for some time after the concentration has returned to normal (Wood, Shinsako & Dallman, 1982; Keller-Wood, Shinsako & Dallman, 1983). This problem might be overcome by means of a two-stage operation, but on the relatively few occasions that it has proved possible to destroy the pituitary stalk as long as 24 h before the experiment, a high basal rate of steroid production has still been encountered in spite of very low plasma ACTH concentrations (A. V. Edwards & C. T. Jones, unpublished observations). Thus, although adrenal medullary activity was not apparently associated with any change in cortisol release, such an effect could have been masked by a high basal output.

Evidence of cortico-medullary interaction was suggested by the observation that the fall in adrenal vascular resistance, which occurred in response to splanchnic nerve stimulation, was abolished by infusing ACTH. This peptide exerts a direct vasodilator effect on the adrenal, which is independent of its action on steroid output or of changes in blood pressure (Edwards *et al.* 1975), but the mechanism of this action is unknown. It seems unlikely that the vasodilator response to splanchnic nerve stimulation was merely obscured by that to ACTH at this dose as the maximum rates of blood flow (< 400 μl min^{-1} kg^{-1}) recorded during these experiments were well below that which occurs with maximal or supramaximal doses of ACTH (700 μl min^{-1} kg^{-1}; Edwards *et al.* 1975). On the other hand, it is tempting to speculate that it may be due to enkephalin release, which was reduced in the presence of ACTH, as these peptides are capable of causing vasodilation under certain conditions (Konturek, Pawlik, Tasler, Thor, Walus, Krol, Jaworek & Schally, 1978). However, secretion of enkephalins was only partially inhibited by ACTH whereas the fall in adrenal vascular resistance was completely abolished. Other peptides are known to be present in the adrenal medulla and vasoactive intestinal polypeptide, which is a very potent vasodilator, is released from the gland in response to splanchnic nerve stimulation (S. R. Bloom & A. V. Edwards, unpublished observations).

The contention that the pituitary–adrenal cortical axis exerts direct short-term effects on the medulla is supported by the finding that the output of enkephalins was modified by ACTH. It is now well established that these peptides are present in adrenal medulla, in a wide range of species, as a proenkephalin precursor possessing six copies of met- and one copy of leu-enkephalin, the processing of which is under neural control (Schultzberg, Lundberg, Hökfelt, Terenius, Brandt, Elde & Goldstein, 1978; Costa, DiGiulio, Fratta, Hong & Yang, 1979; Stern, Lewis, Kimura, Rossier, Gerber, Brink, Stein & Udenfriend, 1979; Viveros, Diliberto, Hazum & Chang, 1979; Chaminade *et al.* 1984; Fleminger, Howells, Kilpatrick & Udenfriend, 1984). They are localized both in chromaffin granules where they are synthesized (Stern *et al.* 1979; Chang, Wilson & Viveros, 1982) and within splanchnic nerve terminals

(Schultzberg *et al.* 1978). The present studies confirm the finding of Costa's group that enkephalin-like immunoreactivity is released in response to splanchnic nerve stimulation (Hexum, Hanbauer, Govoni, Yang & Costa, 1980; Govoni, Hanbauer, Hexum, Yang, Kelly & Costa, 1981). The stimulated isolated cat adrenal produces more met-enkephalin in higher molecular weight forms than free met^5-enkephalin (Chaminade *et al.* 1984) but the ratio between the two was even higher in the present experiments, in which relatively little free met^5-enkephalin was released during nerve stimulation. The use of proteolytic digestion to reveal met-enkephalin-containing peptides has been reported to give good yields of met^5-enkephalin and this was confirmed in the present study (Lewis *et al.* 1980; Chaminade *et al.* 1984; Fleminger *et al.* 1984). Hence the present observations suggest that in the presence of $ACTH_{1-24}$ the observed fall in the release of free met^5-enkephalin from the adrenal does not reflect a fall in total met-enkephalin-containing peptides, whose output is maintained. This leads to the conclusion that ACTH could have reduced the processing of proenkephalin prior to release. It need not be a direct action of ACTH itself but of some cortical product. It is unlikely to be due to a direct effect on the cholinergic release mechanism (Costa, Guidotti & Saiani, 1980; Kumakura, Karoum, Guidotti & Costa, 1980). A glucocorticoid-induced change in processing is plausible as these steroids have been shown to modify post-translational processing in tumour cells, possibly by influencing protein phosphorylation (Firestone, Payvar & Yamamoto, 1982). Alternatively, ACTH administration could have altered the pool of cells contributing to enkephalin production. There are separate adrenaline-containing and noradrenaline-containing chromaffin cells (Hillarp & Hökfelt, 1953) and evidence of differential activation of these depending on the physiological stimulus (Folkow & von Euler, 1954; Douglas & Poisner, 1965; Feuerstein & Gutman, 1971). Hence if a similar heterogeneity was present for enkephalin-containing cells ACTH could have favoured output from those with a lower content of 'free' met-enkephalin.

The physiological significance of this pituitary–adrenal–cortical influence is at present a matter for conjecture. The claim that enkephalins stimulate ACTH release (DeSouza & Van Loon, 1982) raises the possibility that it is implicated in some feed-back control mechanism but this is difficult to reconcile with the studies which indicate that opiate pathways inhibit ACTH secretion (Stubbs, Delitala, Jones, Jeffcoate, Edwards, Besser, Bloom & Alberti, 1978; Del Pozo, Martin-Perez, Stadelman, Girard & Brownwell, 1980; Grossman, Gaillard, McCartney, Rees & Besser, 1982). This would be consistent with the finding that met-enkephalin inhibits directly ACTH-induced steroid output from rat adrenal cortex (Guaza, 1984); however, no inhibitory effect has been observed either in the sheep or guinea-pig (C. T. Jones, unpublished observations). In spite of this confusion evidence is accumulating which suggests that enkephalins may dampen the responses of the adrenal to physiological stimuli (Bouloux, Grossman, Lytras & Besser, 1985), in which case modulation of opiate output from the medulla by ACTH could fulfil a useful biological role.

Whether or not enkephalins from the adrenal medulla exert important physiological effects on peripheral tissues has yet to be established. It has been suggested that they modify the responses of the peripheral autonomic effectors to catecholamines (Costa, Guidotti, Hanbauer & Saiani, 1983). In view of the present observation that free

met^5-enkephalin is a relatively minor component of the total opiate output from the adrenal, the effects of other opiate peptides clearly need to be assessed.

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