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RELEASE OF ADRENOCORTICOTROPHIN FROM THE ADRENAL GLAND IN THE CONSCIOUS CALF

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SUMMARY

1. The effect of stimulation of the splanchnic nerve on the output of ACTH-related peptides from the adrenal gland has been investigated in conscious, functionally hypophysectomized calves, previously fitted with an 'adrenal clamp'.

2. Stimulation of the splanchnic nerve elicited a small, but statistically significant, increase in the output of ACTH-like immunoreactivity at each frequency tested. This response was frequency-dependent over the range 40–70 Hz when stimulating intermittently for 1 s at 10 s intervals and was potentiated by stimulating intermittently. Thus, the average mean output during stimulation in bursts at 70 Hz (25 ± 5 fmol min^{-1} kg^{-1}) was significantly higher than the corresponding value during continuous stimulation at 7 Hz (6 ± 1 fmol min^{-1} kg^{-1}; $P < 0.01$) even though the total number of impulses delivered was identical in each case.

3. There was also a small but significant rise in the output of cortisol from the gland with intermittent stimulation, which was linearly related to the output of ACTH-like immunoreactivity at the lower frequencies (4 and 7 Hz).

4. Separation of the ACTH-related peptides which were extracted from the adrenal effluent plasma of these animals during splanchnic nerve stimulation revealed the existence of two clear forms: ACTH (1–39) accounted for about 60% of total ACTH immunoreactivity and pro-opiomelanocortin (POMC) for about 30%.

5. It is concluded that small amounts of ACTH are released within the adrenal gland during splanchnic nerve stimulation in the functionally hypophysectomized calf and that this may possibly contribute towards the steroidogenic effect of stimulating the splanchnic nerve.

INTRODUCTION

It has been reported previously that stimulation of the peripheral end of the splanchnic nerve potentiates the steroidogenic action of ACTH (Edwards & Jones, 1987). This effect is due in part to increased presentation of ACTH to the gland, secondary to adrenal vasodilatation (Bloom, Edwards & Jones, 1990; Edwards & Jones, 1990). However, neuropeptides such as vasoactive intestinal polypeptide (VIP), which are known to be steroidogenic (Kowal, Horst, Pensky & Alfonzo, 1977; Morera, Cathiard, Laburthe & Saez, 1979; Leboulenger, Leroux,

Delarue, Tonon, Charnay, Dubois, Coy & Vaudry, 1983), are also released within this gland in response to stimulation of the sympathetic innervation (Bloom, Edwards & Jones, 1988). VIP mimics the steroidogenic effect of splanchnic nerve stimulation when administered in small amounts by intra-aortic infusion (Bloom, Edwards & Jones, 1987) and presumably therefore contributes to the steroidogenic effect of splanchnic nerve stimulation.

Corticotrophin-releasing factor (CRF) is also present in the adrenal gland (Suda, Tomori, Tozawa, Mouri, Demura & Shizume, 1984; Hashimoto, Murakami, Hattori, Niimi, Fujino & Ota, 1984; Bruhn, Engeland, Anthony, Gann & Jackson, 1987) and released therefrom in response to haemorrhage (Bruhn *et al.* 1987) or by direct electrical stimulation of the peripheral end of the splanchnic nerve (Edwards & Jones, 1988). Like VIP, CRF exerts a steroidogenic effect on the gland when infused intra-aortically (Edwards & Jones, 1989*a*). The present study was undertaken in order to discover whether or not ACTH is also present in the gland and released from it in response to splanchnic nerve stimulation; the results show that this is so in the calf.

METHODS

Animals

Pedigree Jersey calves were obtained from local farms shortly after birth and used at ages ranging from 22 to 42 days (24–39 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) 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 polytetrafluoroethylene (Teflon) 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. There were used subsequently to monitor aortic blood pressure and heart rate and for collection of arterial blood samples.

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 the peripheral end enclosed in a fluid electrode. A Braunula cannula was inserted into the jugular vein to provide a conduit for I.V. infusions. Following the first operation the animals were maintained by replacement therapy with cortisol (Efcortesol, Glaxo) at a dose of 2·0 mg day^{-1} kg^{-1} and deoxycortisone acetate (Sigma) at a dose of 0·2 mg day^{-1} kg^{-1} following cauterization of the pituitary stalk, with an additional dose of 8·0 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, during which time the animals had made a full recovery from anaesthesia. A standard 20–30 V square-wave stimulus (pulse width 0·5 ms) was employed for 10 min at frequencies of either 4 or 7 Hz continuously or at 40 or 70 Hz for 1 s at 10 s intervals and was invariably below behavioural threshold.

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 packed cell volume per cent (PCV) 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 PCV, glucose, ACTH, and cortisol estimations. Samples of adrenal venous effluent blood were collected in the same way for cortisol, ACTH and POMC estimations. Each was then centrifuged at 4 °C as soon as possible and the plasma stored at −20 or −70 °C.

Glucose was measured enzymatically using a Beckman Mark 2 Glucose Analyzer. ACTH was measured by radioimmunoassay using an antibody against the N-terminal sequence 1–10; it did not cross-react with α, β- or γ-MSH, β-LPH, β-endorphin, ACTH (17–39), CRF-41, met^5-enkephalin or VIP. The details of the assay have been published previously (Jones, Boddy, Robinson & Ratcliffe, 1977). In summary, plasma (1–3 ml) was applied to ^{18}C separation cartridges (Waters Associates), which were subsequently washed with 5 ml of 50 mM-Tris-HCl (pH 8·0) containing 0·2 mM-dithiothreitol. ACTH peptides were eluted with 5 ml 60% (v/v) acetonitrile in 0·1 M-HCl. After lyophilizing the samples ACTH peptides were measured as previously described (Jones *et al.* 1977). The inter- and intra-assay coefficients of variation were about 14% and 12% respectively and the detection limit of the assay was 5–10 pg $tube^{-1}$. Cortisol was measured by radioimmunoassay as described elsewhere (Jones *et al.* 1977); the inter- and intra-assay coefficients of variation were approximately 11% and 9% respectively. In order to check that the steroid being measured by radioimmunoassay was actually cortisol, samples of adrenal effluent plasma collected during control periods and during splanchnic nerve stimulation in half of the experiments were extracted with dichloromethane and analysed by high pressure liquid chromatography (HPLC) 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 UV detector.

High pressure liquid chromatography was used to characterize ACTH immunoreactivity in adrenal venous blood. Plasma for extraction (1–3 ml) was applied to ^{18}C Separation Cartridges (Water Associates), washed with 5 ml of 50 mM-Tris-HCl (pH 8·0) containing 0·2 mM-dithiothreitol then eluted with 4 ml of 60% (v/v) acetonitrile. Lyophilized aliquots were applied to a Bondapak ^{18}C reversed-phase column (0·4 × 25 cm, Water Associates), run at 1200 lbf in^{-2} and 1 ml min^{-1} first with 10 ml of 18% (v/v) acetonitrile followed by a linear gradient (18–45%) of acetonitrile. Fractions were lyophilized and assayed for ACTH immunoreactivity. ACTH fragments were obtained either from Ferring or Peninsula Laboratories. POMC was prepared from anterior pituitaries of sheep, initially by gel filtration as described by Silman, Holland, Chard, Lowry, Hope, Rees, Thomas & Nathanielsz (1979). The fraction representing peptides of 28–35 kDa was applied (*ca* 5 mg protein) to a Biogel anti-ACTH affinity column (2·5 × 15·0 cm) and POMC eluted with 0·2 mg human ACTH (Sigma). ACTH and POMC were then initially separated by gel filtration on a Sephadex G-50 Superfine column (2·5 × 20·0 cm) and the POMC fraction further purified by separation on a Bondapak ^{18}C reversed-phase HPLC column (1·0 × 25·0 cm) eluted as described above. The POMC gave a single peak of immunoreactivity when run on the analytical HPLC column described above and when separated by SDS-gel electrophoresis (Jones & Roebuck, 1980). POMC showed 25·8 ± 3·4% ($n = 8$) of the immunoreactivity of ACTH against the ACTH antibody. For analytical studies, elution of ACTH and POMC standards and fragments was determined by measurement of absorbance at 260 nm. Recovery of ACTH on HPLC separation was 74·6 ± 10·2% ($n = 6$).

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

Post-mortem examinations

After each experiment was concluded the animal was killed by the I.V. injection of a lethal dose of sodium pentobarbitone (Sagatal, May & Baker) and the right adrenal gland together with the adrenal clamp were removed. The position of the clamp was then checked and the gland was inspected to ensure that there was neither haemorrhage nor oedema. The brain was also removed and its base examined to ensure that the pituitary stalk had in fact been destroyed by

thermocautery and without producing significant 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 had not fallen below 20 pg ml^{-1}, or cortisol output below

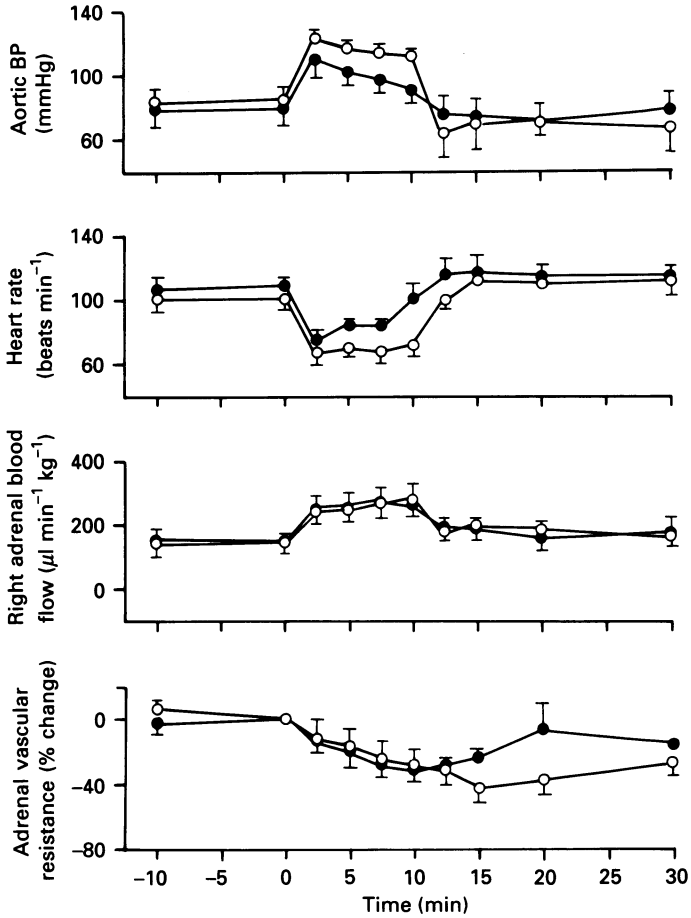

Fig. 1. Changes in mean aortic blood pressure, heart rate, right adrenal blood flow and vascular resistance in five functionally hypophysectomized calves in response to stimulation of the peripheral end of the right splanchnic nerve either at 40 Hz for 1 s at 10 s intervals for 10 min (○) or at 4 Hz continuously for the same period (●), alternately ordered. Horizontal bar: duration of stimulation. Vertical bars: S.E. of each mean value.

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.

RESULTS

Cardiovascular and metabolic responses

Stimulation of the peripheral end of the right splanchnic nerve at either 4 Hz continuously for 10 min, or at 40 Hz for 1 s at 10 s intervals, produced the expected rise in mean aortic blood pressure and fall in mean heart rate and both responses were within the range reported previously in functionally hypophysectomized calves

(Edwards & Jones, 1987). The rise in mean aortic blood pressure and the fall in mean heart rate were both consistently greater in response to intermittent than continuous stimulation but no physiological significance can be attached to these differences because the initial values were not identical (Fig. 1). More germane to the purpose

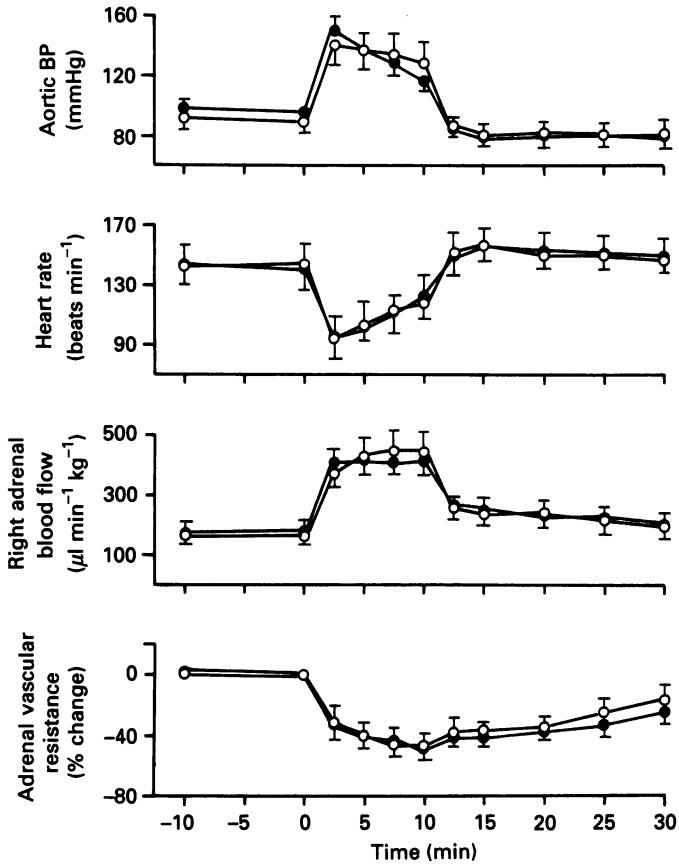

Fig. 2. Changes in mean aortic blood pressure, heart rate, right adrenal blood flow and vascular resistance in six functionally hypophysectomized calves in response to stimulation of the peripheral end of the right splanchnic nerve at either 70 Hz for 1 s at 10 s intervals for 10 min (○) or at 7 Hz continuously for the same period (●), alternately ordered. Horizontal bar: duration of stimulation. Vertical bars: S.E. of each mean value.

of the study was the finding that the changes in both mean right adrenal blood flow and adrenal vascular resistance were closely similar in these two groups of animals (Fig. 1). Thus, mean adrenal blood flow rose from 152 ± 24 μl min^{-2} kg^{-1} during continuous stimulation at 4 Hz and from 151 ± 37 to 260 ± 9 μl min^{-1} kg^{-1} during intermittent stimulation at 40 Hz; mean right adrenal vascular resistance fell by an average mean value of 23 ± 4% during continuous stimulation at 4 Hz and 20 ± 4% during intermittent stimulation at 40 Hz.

PCV and arterial plasma glucose concentration were also monitored during these experiments and the expected increase in both occurred in response to splanchnic

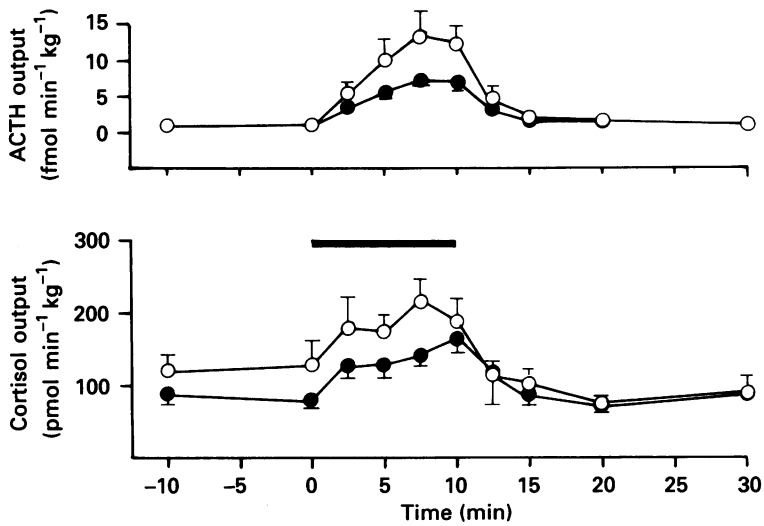

Fig. 3. Changes in mean right adrenal ACTH and cortisol output in five functionally hypophysectomized calves in response to stimulation of the peripheral end of the right splanchnic nerve either at 40 Hz for 1 s at 10 s intervals (○) or at 4 Hz continuously for the same period (●), alternately ordered. Horizontal bar: duration of stimulation. Vertical bars: S.E. of each mean value.

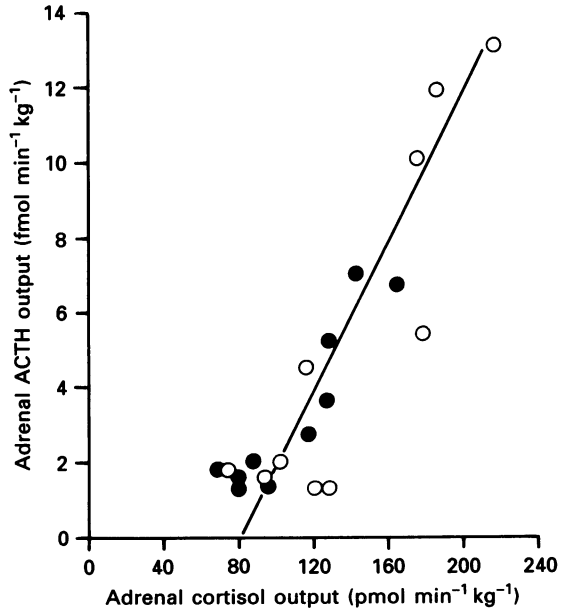

Fig. 4. Relation between mean right adrenal ACTH and cortisol output in five functionally hypophysectomized calves in which the peripheral end of the right splanchnic nerve was stimulated either at 40 Hz for 1 s continuously for 10 min (○) or at 4 Hz continuously for the same period (●), alternately ordered. The regression line was calculated by the method of least squares; $r = 0{\cdot}89$.

nerve stimulation in these two groups of animals. Neither response was significantly affected by the pattern of stimulation employed (data not shown).

Stimulation of the peripheral end of the right splanchnic nerve at higher frequencies (70 Hz for 1 s at 10 s intervals for 10 min or 7 Hz continuously for the

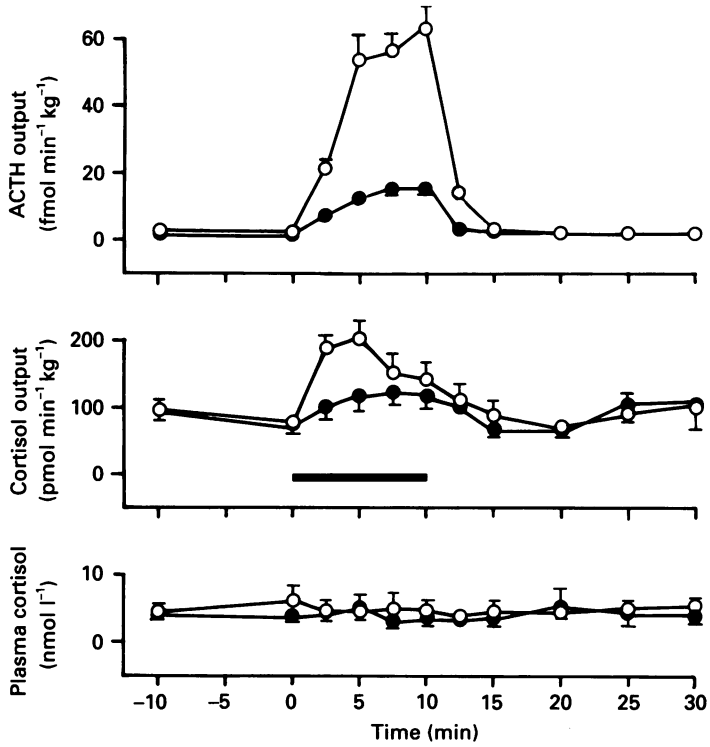

Fig. 5. Changes in mean right adrenal ACTH and cortisol output and mean plasma cortisol concentration in six functionally hypophysectomized calves in response to stimulation of the peripheral end of the right splanchnic nerve either at 70 Hz for 1 s at 10 s intervals for 10 min (○) or at 7 Hz continuously for the same period (●), alternately ordered. Horizontal bar: duration of stimulation. Vertical bars: S.E. of each mean value wherever these exceed the size of the symbol.

same period) produced closely similar changes in mean aortic blood pressure, heart rate, mean right adrenal blood flow and vascular resistance, whichever pattern of stimulation was employed (Fig. 2). Changes in PCV and mean plasma glucose concentration were also closely similar in these two groups of animals (data not shown).

Adrenocortical responses

Stimulation of the peripheral end of the splanchnic nerve at 4 Hz continuously for 10 min produced a small, but steady, rise in the output of ACTH-like immunoreactivity from the right adrenal gland during this 10 min period (Fig. 3). The average mean output during stimulation ($5{\cdot}6 \pm 0{\cdot}8$ fmol min^{-1} kg^{-1}) was significantly higher than the mean right adrenal output of ACTH-like immunoreactivity before

and after stimulation ($1{\cdot}8 \pm 0{\cdot}2$ fmol min^{-1} kg^{-1}; $P < 0{\cdot}01$). Stimulation at 40 Hz for 1 s at 10 s intervals for the same period increased the response roughly twofold (Fig. 3) but the difference did not achieve statistical significance. Splanchnic nerve stimulation also caused a small, but statistically significant, increase in cortisol

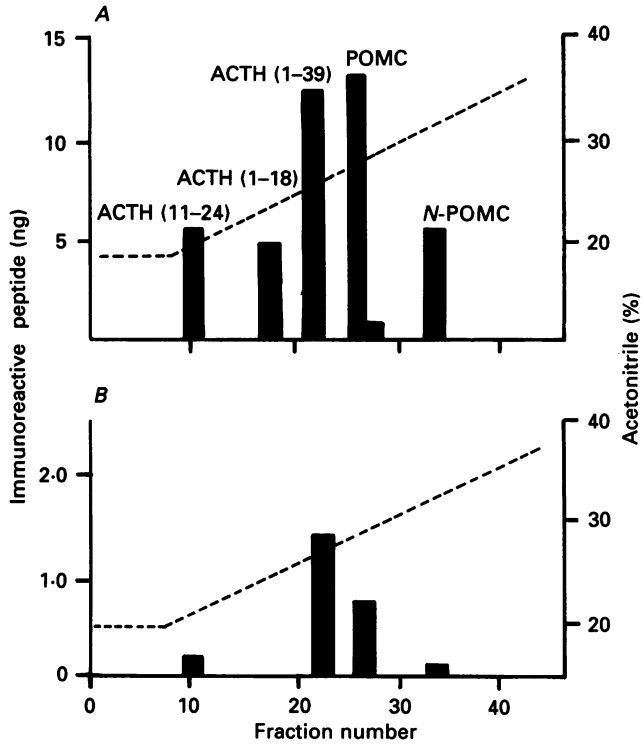

Fig. 6. Separation of ACTH-related peptides on a Bondapak ^{18}C column ($0{\cdot}4 \times 25$ cm) eluted at 1200 lbf in^{-2} and 1 ml min^{-1} with acetonitrile (dashed line). *A*, ACTH standards and fragments. *B*, extract of adrenal venous effluent plasma from a functionally hypophysectomized calf during splanchnic nerve stimulation at 70 Hz for 1 s at 10 s intervals for 10 min.

output from the gland ($P < 0{\cdot}01$ in each group). This response was not affected by the pattern of stimulus employed as can be seen if allowance is made for the disparity between the initial values. Thus, the average mean incremental output during stimulation in bursts (61 ± 10 pmol min^{-1} kg^{-1}) was actually identical with the corresponding incremental value during continuous stimulation at 4 Hz (61 ± 9 pmol min^{-1} kg^{-1}). The mean right adrenal cortisol outputs from both these groups of animals were found to be related to the mean right adrenal immunoreactive ACTH outputs in strictly linear fashion throughout these experiments (Fig. 4; $r = 0{\cdot}89$).

These responses were also assessed during stimulation at higher frequency. Stimulation at 7 Hz continuously for 10 min produced a slow, but steady, rise in the mean output of ACTH-like immunoreactivity from the right adrenal gland, the extent of which was closely similar to that which occurred in response to stimulation at 4 Hz continuously (Figs 3 and 5). In contrast, stimulation at the corresponding

intermittent frequency (70 Hz for 1 s at 10 s intervals) substantially increased the ACTH response. Thus the average mean right adrenal ACTH output during stimulation at 70 Hz in bursts (25 ± 5 fmol min^{-1} kg^{-1}) was significantly higher than the corresponding average mean value during splanchnic nerve stimulation at 7 Hz continuously (6 ± 1 fmol min^{-1} kg^{-1}; $P < 0{\cdot}01$; Fig. 5).

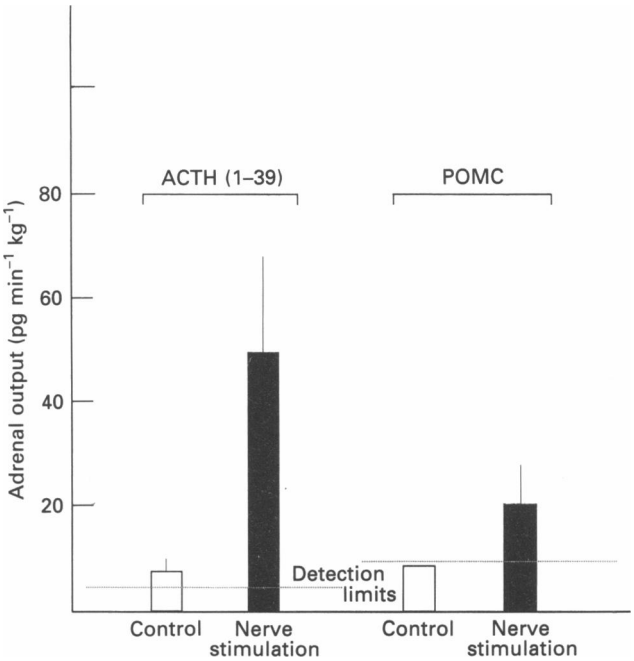

Fig. 7. Mean outputs of ACTH and POMC from the right adrenal gland of six conscious, functionally hypophysectomized calves in response to stimulation of the peripheral end of the right splanchnic nerve at 70 Hz for 1 s at 10 s intervals for 10 min.

Separation of the ACTH-related peptides which were extracted from the adrenal effluent plasma of these animals during splanchnic nerve stimulation revealed the fact that two clear forms were released from the gland (Fig. 6). ACTH (1–39) was the predominant form accounting for $60{\cdot}2 \pm 3{\cdot}6$ % of total ACTH immunoreactivity, and pro-opiomelanocortin (POMC) accounted for $30{\cdot}1 \pm 4{\cdot}3$ % (Fig. 7).

DISCUSSION

The results of these experiments provide clear evidence that ACTH (1–39) and POMC is released from the adrenal gland in response to stimulation of the splanchnic sympathetic innervation. Since CRF is also released from the gland under these conditions (Edwards & Jones, 1988) it is tempting to conclude that the hypothalamo-anterior pituitary CRF–ACTH system for controlling cortisol secretion is replicated in the adrenal gland itself, under sympathetic control. Such a control, linking cortisol output with any increase in sympathetic activity would be in complete accord with the sensitivity with which cortisol output increases in response to stress, together

with the fact that it can do so with no appropriate change in the concentration of ACTH in the circulating plasma (see Edwards & Jones, 1989*b* for recent review). However, it is by no means clear that the increase in the output of ACTH from the adrenal gland is a consequence of the CRF which is also mobilized during splanchnic nerve stimulation, or that is in any way related to the cortisol which is also secreted. CRF has been localized to cells in the adrenal medulla (Hashimoto *et al.* 1984; Bruhn *et al.* 1987) but the distribution of ACTH within the gland has yet to be determined. It has been reported, albeit tentatively, that ACTH-like immunoreactivity is present in a population of enteric nerves (Sundler, Håkanson & Leander, 1980) and so it is possible that it is released directly from nerve terminals in the adrenal gland. The concensus now is that that cortex and medulla are supplied with separate capillary beds with no communicating portal system (see for instance Sparrow & Coupland, 1987). Accordingly, ACTH would need to be released within the cortex if the peptide were to have any chance of reaching the cells in the zona fasciculata and so stimulate steroidogenesis.

Preliminary experiments suggest that CRF is capable of releasing ACTH within the adrenal gland in this conscious, hypophysectomized calf model (A. V. Edwards & C. T. Jones, unpublished observations) but it is by no means clear that this occurs during splanchnic nerve stimulation. The fact that the output of ACTH from the adrenal of about 12·5 fmol min^{-1} kg^{-1} during stimulation of the splanchnic nerve at 40 Hz for 1 s at 10 s intervals was of the same order of magnitude as that of CRF under precisely similar conditions (8 fmol min^{-1} kg^{-1}; Edwards & Jones, 1988) is suggestive of common causality; both peptides possibly being discharged from splanchnic nerve terminals. Much higher 'gearing' would be expected if the release of ACTH was a consequence of the release of CRF, at least by analogy with the anterior pituitary.

When the splanchnic nerve was stimulated at either 4 Hz continuously, or at 40 Hz in bursts, the mean output of ACTH from the gland was linearly related to mean cortisol output (Fig. 4; $r = 0{\cdot}89$). Furthermore, cortisol output exceeded ACTH output by well over 4 orders of magnitude over the whole range of values that were obtained. At the higher stimulus frequencies which were employed (7 Hz continuously and 70 Hz in bursts; data not shown) the same relation between the mean adrenal output of ACTH was found to apply, although the linearity was less convincing. It is possible that the secretion of glucocorticoids from the adrenal cortex, which occurs in response to stimulation of the splanchnic nerve, might be mediated, at least in part, by ACTH which is released locally within the adrenal cortex, independent of the release of CRF, which is probably restricted to the medulla.

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