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ADRENAL RESPONSES TO SPLANCHNIC NERVE STIMULATION IN CONSCIOUS CALVES GIVEN NALOXONE

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SUMMARY

1. The effects of stimulating the peripheral end of the right splanchnic nerve in the presence of naloxone (2 mg kg^{-1}) have been investigated in conscious 3 to 6-week-old calves.

2. Mean aortic blood pressure rose to significantly higher levels during splanchnic stimulation in bursts at 40 Hz for 1 s at 10 s intervals than it did during stimulation at the corresponding continuous frequency (4 Hz). Furthermore, naloxone significantly reduced the fall in mean vascular resistance in response to both patterns of stimulation.

3. The output of catecholamines from the adrenal gland, together with the proportion of noradrenaline released, was significantly enhanced by stimulating the splanchnic nerves in bursts in animals pre-treated with naloxone and the proportion of noradrenaline released also increased. In both cases the output of adrenaline and noradrenaline was within the same range as that reported previously in normal control animals.

4. Naloxone significantly increased the amounts of enkephalin-like immunoreactivity and corticotrophin-releasing factor (CRF)-like immunoreactivity released from the adrenal gland in response to splanchnic nerve stimulation and raised the proportion of total to free met^5-enkephalin that was secreted.

5. Naloxone also inhibited the rise in plasma adrenocorticotrophic hormone (ACTH) concentration during continuous stimulation at 4 Hz, but not during stimulation at 40 Hz in bursts. Under these latter conditions the output of cortisol apparently directly from the adrenal gland was inhibited. The finding that splanchnic nerve stimulation can potentiate the output of cortisol in response to ACTH was confirmed.

6. These results provide evidence that release of enkephalins and of CRF from the adrenal is inhibited by activating opioid receptors within the gland itself.

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INTRODUCTION

Opioid peptides are present in the 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, DiGiuilo, Fratta, Hong & Yang, 1979; Stern, Lewis, Kimura, Rossier, Gerber, Brink, Stein & Udenfriend, 1979; Viveros, Diliberto, Hazum & Chang, 1979; Chaminade, Foutz & Rossier, 1984; Fleminger, Howells, Kilpatrick & Udenfriend, 1984). They are localized both in the chromaffin granules where they are synthesized (Stern *et al.* 1979; Chang, Wilson & Viveros, 1982) and within splanchnic nerve terminals (Schultzberg *et al.* 1978). Electrical stimulation of the peripheral end of the splanchnic nerve causes the release of enkephalins from the adrenal gland as first reported by Costa and his colleagues (Hexum, Hanbauer, Govoni, Yang & Costa, 1980; Govoni, Hanbauer, Hexum, Yang, Kelly & Costa, 1981). In the conscious calf the process is potentiated by intermittent, high-frequency stimulation in bursts and release is mainly, but not entirely, in the form of a high molecular weight precursor (Edwards, Hansell & Jones, 1986; Bloom, Edwards & Jones, 1988; Rossier, Barres, Cupo & Edwards, 1988). Accordingly, it is possible that enkephalins fulfil some functional role within the gland itself, in addition to any action that they may exert peripherally. Accordingly, this possibility has been investigated by examining selected responses of conscious calves to splanchnic nerve stimulation in the presence of naloxone.

METHODS

Animals

Pedigree Jersey calves were obtained from local farms shortly after birth and used at ages ranging between 21 and 39 days (25–37 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 the insertion of narrow-bore polytetrafluoroethylene (Teflon) catheters 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 and for collection of arterial blood samples. 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 designed to minimize spread of stimulus to surrounding tissues. A Braunula cannula was inserted into the jugular vein to provide a conduit for I.V. injections.

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, 2·0 ms) was employed at a frequency of either 4 Hz continuously for 10 min or at 40 Hz for 1 s at 10 s intervals for the same period. 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 outputs of catecholamines and peptides from the gland were calculated. Adrenal vascular resistance was estimated by dividing the perfusion pressure (mean aortic blood pressure) by the right adrenal blood flow. Naloxone hydrochloride (Sigma) was administered by intravenous injection 5 min before each period of splanchnic nerve stimulation.

Analytical procedures

Samples of arterial blood were collected at intervals into heparinized tubes containing phenylmethylsulphonyl fluoride (PMSF; final concentration, 0·1 mM; Sigma) for haematocrit, glucose, adrenocorticotrophic hormone (ACTH), cortisol and enkephalin estimations. Samples of adrenal venous effluent blood were collected in the same way for cortisol and enkephalin estimations and into tubes containing 2–3 mg EDTA for catecholamine 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 by means of a Beckman Mark 2 Glucose Analyzer. Adrenaline and noradrenaline were measured by high performance liquid chromatography with electrochemical detection (Arkinstall & Jones, 1985). ACTH and cortisol were measured by radioimmunoassay (Jones, Boddy, Robinson & Ratcliffe, 1977). Corticotrophin-releasing factor (CRF) was determined by radioimmunoassay as described previously (Edwards & Jones, 1988); the inter- and intra-assay coefficients of variation were 8·6 and 7·2% respectively.

Met^5-enkephalin was measured by radioimmunoassay as described previously (Edwards *et al.* 1986). The assay was carried out either on samples of untreated adrenal venous effluent plasma to provide a value for free met^5-enkephalin or after proteolytic digestion to liberate met^5-enkephalin from any released precursor, as a measure of total met^5-enkephalin, essentially as described by Lewis, Stern, Kimura, Rossier, Stein & Udenfriend (1978) and by Chaminade *et al.* (1984) (see Edwards *et al.* 1986, for further details).

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

Post-mortem examinations

After each experiment was concluded the animal was killed by the injection of a lethal dose of sodium pentobarbitone (Sagatal; May & Baker) and the right adrenal gland together with the adrenal clamp was removed. The positioning of the clamp was then checked and the gland was inspected to ensure that there was no haemorrhage or oedema.

RESULTS

Cardiovascular 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 for the same period produced closely similar falls in mean heart rate and adrenal vascular resistance, and similar rises in mean right adrenal blood flow in conscious calves that were pretreated with naloxone (2 mg kg^{-1}; Fig. 1). However the fall in adrenal vascular resistance in response to both patterns of stimulation was significantly less than that obtained previously in the absence of naloxone (Bloom *et al.* 1988). Thus the mean average fall in adrenal vascular resistance over the period 0–20 min in the groups stimulated in bursts was 12 ± 1% in the presence of naloxone and 32 ± 3% in its absence ($P < 0·001$). The corresponding figures for the groups stimulated continuously were 11 ± 3% with naloxone and 28 ± 3% in its absence ($P < 0·01$). Mean aortic blood pressure rose to significantly higher levels during stimulation in bursts at 40 Hz than during continuous stimulation (Fig. 1). Thus the average mean value during stimulation in bursts was 137 ± 4 and 117 ± 5 mmHg during continuous stimulation ($P < 0·05$). The corresponding values obtained previously in normal control animals were 137 ± 2 (bursts) and 128 ± 4 mmHg (continuous; Bloom *et al.* 1988).

Haematocrit and arterial plasma glucose concentration were also monitored and the expected increase in both occurred in response to splanchnic nerve stimulation.

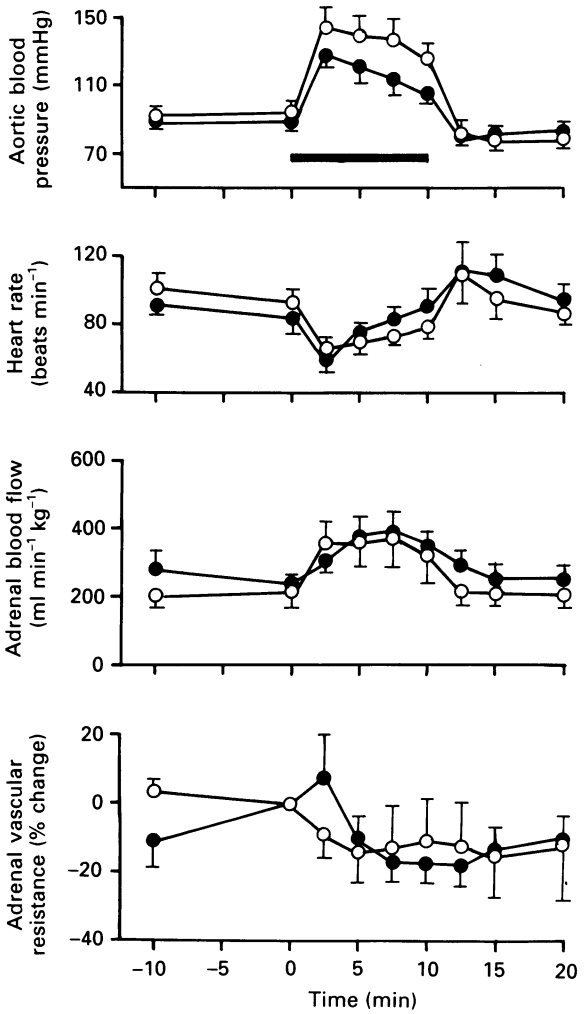

Fig. 1. Changes in mean aortic blood pressure, heart rate, adrenal blood flow and adrenal vascular resistance, in conscious calves given naloxone (2·0 mg kg^{-1}), in response to stimulation of the peripheral end of the right splanchnic nerve at either 4 Hz continuously for 10 min (●: $n = 5$) or at 40 Hz for 1 s at 10 s intervals (○: $n = 6$) for the same period. Horizontal bar, duration of stimulus. Vertical bars, S.E. of each mean value.

Neither response was affected significantly by naloxone or by the pattern of the stimulus.

Adrenal medullary responses

In the presence of naloxone (2 mg kg^{-1} I.V.) the release of both adrenaline and noradrenaline from the right adrenal gland, in response to stimulation of the peripheral end of the right splanchnic nerve, was significantly increased, and in fact more than doubled by stimulating intermittently in bursts at high frequency. Thus

the mean average output of noradrenaline during stimulation at 4 Hz continuously was 76 ± 5 ng min^{-1} kg^{-1} compared with an output of 224 ± 7 ng min^{-1} kg^{-1} during stimulation at 40 Hz for 1 s at 10 s intervals ($P < 0{\cdot}001$; Fig. 2). The corresponding values for adrenaline were 106 ± 6 and 234 ± 9 ng min^{-1} kg^{-1} ($P < 0{\cdot}001$). As has been

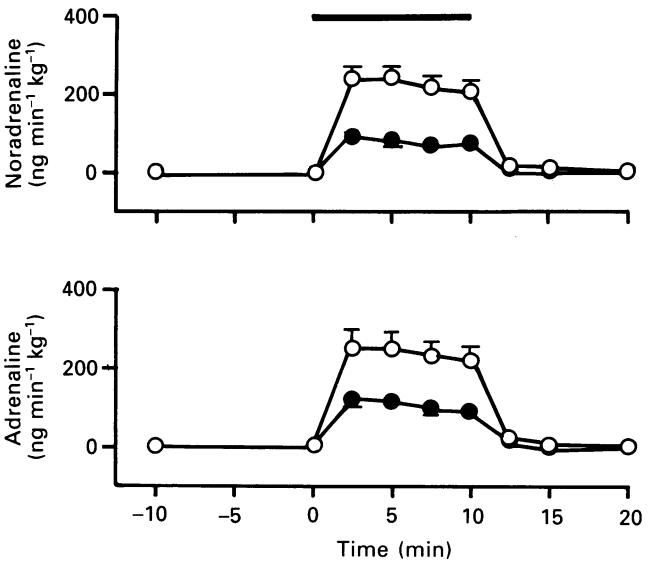

Fig. 2. Changes in mean right adrenal catecholamine output, in conscious calves given naloxone (2·0 mg kg^{-1}), in response to stimulation of the peripheral end of the right splanchnic nerve at either 4 Hz continuously for 10 min (●; $n = 5$) or at 40 Hz for 1 s at 10 s intervals (○; $n = 6$) for the same period. Horizontal bar, duration of stimulation. Vertical bars, S.E. of each mean value.

reported previously in normal calves of the same age (Bloom *et al.* 1988) the output of noradrenaline was increased more than that of adrenaline when the splanchnic nerve was stimulated in bursts.

Similarly, the outputs of both free and total met^5-enkephalin-like immunoreactivity were substantially enhanced by stimulating in bursts. The mean average right adrenal output of free met^5-enkephalin was increased thereby roughly twofold, from $9{\cdot}2 \pm 0{\cdot}5$ to $19{\cdot}8 \pm 0{\cdot}9$ ng min^{-1} kg^{-1} ($P < 0{\cdot}001$), and that of total met^5-enkephalin some 2·5 times from 90 ± 5 to 234 ± 11 ng min^{-1} kg^{-1} ($P < 0{\cdot}001$; Fig. 3). The outputs of both free and total met^5-enkephalin were significantly higher than the corresponding values previously obtained in calves of the same age in the absence of naloxone under both conditions of stimulation (Bloom *et al.* 1988; Table 1). Furthermore, the ratio of free to total met^5-enkephalin was increased by stimulating in bursts in the presence of naloxone, just as it is in the absence of the drug (Bloom *et al.* 1988).

Naloxone also significantly increased the output of CRF-like immunoreactivity from the adrenal gland in response to both patterns of stimulation (Table 1) and, as with the catecholamines and the enkephalins, secretion of this peptide was significantly enhanced by stimulating in bursts both in the presence and the absence of naloxone (Fig. 3; Edwards & Jones, 1988).

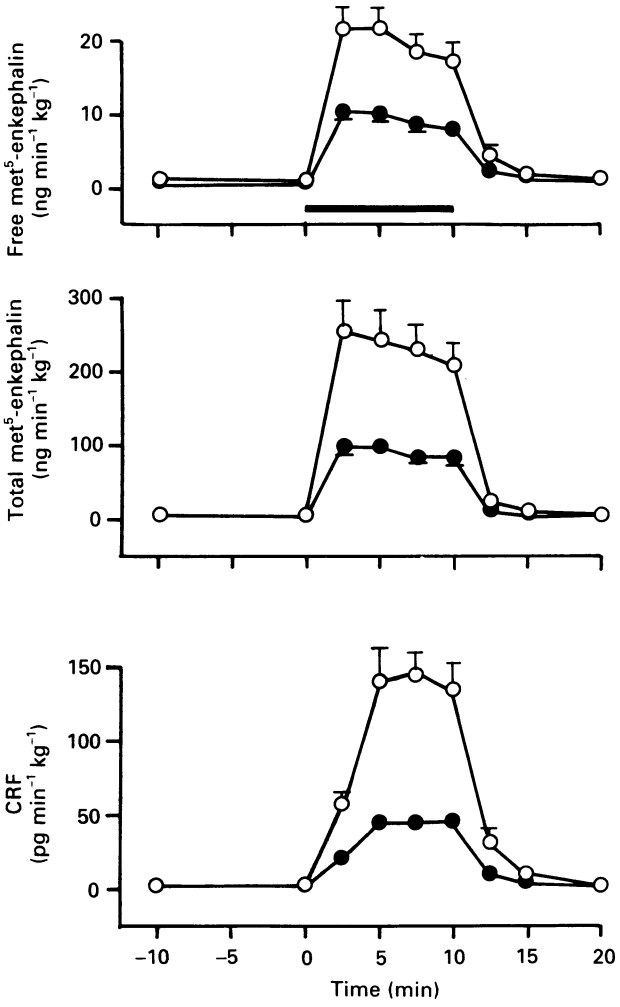

Fig. 3. Changes in mean right adrenal met⁵-enkephalin-like and corticotrophin-releasing factor (CRF)-like immunoreactivity, in conscious calves given naloxone (2·0 mg kg^{-1}), in response to stimulation of the peripheral end of the right splanchnic nerve at either 4 Hz continuously for 10 min (●; $n = 5$) or at 40 Hz for 1 s at 10 s intervals (○; $n = 6$) for the same period. Horizontal bar, duration of stimulation. Vertical bars, S.E. of each mean value.

Adrenal cortical responses

Stimulation of the peripheral end of the splanchnic nerve caused a steady rise in the concentration of ACTH in the arterial plasma which was associated with an increase in adrenal cortisol output and a rise in the concentration of cortisol in the peripheral plasma (Fig. 4). Each of these responses was significantly enhanced by stimulating in bursts. Thus the mean averages of plasma ACTH concentration, cortisol output and plasma cortisol concentration during stimulation at 40 Hz for 1 s at 10 s intervals were 160 ± 37 pg ml^{-1}, 87 ± 15 ng min^{-1} kg^{-1} and 55 ± 11 ng ml^{-1}

TABLE 1. Comparison of the mean average outputs from the right adrenal gland of free and total met^5-enkephalin-like immunoreactivity and of corticotrophin-releasing factor (CRF)-like immunoreactivity during stimulation of the peripheral end of the right splanchnic nerve in calves given naloxone (2·0 mg kg^{-1} I.V.) with those obtained previously in the absence (—) of naloxone (Bloom *et al.* 1988; Edwards & Jones, 1988).

Total met^5-enkephalin (ng min^{-1} kg^{-1})				Free met^5-enkephalin (ng min^{-1} kg^{-1})				CRF (pg min^{-1} kg^{-1})			
4 Hz continuous		40 Hz bursts		4 Hz continuous		40 Hz bursts		4 Hz continuous		40 Hz bursts	
(Naloxone)	(—)	(Naloxone)	(—)	(Naloxone)	(—)	(Naloxone)	(—)	(Naloxone)	(—)	(Naloxone)	(—)
90±4	22±1	234±11	65±6	9·2±0·5	3·1±0·1	19·5±0·9	6·0±0·4	40±6	16±4	119±21	27±8
$P < 0·001$		$P < 0·001$		$P < 0·001$		$P < 0·001$		$P < 0·02$		$P < 0·01$	

TABLE 2. Comparison of the mean average plasma ACTH concentrations together with the output of cortisol from the right adrenal gland during stimulation of the peripheral end of the right splanchnic nerve in calves given naloxone (2·0 mg kg^{-1} I.V.) with those obtained previously in the absence of naloxone (Bloom *et al.* 1988)

Plasma ACTH (pg ml^{-1})				Cortisol output (ng min^{-1} kg^{-1})			
4 Hz continuous		40 Hz bursts		4 H continuous		40 Hz bursts	
(Naloxone)	(—)	(Naloxone)	(—)	(Naloxone)	(—)	(Naloxone)	(—)
39±8	115±6	160±57	162±16	31±4	124±8	87±15	152±19
$P < 0·001$		n.s.		$P < 0·001$		n.s.	

respectively; the corresponding values during stimulation at 4 Hz continuously were 39 ± 8 pg ml^{-1} ($P < 0{\cdot}02$), 31 ± 4 ng min^{-1} kg^{-1} ($P < 0{\cdot}02$) and 21 ± 4 ng ml^{-1} ($P < 0{\cdot}05$). These differences were all considerably greater than those recorded previously in the absence of naloxone (Table 2). During stimulation at 4 Hz continuously,

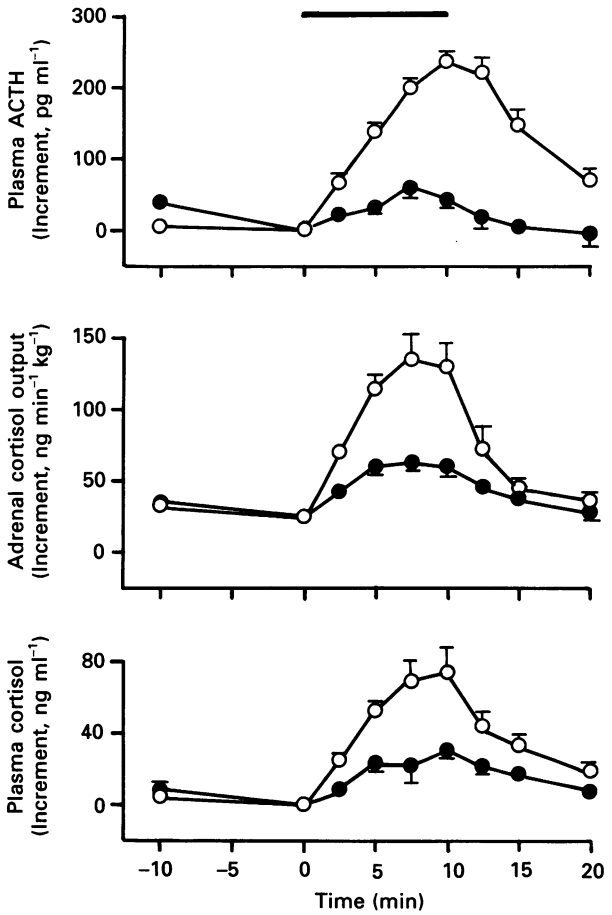

Fig. 4. Changes in mean plasma ACTH and cortisol concentration and right adrenal cortisol output, in conscious calves given naloxone (2·0 mg kg^{-1}), in response to stimulation of the peripheral end of the right splanchnic nerve either at 4 Hz continuously for 10 min (●; $n = 5$) or at 40 Hz for 1 s at 10 s intervals (○; $n = 6$) for the same period. Horizontal bar, duration of stimulation. Vertical bars, S.E. of each mean value. Absolute mean values prior to continuous stimulation: plasma ACTH, 63 ± 24 pg ml^{-1}; cortisol output, 25 ± 6 ng min^{-1} kg^{-1}; plasma cortisol, 22 ± 4 ng ml^{-1}. Prior to stimulation in bursts: plasma ACTH, 34 ± 4 pg ml^{-1}; cortisol output, 19 ± 1 ng min^{-1} kg^{-1}; plasma cortisol, 17 ± 2 ng ml^{-1}.

naloxone was found to inhibit the rise in plasma ACTH substantially, and sufficed to account for the reduced output of cortisol from the adrenal gland. This inhibition was overcome, however, by stimulating in bursts, under which conditions naloxone directly inhibited cortisol output.

Within 2·5 min after stimulation of the splanchnic nerve in bursts of 40 Hz, mean plasma ACTH concentration had fallen proportionately more than right adrenal

cortisol output suggesting that some other factor might have contributed to the preceding rise in cortisol output. Plotting the mean plasma presentation rate of ACTH (calculated from right adrenal plasma flow and adrenal venous effluent plasma ACTH concentration) during these experiments against mean plasma cortisol concentration revealed a linear relationship between the two for all the samples before and after stimulation. However, all the values obtained during

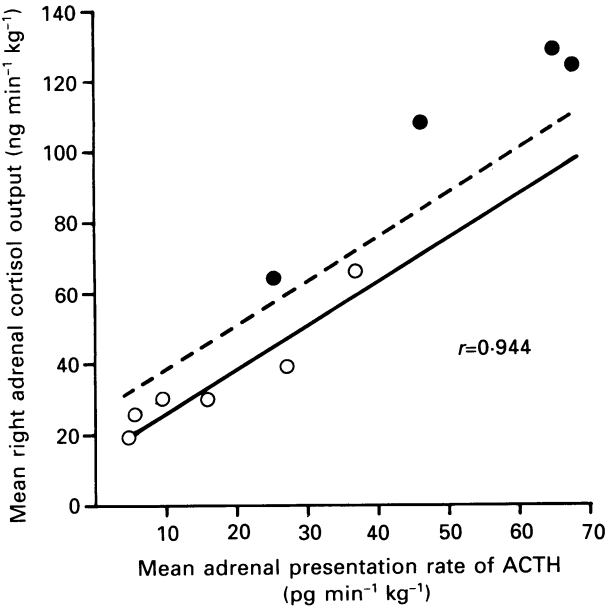

Fig. 5. Relation between mean right adrenal ACTH presentation rate and right adrenal cortisol output, in conscious calves given naloxone (2·0 mg kg^{-1}), in response to stimulation of the peripheral end of the right splanchnic nerve at 40 Hz for 1 s at 10 s intervals, ○, values obtained before and after stimulation. ●, values obtained during stimulation. The regression line was calculated by the method of least squares applied to the values before and after stimulation. The dashed line is at a distance of 2 × S.D.

splanchnic nerve stimulation fell to the left of this regression and each was more distant than two standard deviations (Fig. 5). It follows that, during splanchnic nerve stimulation in the presence of naloxone, adrenal cortisol output exceeds that which is appropriate to ACTH alone. No such finding emerged from the experiments involving continuous stimulation at 4 Hz (results not shown).

The administration of naloxone had no detectable effect on any parameter monitored during the course of this study prior to splanchnic nerve stimulation.

DISCUSSION

The finding that the output of enkephalins from the adrenal gland in response to splanchnic nerve stimulation was so greatly enhanced by pre-treatment with naloxone provides compelling evidence that these peptides normally exert an inhibitory effect on their own release in the absence of such a blocking agent, as first

suggested by Costa and his colleagues (Costa, Guidotti, Hanbauer, Kageyama, Kataoka, Panula, Quach & Schwartz, 1984). However, the fact that these peptides are present both in splanchnic nerve terminals and in the chromaffin cells (Schultzberg *et al.* 1978) precludes further characterization of this mechanism at present. High-affinity binding sites for opioid ligands have been identified in the membranes of bovine chromaffin cells (Kumakura, Karoum, Guidotti & Costa, 1980) but this does not eliminate the possibility that the effect might be mediated presynaptically, at least in part; the fact that the pool of enkephalins in the chromaffin cells is much larger than the amount stored in splanchnic nerve terminals (Schultzberg *et al.* 1978) suggests that the former store must be subject to the inhibition because the enhancement of release in the presence of naloxone was so large. The output of free met^5-enkephalin which occurred in response to either pattern of splanchnic nerve stimulation was more than three times greater than in its absence, and that of total met^5-enkephalin more than four times greater (Table 1).

It has also been shown that enkephalins of adrenal medullary origin are co-released with catecholamines both *in vitro* (Livett, Dean, Whelan, Udenfriend & Rossier, 1981) and *in vivo*, in response to haemmorhage in conscious dogs (Engeland, Bereiter & Gann, 1986) and splanchnic nerve stimulation in conscious calves (Bloom *et al.* 1988).

The proposition that the two types of agonist are also co-released in response to splanchnic nerve stimulation is supported by the finding that the output of both was increased roughly in proportion by stimulating intermittently at high frequency. Thus the ratios of the mean average noradrenaline and adrenaline outputs during splanchnic nerve stimulation in bursts compared with those during continuous stimulation were 2·9:1 and 2·2:1 respectively whereas the corresponding ratios for free and total met^5-enkephalin were 2·2 and 2·6 respectively. This would appear to provide further evidence of co-release of these agonists. The catecholamine outputs during splanchnic nerve stimulation in the presence of naloxone in the present study fell within the range previously reported (Bloom *et al.* 1988; Edwards & Jones, 1988). The further suggestion that enkephalins act as neuromodulators within the gland and exert an inhibitory effect on catecholamine secretion (Kumakura *et al.* 1980) is therefore difficult to reconcile with the present results.

Another consequence of pre-treatment with naloxone was a highly significant increase in the output of CRF in response to both patterns of splanchnic nerve stimulation but which was particularly pronounced during stimulation in bursts, when it amounted to an increase of 441 % over the values obtained without naloxone (Edwards & Jones, 1988). The pathways modulating the CRF output induced by splanchnic nerve stimulation of the adrenal are not clear. However, the naloxone effect implies that here, as in the pituitary, opioid receptors are inhibitory.

We have previously reported the fact that ACTH, at somewhat higher concentrations than those reported here, reduces the output of CRF from the adrenal during splanchnic nerve stimulation (Edwards & Jones, 1988). Hence, the potentiation of this response in the presence of naloxone is the more impressive.

It is noteworthy that the response of the adrenal cortex to ACTH, either in the presence or the absence of splanchnic nerve stimulation, was not affected by naloxone. The slope of the regression line relating adrenal cortisol output to the

presentation rate of ACTH was closely similar to that calculated for previous data obtained in the absence of ACTH (Edward & Jones, 1987) as was the enhancement during splanchnic nerve stimulation. Hence it would appear that endogenous enkephalins do not influence the response.

As the protocol involved collecting virtually the whole of the adrenal venous effluent blood throughout the period of stimulation, little enkephalin peptide, catecholamine or CRF would have passed from the adrenal into the circulation. Intra-aortic infusions of CRF at a dose as low as 8 pmol min^{-1}, which raise the concentration of the peptide in the adrenal venous effluent plasma to a maximum of 75 pmol l^{-1}, causes significant adrenal vasoconstriction. During splanchnic nerve stimulation in bursts in the presence of naloxone the concentration of CRF in the adrenal venous effluent plasma rose by up to 115 pmol l^{-1}. However, it seems unlikely that CRF contributed much to the reduced fall in adrenal vascular resistance that was observed during splanchnic nerve stimulation because it was unaffected by the pattern of stimulation. It could have been due to inhibition of enkephalinergic vasodilatation as it has been reported that enkephalins exert vasodilator effects under certain conditions (Hanbauer, Govoni, Majane, Yang & Costa, 1982; Lang, Bruckner, Hermann, Kempf, Rascher, Sturm, Unger & Ganten, 1982). Alternatively, it may have been due to naloxone blockade of vasoactive intestinal polypeptide (VIP)- ergic vasodilatation as VIP has been implicated in both adrenal cortical and medullary function (Bloom, Edwards & Jones, 1987; Malhotra, Wakade & Wakade, 1988) and its effect on catecholamine secretion is reportedly blocked by naloxone (Wakade, 1988). In the calf, VIP release is thought to be associated with potentiating the output of cortisol in response to ACTH and clear evidence of this phenomenon was obtained during splanchnic nerve stimulation in bursts.

In summary, the present results indicate that enkephalins modulate their own release and that of CRF within the adrenal gland.

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REFERENCES

ARKINSTALL, S. & JONES, C. T. (1985). Regional changes in catecholamine content of pregnant uterus. *Journal of Reproduction and Fertility* **73**, 547–557.

BLOOM, S. R., EDWARDS, A. V. & JONES, C. T. (1987). Adrenal cortical responses to vasoactive intestinal peptide in conscious hypophysectomized calves. *Journal of Physiology* **300**, 41–53.

BLOOM, S. R., EDWARDS, A. V. & JONES, C. T. (1988). The adrenal contribution to the neuroendocrine responses to splanchnic nerve stimulation in conscious calves. *Journal of Physiology* **397**, 513–526.

CHAMINADE, M., FOUTZ, A. S. & ROSSIER, J. (1984). Co-release of enkephalins and precursors with catecholamines from the perfused cat adrenal gland. *Journal of Physiology* **353**, 157–169.

CHANG, K. J., WILSON, S. P. & VIVEROS, O. H. (1982). Co-storage and co-secretion of opioid peptides and catecholamines. *Advances in Biosciences* **36**, 243–248.

COSTA, E., DIGIULIO, A., FRATTA, W., HONG, J. & YANG, H.-Y. T. (1979). Interactions of enkephalinergic and catecholaminergic neurones in CNS and periphery. In *Catecholamines: Basic and Clinical Frontiers*, ed. USDIN, E., KOPIN, I. J. & BARCHAS, J., pp. 1020–1025. Oxford: Pergamon Press.

COSTA, E., GUIDOTTI, A., HANBAUER, I., KAGEYAMA, H., KATAOKA, Y., PANULA, P., QUACH, T. T. & SCHWARTZ, J. P. (1984). Adrenal medulla: regulation of biosynthesis and secretion of catecholamines and enkephalins. In *Neurology and Neurobiology 8A, Catecholamines: Basic and Peripheral Mechanisms*, ed. USDIN, E., CARLSSON, A., DAHLSTROHM, A. & ENGEL, J., pp. 153–161. New York: Alan R. Liss.

EDWARDS, A. V., FURNESS, P. N. & HELLE, K. B. (1980). Adrenal medullary responses to stimulation of the splanchnic nerve in the conscious calf. *Journal of Physiology* **308**, 15–27.

EDWARDS, A. V., HANSELL, D. & JONES, C. T. (1986). Effects of synthetic adrenocorticotrophin on adrenal medullary responses to splanchnic nerve stimulation in conscious calves. *Journal of Physiology* **379**, 1–16.

EDWARDS, A. V., HARDY, R. N. & MALINOWSKA, K. W. (1974). The effects of infusions of synthetic adrenocorticotrophin in the conscious calf. *Journal of Physiology* **239**, 477–498.

EDWARDS, A. V. & JONES, C. T. (1987). Effect of splanchnic nerve section on the sensitivity of the adrenal cortex to adrenocorticotrophin in the calf. *Journal of Physiology* **390**, 23–31.

EDWARDS, A. V. & JONES, C. T. (1988). Secretion of corticotrophin releasing factor from the adrenal during splanchnic nerve stimulation in the conscious calf. *Journal of Physiology* **400**, 89–100.

ENGELAND, W. C., BEREITER, D. F. & GANN, D. S. (1986). Sympathetic control of adrenal secretion of enkephalins after hemorrhage in awake dogs. *American Journal of Physiology* **251**, R341–348.

FLEMINGER, G., HOWELLS, R. D., KILPATRICK, R. D. & UDENFRIEND, S. (1984). Intact proenkephalin is the major enkephalin-containing peptide produced in rat adrenal glands after denervation. *Proceedings of the National Academy of Sciences of the USA* **81**, 7985–7988.

GOVONI, S., HANBAUER, I., HEXUM, T. D., YANG, H.-Y. T., KELLY, G. D. & COSTA, E. (1981). *In vivo* characterization of the mechanisms that secrete enkephalin-like peptides stored in dog adrenal medulla. *Neuropharmacology* **20**, 639–645.

HANBAUER, I., GOVONI, S., MAJANE, E. A., YANG, H.-Y. T. & COSTA, E. (1982). *In vivo* regulation of the release of met-enkephalin-like peptides from dog adrenal medulla. In *Regulatory Peptides: From Molecular Biology to Function*, ed. COSTA, E. & TRABUCCI, M., pp. 209–215. New York: Raven Press.

HEXUM, T. D., HANBAUER, I., GOVONI, S., YANG, H.-Y. T. & COSTA, E. (1980). Secretion of enkephalin-like peptide from canine adrenal gland following splanchnic nerve stimulation. *Neuropeptides* **1**, 137–142.

JONES, C. T., BODDY, K., ROBINSON, J. S. & RATCLIFFE, J. G. (1977). Developmental changes in the response of the adrenal glands of the fetal sheep to endogenous corticotrophin, as indicated by hormone response to hypoxaemia. *Journal of Endocrinology* **72**, 279–292.

KUMAKURA, K., KAROUM, F., GUIDOTTI, A. & COSTA, E. (1980). Modulation of nicotinic receptors by opiate receptor agonists in cultured adrenal chromaffin cells. *Nature* **283**, 489–492.

LANG, R. E., BRUCKNER, U. B., HERMANN, K., KEMPF, B., RASCHER, W., STURM, V., UNGER, TH. & GANTEN, D. (1982). Effect of hemorrhagic shock on the concomitant release of endorphin and enkephalin-like peptides from the pituitary and adrenal gland in the dog. In *Regulatory Peptides: From Molecular Biology to Function*, ed. COSTA, E. & TRABUCCI, M., pp. 363–368. New York: Raven Press.

LEWIS, R. V., STERN, A. S., KIMURA, S., ROSSIER, J., STEIN, S. & UDENFRIEND, S. (1980) An about 50,000-dalton protein in adrenal medulla: a common precursor of [met]- and [leu]-enkephalin. *Science* **208**, 1459–1461.

LIVETT, B. G., DEAN, D. M., WHELAN, L. G., UDENFRIEND, S. & ROSSIER, J. (1981). Co-release of enkephalin and catecholamines from cultured adrenal chromaffin cells. *Nature* **289**, 317–319.

MALHOTRA, R. K., WAKADE, T. D. & WAKADE, A. R. (1988). Vasoactive intestinal polypeptide and muscarine mobilize intracellular Ca^{2+} through breakdown of phosphoinositides to induce catecholamine secretion. *Journal of Biological Chemistry* **263**, 2123–2126.

ROSSIER, J., BARRES, E., CUPO, A. & EDWARDS, A. V. (1988). The release of enkephalin-containing peptides from the adrenal gland in conscious calves. In *Neurosecretion; Cellular Aspects of the Production and Release of Neuropeptides*, ed. PICKERING, B. T., WAKERLEY, J. B. & SUMMERLEE, A. J. S., pp. 53–58. New York: Plenum Press.

SCHULTZBERG, M., LUNDBERG, J. M., HÖKFELT, T., TERENIUS, L., BRANDT, J., ELDE, R. P. & GOLDSTEIN, M. (1978). Enkephalin-like immunoreactivity in gland cells and nerve terminals of the adrenal medulla. *Neuroscience* **3**, 1169–1186.

SNEDECOR, G. W. & COCHRAN, W. G. (1967). *Statistical Methods*, 6th edn. Ames, IA, USA: Iowa State College Press.

STERN, A. S., LEWIS, R. V., KIMURA, S., ROSSIER, J., GERBER, L. D., BRINK, L., STEIN, S. & UDENFRIEND, S. (1979). Isolation of the opioid heptapeptide met-enkephalin [arg^6, phe^7] from bovine adrenal medullary granules and striatum. *Proceedings of the National Academy of Sciences of the USA* **76**, 6680–6683.

VIVEROS, O. H., DILIBERTO, E. J. JR, HAZUM, E. & CHANG, K.-J. (1979). Opiate-like materials in the adrenal medulla: evidence for storage and secretion with catecholamines. *Molecular Pharmacology* **16**, 1101–1108.

WAKADE, A. R. (1988). Noncholinergic transmitter(s) maintain secretion of catecholamines from rat adrenal medulla for several hours of continuous stimulation of splanchnic neurons. *Journal of Neurochemistry* **50**, 1302–1308.