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ENDOCRINE RESPONSES TO INTRA-AORTIC INFUSIONS OF ACETYLCHOLINE IN CONSCIOUS CALVES

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SUMMARY

1. Adrenal responses to intra-aortic infusions of acetylcholine (4·5 nmol min^{-1} kg^{-1} for 10 min) have been investigated in conscious, functionally hypophysectomized, 3- to 6-week-old calves, in the presence and absence of exogenous ACTH (2 ng min^{-1} kg^{-1}, I.V.).

2. Acetylcholine produced a substantial fall in adrenal vascular resistance, which was significantly reduced in the presence of exogenous ACTH, while producing minimal changes in aortic blood pressure and heart rate.

3. There was also a significant rise in right adrenal cortisol output which was sufficient to produce a measurable rise in plasma cortisol concentration. The effect could be accounted for by the increase in adrenal ACTH presentation. It was abolished by pre-treatment with atropine (0·2 mg kg^{-1}). A small but significant rise in aldosterone output during acetylcholine infusions was also abolished in the presence of ACTH.

4. Both adrenaline and noradrenaline were released during intra-aortic acetylcholine infusions and these responses were substantially reduced, but not abolished, by pre-treatment with atropine.

5. Acetylcholine also stimulated the release of corticotrophin-releasing factor (CRF) and [Met^5]enkephalins from the gland. The output of CRF was enhanced and that of free [Met^5]enkephalin was significantly reduced in the presence of exogenous ACTH. All these responses were largely, but not completely, suppressed by atropine.

6. Acetylcholine also promoted the release of the pancreatic hormones glucagon, insulin and pancreatic polypeptide (PP). The amounts of pancreatic glucagon and insulin that were released were highly dependent on the concentration of glucose in the circulating plasma and all these responses were abolished by atropine.

7. It is concluded that acetylcholine is capable of stimulating the release of a wide variety of agonists from the adrenal gland when infused intra-aortically at a dose of 4·5 nmol min^{-1} kg^{-1}. The increase in cortisol output appears to be secondary to an

increase in blood flow whereas the adrenal medullary responses are not, and appear to be due largely, but not entirely, to activation of muscarinic receptors.

INTRODUCTION

It has been reported previously that stimulation of the splanchnic sympathetic innervation to the adrenal gland, in conscious calves, potentiates the steroidogenic effect of ACTH (Edwards & Jones, 1987). It also leads to the release of corticotrophin releasing factor (CRF) and enkephalin peptides, in addition to catecholamines, from the adrenal medulla (Bloom, Edwards & Jones, 1988). It has long been known that the preganglionic sympathetic fibres which supply the gland release acetylcholine (although it has yet to be established what peptides may be co-released and what their actions may be). Accordingly, the present study was undertaken to discover whether adrenal responses to splanchnic nerve stimulation could be reproduced by intra-arterial infusions of acetylcholine. Pancreatic endocrine responses were also monitored.

The protocol involved intra-aortic infusions of quite low doses of acetylcholine (4·5 nmol min^{-1} kg^{-1} for 10 min) in conscious calves in which adrenal clamps had previously been emplaced. The animals were functionally hypophysectomized to prevent spontaneous fluctuations in endogenous arterial plasma ACTH concentration and the effects of acetylcholine were tested both in the presence and the absence of exogenous ACTH.

The results show that acetylcholine is capable of stimulating the release of a wide variety of agonists from both the adrenal gland and the pancreas and, in the case of the adrenal, can act both directly and indirectly.

METHODS

Animals

Pedigree Jersey calves were obtained from local farms shortly after birth and used at ages ranging between 22 and 40 days (24–36 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. These were used subsequently to monitor aortic blood pressure and heart rate and for collection of arterial blood samples. The catheter with the tip in the thorax was employed for intra-aortic infusions of acetylcholine above the level of the adrenal gland.

During the second operation the right kidney was removed, the right renal vein was cannulated and an adrenal clamp emplaced (Edwards, Hardy & Malinowska, 1974; Edwards, Furness & Helle, 1980). The right splanchnic nerve was cut immediately below the diaphragm and a Braunula cannula inserted into the jugular vein to provide a conduit for I.V. infusions of ACTH.

The animals were maintained by replacement therapy with cortisol (Efcortesol; Glaxo) at a dose of 2·0 mg day^{-1} kg^{-1} and 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} cortisol 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. Acetylcholine (acetylcholine chloride; Sigma) was made up as a stock solution, 1 mg ml^{-1} in 0·25 M-sodium dihydrogen orthophosphate which was then diluted with an appropriate volume of sterile physiological saline for infusion at a dose of 4·5 nmol min^{-1} kg^{-1} (1 ml min^{-1}) for 10 min. $ACTH_{1-24}$ (Synacthen; CIBA) was dissolved in saline and infused I.V. at 2 ng min^{-1} kg^{-1} (2·5 ml min^{-1}) for 50 min and the effects of acetylcholine, in the presence of ACTH, were tested by infusing it intra-aortically for 10 min after ACTH had been infused I.V. for 20 min. Assay of ACTH in the infusate emerging from the catheter at the end of the infusion showed that the concentration was 90±10% of that expected. Aortic blood pressure was monitored continuously by means of a Devices M19 recorder. Right adrenal blood flow was estimated gravimetrically and corrected for packed cell volume (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, ACTH, glucose, pancreatic glucagon, insulin, pancreatic polypeptide (PP) and cortisol estimations. Samples of adrenal venous effluent blood were collected in the same way for cortisol, aldosterone, [Met^5]enkephalin and CRF 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.

Adrenaline and noradrenaline, were measured by high-pressure liquid chromatography (HPLC) with electrochemical detection (Arkinstall & Jones, 1985). ACTH and cortisol were measured by radioimmunoassay (Jones, Boddy, Robinson & Ratcliffe, 1977). In some instances steroids in the adrenal effluent plasma were extracted with dichloromethane and analysed by HPLC involving separation on a Zorbax-ODS column (25 × 0·4 cm, 5 μm, Dupont 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. Aldosterone was measured by radioimmunoassay (D-aldosterone (3H) kit; Radioassay Systems Laboratories Inc.). Corticotrophin releasing factor (CRF) was determined by radioimmunoassay, essentially as described by Vale, Vaughan, Yamamoto, Bruhn, Douglas, Dalton, Rivier & Rivier (1983). The antibody used cross-reacted with bovine, ovine and human CRF. It showed no cross-reactivity with ACTH, β-lipotropin, pro-opiomelanocortin, β-melanocyte-stimulating hormone, β-endorphin, [Met^5]enkephalin, vasoactive intestinal peptide, neuropeptide Y, sauvagine, vasopressin or oxytocin. [Met^5]enkephalin was measured by a specific radioimmunoassay as described previously (Edwards *et al.* 1986; Edwards & Jones, 1987). Briefly, untreated plasma was assayed in order to determine the content of the free peptide, and other samples of plasma were assayed after proteolytic digestion to liberate all the [Met^5]enkephalin from any high molecular weight precursor molecules that were present; these values are referred to as total [Met^5]enkephalin.

Glucose was measured by means of a Mark 2 Beckman Glucose Analyzer. Pancreatic glucagon was measured by a radioimmunoassay using an antiserum relatively specific for pancreatic glucagon which was C-terminal reacting (Assan & Slusher, 1972) and gave zero values in human plasma after total pancreatectomy, reacting less than 5% with 'glucagon of ileal origin' (enteroglucagon). Insulin and pancreatic polypeptide were also measured by radioimmunoassay (Albano, Ekins, Maritz & Turner, 1972; Adrian, Bloom, Bryant, Polak, Heitz & Barnes, 1976).

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 were removed. The positioning of the clamp was then checked and the gland was inspected to ensure that there was no haemorrhage or oedema. The brain was also removed and its base examined to ensure that the pituitary stalk had in fact been destroyed by 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 10–15 pg ml^{-1} (the detection limit of the assay) or cortisol output below 200 ng min^{-1} kg^{-1}, were excluded from the series on those grounds alone, as were any in which the adrenal was found to be haemorrhagic.

RESULTS

Cardiovascular responses

Intra-aortic infusions of acetylcholine (4·5 nmol min^{-1} kg^{-1} for 10 min) caused a small but significant fall in mean aortic blood pressure in functionally hypophysectomized calves which were given a continuous infusion of exogenous ACTH (2 ng min^{-1} kg^{-1}, I.V.) but not in the control group. Thus, in the presence of ACTH the average mean aortic blood pressure during the infusion of acetylcholine was 81 ± 1 mmHg compared with an average mean value of 87 ± 2 before and after that infusion ($P < 0{\cdot}05$; Fig. 1). However, there were no significant changes in mean heart rate in either group (Fig. 1). Both groups responded to acetylcholine with a significant fall in adrenal vascular resistance ($P < 0{\cdot}01$) which was reflected by a rise in mean adrenal blood flow ($P < 0{\cdot}01$) but the extent of these changes was greatest in the absence of exogenous ACTH (Fig. 1). All these cardiovascular effects were abolished by pre-treatment with atropine (0·2 mg kg^{-1}; data not shown) and so attributable to activation of muscarinic receptors.

Adrenal cortical responses

Acetylcholine produced no detectable effect on plasma ACTH concentration, right adrenal cortisol output or mean plasma cortisol concentration in the absence of exogenous ACTH (Fig. 2). However, when it was infused intra-aortically during the course of a continuous infusion of ACTH (2 ng min^{-1} kg^{-1}, I.V.) there was a steady rise in mean right adrenal cortisol output from 440 ± 61 immediately before acetylcholine was infused, to 755 ± 76 ng min^{-1} kg^{-1} at 10 min ($P < 0{\cdot}02$; Fig. 2). This response was associated with a steady and significant rise in mean plasma cortisol concentration, from $20{\cdot}7 \pm 1{\cdot}3$ ng ml^{-1} initially to $33{\cdot}9 \pm 2{\cdot}1$ ng ml^{-1} at 10 min, but no change in mean plasma ACTH concentration (Fig. 2). These responses were effectively suppressed by pre-treatment with atropine (0·2 mg kg^{-1}, I.V.; data not shown).

The rate at which ACTH was presented to the right adrenal gland was estimated from mean arterial plasma ACTH concentration and mean right adrenal plasma flow and found to be linearly related to mean right adrenal cortisol output both before and after intra-aortic infusions of acetylcholine ($r = 0{\cdot}841$; Fig. 3). A closely similar relation between these two variables was preserved during intra-aortic acetylcholine infusions, all values falling within 2 S.D.s of the basal regression line (Fig. 3). Mean right adrenal cortisol output was also found to be linearly related to mean plasma

cortisol concentration before and after intra-aortic infusions of acetylcholine (r = 0·923; data not shown) and the values obtained during infusions of acetylcholine appeared to exhibit the same relation.

Intra-arterial infusions of acetylcholine (4·5 nmol min^{-1} kg^{-1} for 10 min) were found to cause a steady rise in mean right adrenal aldosterone output to a peak

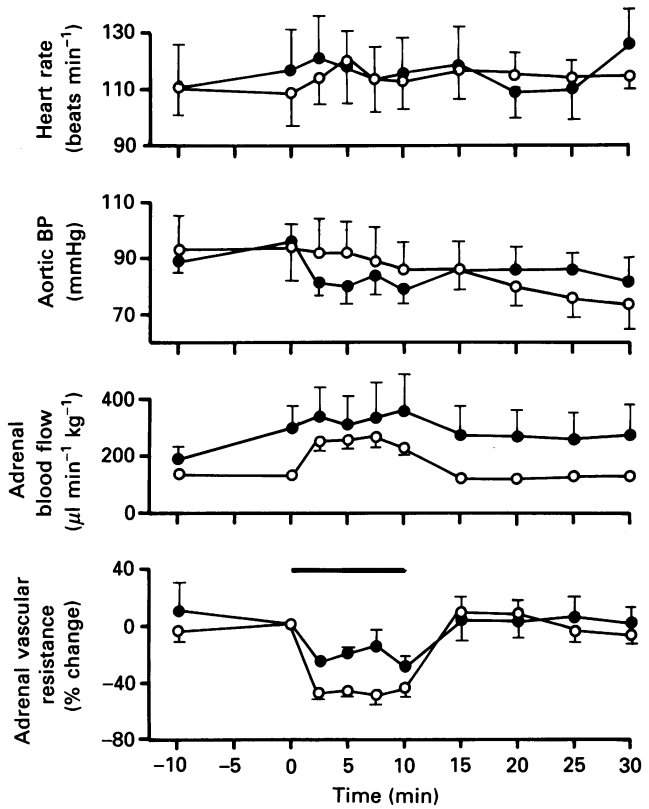

Fig. 1. Changes in mean heart rate, aortic blood pressure (BP), right adrenal blood flow and vascular resistance in four conscious, functionally hypophysectomized calves given intra-aortic infusions of acetylcholine (4·5 nmol min^{-1} kg^{-1}) in the presence (●) and absence (○) of exogenous ACTH (2 ng min^{-1} kg^{-1}, I.V.). Horizontal bar, duration of acetylcholine infusion. Vertical bars, S.E. of each mean value wherever greater than the size of the symbol.

incremental value of $2·7 \pm 1·5$ ng min^{-1} kg^{-1} at 10 min. The average mean incremental aldosterone output during the acetylcholine infusion ($1·5 \pm 0·5$ ng min^{-1} kg^{-1}) was significantly higher than the corresponding average mean incremental value before and after that infusion ($-0·3 \pm 0·1$ ng min^{-1} kg^{-1}; $P < 0·02$; Fig. 4). This effect was completely abolished in the presence of exogenous ACTH (Fig. 4). The mean peak rise in right adrenal aldosterone output in response to acetylcholine output represented an increase of about threefold and so could not be accounted for simply by the (twofold) increase in right adrenal blood flow which occurred under these conditions. The two responses also had quite different time courses (Figs 1 and 4).

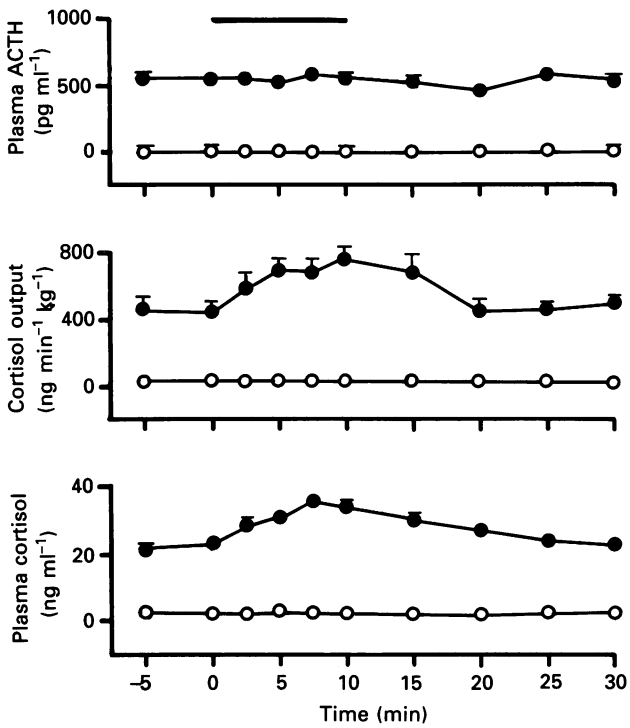

Fig. 2. Changes in mean plasma ACTH and cortisol concentration and right adrenal cortisol output in four conscious, functionally hypophysectomized calves given intra-aortic infusions of acetylcholine (4·5 nmol min^{-1} kg^{-1}) in the presence (●) and absence (○) of exogenous ACTH (2 ng min^{-1} kg^{-1}, I.V.). Horizontal bar, duration of acetylcholine infusion. Vertical bars, S.E. of each mean value wherever greater than the size of the symbol.

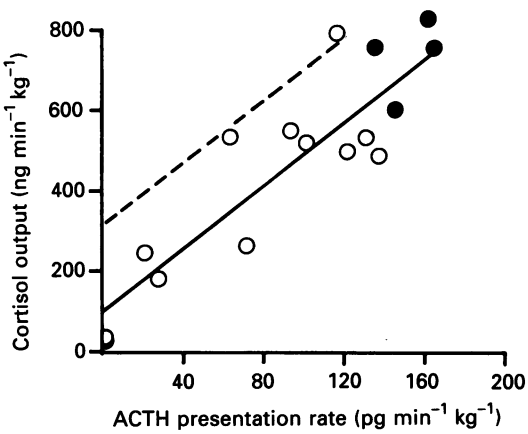

Fig. 3. Relation between mean right adrenal ACTH presentation rate and cortisol output in four conscious, functionally hypophysectomized calves given an intra-aortic infusion of acetylcholine (4·5 nmol min^{-1} kg^{-1}) during an intravenous infusion of exogenous ACTH (2 ng min^{-1} kg^{-1}). ○, before and after acetylcholine. ●, during acetylcholine. Regression line calculated by the method of least squares applied to the values obtained before and after acetylcholine ($r = 0{\cdot}841$). Hatched line, regression + 2 S.D.S.

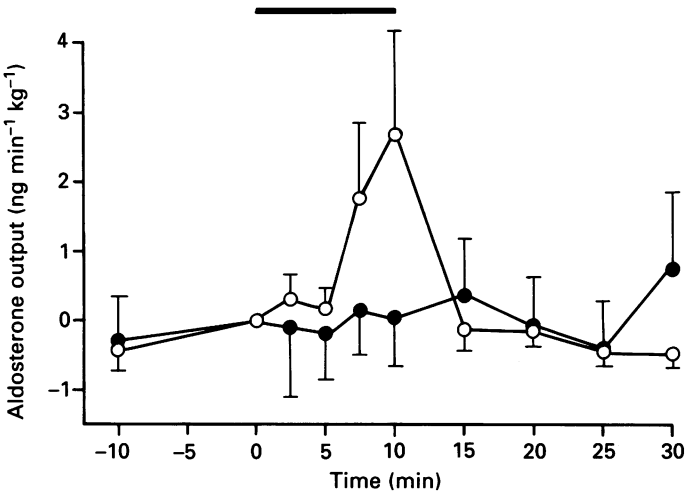

Fig. 4. Changes in mean right adrenal aldosterone output in four conscious, functionally hypophysectomized calves given intra-aortic infusions of acetylcholine (4·5 nmol min^{-1} kg^{-1}) in the presence (●) and absence (○) of exogenous ACTH (2 ng min^{-1} kg^{-1}, I.V.). Horizontal bar, duration of acetylcholine infusion. Vertical bars, S.E. of each mean value wherever greater than the size of the symbol. Absolute value at time = 0 with ACTH, $2·4 \pm 0·7$ ng min^{-1} kg^{-1}; without ACTH, $1·4 \pm 0·5$ ng min^{-1} kg^{-1}.

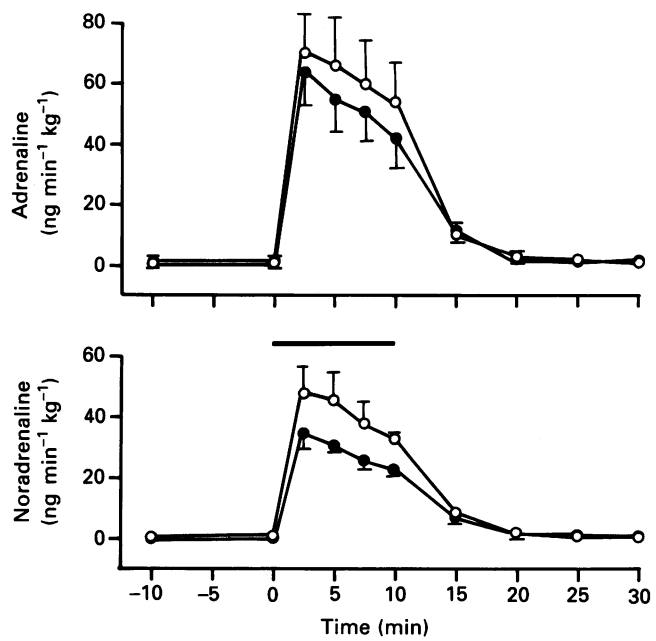

Fig. 5. Changes in mean right adrenal catecholamine output in four conscious, functionally hypophysectomized calves given intra-aortic infusions of acetylcholine (4·5 nmol min^{-1} kg^{-1}) in the presence (●) and absence (○) of exogenous ACTH (2 ng min^{-1} kg^{-1}, I.V.). Horizontal bar, duration of acetylcholine infusion. Vertical bars, S.E. of each mean value wherever greater than the size of the symbol.

Adrenal medullary responses

Intra-aortic infusions of acetylcholine caused an abrupt increase in the output of both adrenaline and noradrenaline from the right adrenal gland of functionally hypophysectomized calves (Fig. 5). Mean adrenaline output rose from $0{\cdot}8 \pm 0{\cdot}3$ to a

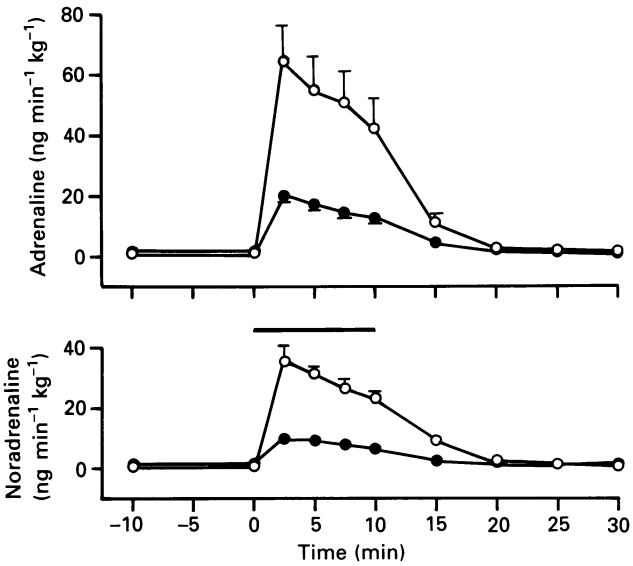

Fig. 6. Changes in mean right adrenal catecholamine output in four conscious, functionally hypophysectomized calves given intra-aortic infusions of acetylcholine (4·5 nmol min^{-1} kg^{-1}) during an intravenous infusion of exogenous ACTH (2 ng min^{-1} kg^{-1}, I.V.) in the presence (●) and absence (○) of atropine (0·2 mg kg^{-1}). Horizontal bar, duration of acetylcholine infusion. Vertical bars, S.E. of each mean value wherever greater than the size of the symbol.

mean peak value of $70{\cdot}0 \pm 13{\cdot}0$ ng min^{-1} kg^{-1} at 2·5 min after which it fell steadily during the remaining period of infusion and then abruptly thereafter (Fig. 5). Mean noradrenaline output exhibited a very similar pattern but had risen from $0{\cdot}7 \pm 0{\cdot}2$ to a somewhat lower mean peak value at 2·5 min ($48{\cdot}0 \pm 8{\cdot}5$ ng min^{-1} kg^{-1}). The average mean output of adrenaline during the acetylcholine infusion ($62{\cdot}6 \pm 3{\cdot}4$ ng min^{-1} kg^{-1}) was significantly higher than that of noradrenaline ($41{\cdot}6 \pm 3{\cdot}3$ ng min^{-1} kg^{-1}; $P < 0{\cdot}01$). The outputs of both catecholamines, in response to acetylcholine, were consistently lower in the presence of exogenous ACTH but the difference between the average mean values during the course of the infusions only achieved statistical significance in the case of noradrenaline ($P < 0{\cdot}05$). Both the adrenaline and the noradrenaline responses to acetylcholine were substantially reduced (roughly in the same proportion) by prior administration of atropine (0·2 mg kg^{-1}; Fig. 6) but were by no means abolished thereby.

Acetylcholine also produced an abrupt increase in the output of corticotrophin releasing factor (CRF) from the right adrenal gland which rose from $0{\cdot}8 \pm 0{\cdot}1$ to a peak mean value of $15{\cdot}2 \pm 1{\cdot}4$ pg min^{-1} kg^{-1} at 2·5 min. This response was potentiated in the presence of exogenous ACTH and the values during the infusion were

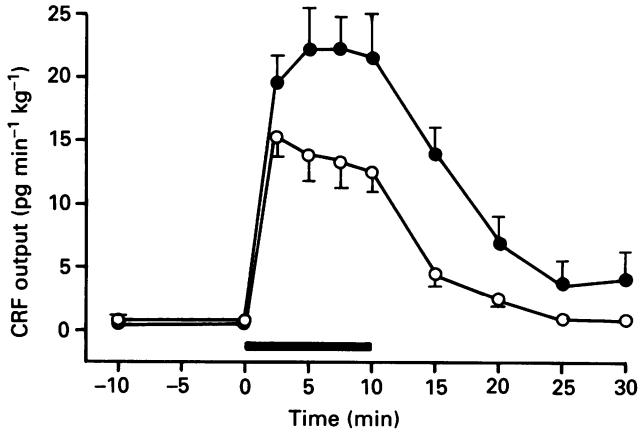

Fig. 7. Changes in mean right adrenal CRF output in four conscious, functionally hypophysectomized calves given intra-aortic infusions of acetylcholine (4·5 nmol min^{-1} kg^{-1}) in the presence (●) and absence (○) of exogenous ACTH (2 ng min^{-1} kg^{-1}, I.V.). Horizontal bar, duration of acetylcholine infusion. Vertical bars, S.E. of each mean value wherever greater than the size of the symbol.

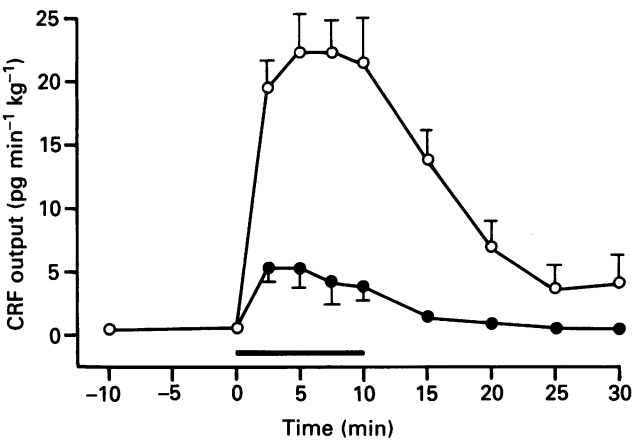

Fig. 8. Changes in mean right adrenal CRF output in four conscious, functionally hypophysectomized calves given intra-aortic infusions of acetylcholine (4·5 nmol min^{-1} kg^{-1}) during an intravenous infusion of ACTH (2 ng min^{-1} kg^{-1}, I.V.) in the presence (●) and absence (○) of atropine (0·2 mg kg^{-1}). Horizontal bar, duration of acetylcholine infusion. Vertical bars, S.E. of each mean value wherever greater than the size of the symbol.

consistently higher under these latter conditions (Fig. 7). The average mean value during acetylcholine infusion in the presence of ACTH (21·4 ± 0·6) was significantly higher than the corresponding value in the absence of ACTH (13·7 ± 0·6 pg min^{-1} kg^{-1}; $P < 0·001$; Fig. 9). This response was also substantially reduced, but not entirely abolished, by pre-treatment with atropine (0·2 mg kg^{-1}), both in the presence and absence of exogenous ACTH. Values from the group given I.V. infusions of ACTH are shown in Fig. 8.

There was an abrupt increase in the output of enkephalin peptides from the right

adrenal gland in response to intra-aortic infusions with peak mean values attained within 2·5 min (Fig. 9). The average mean output of free [Met^5]enkephalin during the infusion of acetylcholine in the presence of exogenous ACTH (14·4 ± 0·7 ng min^{-1} kg^{-1}) was significantly higher than the corresponding value in the absence of

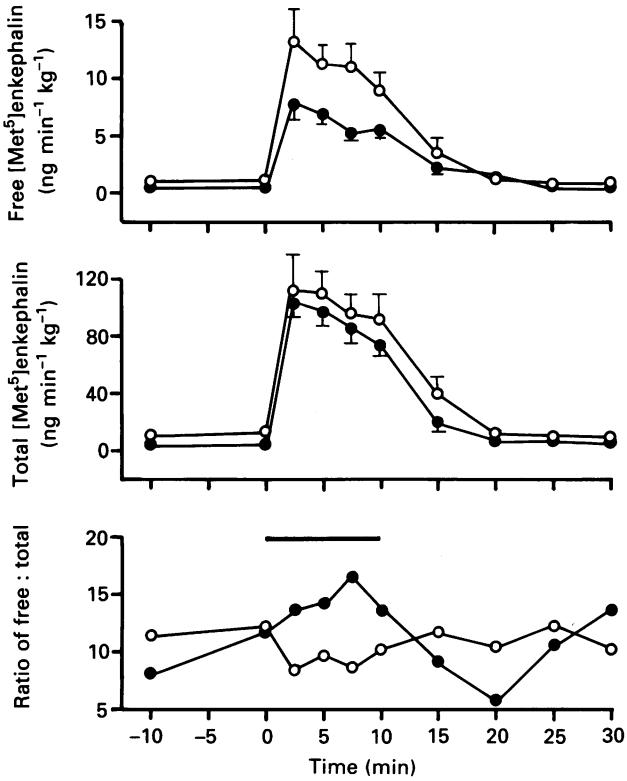

Fig. 9. Changes in mean right adrenal [Met^5]enkephalin output, and in the ratio between free and total peptide in four conscious, functionally hypophysectomized calves given intra-aortic infusions of acetylcholine (4·5 nmol min^{-1} kg^{-1}) in the presence (●) and absence (○) of exogenous ACTH (2 ng min^{-1} kg^{-1}, I.V.). Horizontal bar, duration of acetylcholine infusion. Vertical bars, S.E. of each mean value wherever greater than the size of the symbol.

ACTH (9·3 ± 0·4 ng min^{-1} kg^{-1}; $P < 0·01$). ACTH had no significant effect on the output of the larger molecular weight precursor forms of [Met^5]enkephalin (total [Met^5]enkephalin; Fig. 9), which were also released from the gland in far greater amounts. A consequence of the fact that ACTH inhibited the release of free [Met^5]enkephalin from the gland more effectively than that of the larger forms was the fact that the ratio of free:total [Met^5]enkephalin rose during acetylcholine infusions in the presence of ACTH and fell in its absence (Fig. 9). The difference between the mean average ratios during the infusion of acetylcholine was highly significant statistically ($P < 0·01$).

The output of both forms of [Met^5]enkephalin from the adrenal gland, which occurred in response to acetylcholine, was substantially reduced, but not completely

suppressed, by pre-treatment with atropine (0·2 mg kg^{-1}) both in the presence and absence of ACTH. The effect of atropine in the group tested in the presence of ACTH is illustrated in Fig. 10, which also shows that the output of total [Met^5]enkephalin was reduced proportionately more than that of the free peptide, reversing the change in the ratio of free:total [Met^5]enkephalin output.

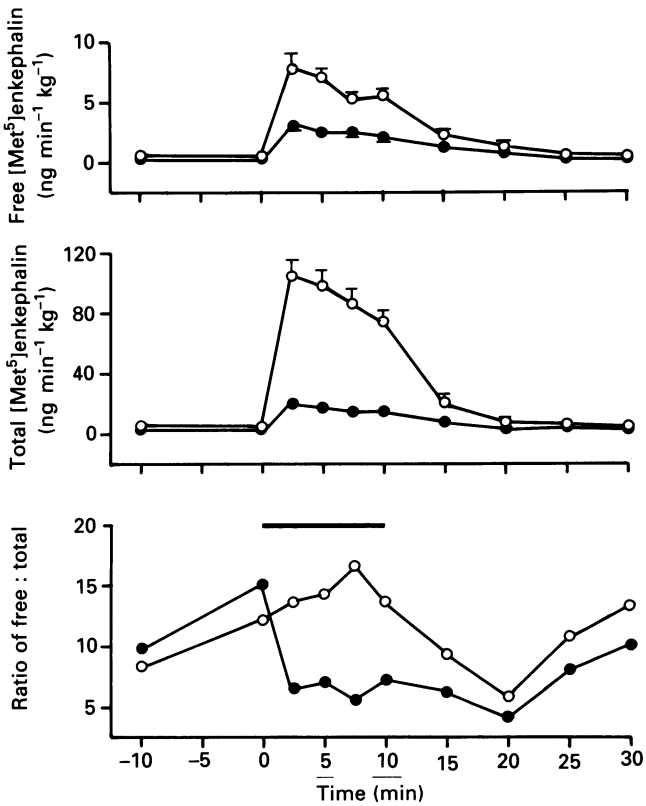

Fig. 10. Changes in mean right adrenal [Met^5]enkephalin output in four conscious, functionally hypophysectomized calves given intra-aortic infusions of acetylcholine (4·5 nmol min^{-1} kg^{-1}) during an intravenous infusion of exogenous ACTH (2 ng min^{-1} kg^{-1}) in the presence (●) and absence (○) of atropine (0·2 mg kg^{-1}). Horizontal bar, duration of acetylcholine infusion. Vertical bars, S.E. of each mean value wherever greater than the size of the symbol.

Pancreatic endocrine responses

Intra-aortic infusions of acetylcholine also elicited the release of glucagon, insulin and pancreatic polypeptide (PP) from the pancreas in these animals, irrespective of the presence or absence of ACTH. Rises in plasma insulin and pancreatic glucagon were found to be highly glucose-dependent whereas the PP response was not. Thus, in animals in which the initial arterial plasma glucose concentration exceeded 4·5 mmol l^{-1} there was a substantial rise in plasma insulin concentration, with little or no rise in plasma glucagon concentration, and a consequential fall in plasma glucose concentration. In contrast, in animals with an initial arterial plasma glucose

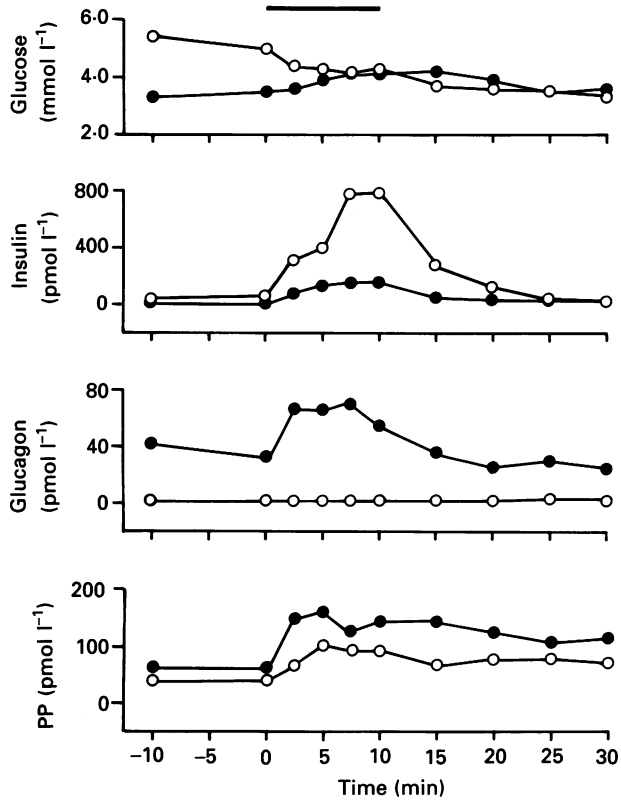

Fig. 11. Changes in arterial plasma glucose, insulin, glucagon and PP concentration in response to intra-aortic infusions of acetylcholine (4·5 nmol min^{-1} kg^{-1} for 10 min) in two individual, functionally hypophysectomized calves, one of which had a relatively high initial plasma glucose concentration (○; 5·0 mmol l^{-1}) while the other had a relatively low initial plasma glucose concentration (●; 3·5 mmol l^{-1}).

concentration below 4·0 mmol l^{-1}, the insulin response was blunted and the rise in plasma glucagon enhanced, these changes being reflected in a rise in plasma glucose concentration in response to acetylcholine. Typical results from individual animals with initial arterial plasma glucose concentrations above, and below, 4·0–4·5 mmol l^{-1} exemplify these findings (Fig. 11). All these pancreatic endocrine responses were abolished by atropine (0·2 mg kg^{-1}).

DISCUSSION

Acetylcholine was first shown to exert a steroidogenic effect in isolated calf adrenal glands by Rosenfeld (1955) and this was confirmed more recently in suspensions of bovine fasciculata cells by Hadjian, Guidicelli & Chambaz (1982) and also in the isolated adrenal gland of the frog by Benyamina, Leboulenger, Lirhmann, Delarue, Feuilloley & Vaudry (1987). However, the cortisol response to intra-aortic infusions of acetylcholine at a dose of 4·5 nmol min^{-1} kg^{-1} in these conscious calves resembled

that to splanchnic nerve stimulation reported previously (Edwards & Jones, 1987) in that it only occurred, in functionally hypophysectomized calves, when exogenous ACTH was also infused and not otherwise. It differed from the response to splanchnic nerve stimulation in that the increase in adrenal cortisol output could be fully accounted for by the estimated increase in the rate at which ACTH was presented to the gland consequent upon adrenal vasodilatation, presumably due to activation of vascular muscarinic receptors. This provides confirmatory evidence that the potentiation of the steroidogenic effect of ACTH which occurs during splanchnic nerve stimulation does not depend entirely on an increase in ACTH presentation secondary to adrenal vasodilatation. No doubt such an effect contributes to the response because adrenal cortisol output has been shown to fall when adrenal blood flow, and consequently the estimated ACTH presentation rate, is reduced artificially by intra-aortic infusions of endothelin and the experiments carried out in the same species under directly comparable conditions (Bloom, Edwards & Jones, 1990); such blood flow-dependent adrenal cortical responses were originally described by Urquhart (1965) in isolated perfused canine adrenal glands. However, in the calf, there must clearly be some additional factor, such as vasoactive intestinal polypeptide (VIP), which is released within the gland in response to splanchnic nerve stimulation and further enhances the effect of ACTH somehow (Bloom, Edwards & Jones, 1987, 1988). Corticotrophin releasing factor (CRF) is also released within the gland during splanchnic nerve stimulation (Edwards & Jones, 1988) and exerts a steroidogenic effect when administered intra-arterially (Jones & Edwards, 1990). However, it is not thought to mediate enhanced steroidogenesis during splanchnic nerve stimulation because release is inhibited in the presence of ACTH (Edwards & Jones, 1988) and roughly similar amounts of CRF were released in the present study in response to acetylcholine as were previously during splanchnic nerve stimulation (Edwards & Jones, 1988).

These results are also illuminating with regard to adrenal cortical responses to calcitonin gene-related peptide (CGRP) in the calf, which have been reported previously (Bloom, Edwards & Jones, 1989). Unlike splanchnic nerve stimulation or intra-aortic infusions of VIP, CGRP stimulates the output of cortisol from the adrenal gland in the absence of exogenous ACTH and apparently by a direct action of its own. However, it is also a potent vasodilator agonist and caused a rise in adrenal blood flow of about 30% at a dose of about 30 pmol min^{-1} kg^{-1} (Bloom *et al.* 1989). It seemed unlikely that a 30% increase in ACTH presentation could have accounted for the observed increase in cortisol output of about 500% which occurred but it may have made some contribution. That interpretation is confirmed by the present results in which a considerably greater rise in adrenal blood flow (of about 100%) produced no detectable change in cortisol output at very low plasma ACTH concentrations.

The observation that acetylcholine stimulates the release of aldosterone from the adrenal cortex is in accord with findings in isolated bovine glomerulosa cells in which the calcium messenger system has been implicated in the response (Kojima, Kojima, Shibata & Ogata, 1986). It has been reported recently that chronic treatment with ACTH inhibits the biosynthesis of aldosterone (Abayesekara, Vazir, Whitehouse, Price, Hinson & Vinson, 1989), but the orthodoxy is that under acute conditions,

such as those employed in the present experiments, ACTH is a potent stimulant to aldosterone secretion. It was therefore surprising to find that the aldosterone response to acetylcholine was inhibited by ACTH at a low dose (2 ng min^{-1} kg^{-1}, I.V.) under these particular experimental conditions. The speculation that cholinergic stimulation of aldosterone release might fulfil a physiological function is supported

TABLE 1. Comparison of the average mean outputs of catecholamines, [Met^5]enkephalins and of CRF in response to an intra-aortic infusion of acetylcholine (4·5 nmol min^{-1} kg^{-1} for 10 min) in conscious, functionally hypophysectomized calves given ACTH (2 ng min^{-1} kg^{-1}; I.V.), in the presence and absence of atropine (0·2 mg kg^{-1}; $n = 4$)

	−Atropine (ng min^{-1} kg^{-1})	+Atropine (ng min^{-1} kg^{-1})	Percentage fall
Adrenaline	54±5	16±2	70
Noradrenaline	29±3	8±1	72
Free [Met^5]enkephalin	14±1	4±1	71
Total [Met^5]enkephalin	91±7	17±1	81
	(pg min^{-1} kg^{-1})	(pg min^{-1} kg^{-1})	
CRF	21·4±0·6	4·8±0·4	78

by the finding that the adrenal gland receives a vagal motor innervation (Coupland, Parker, Kesse & Mohamed, 1989), together with the fact that the output of aldosterone from the adrenal gland increased as much as threefold in just 10 min in response to acetylcholine. This would appear to be a sufficiently large response to produce changes in electrolyte excretion if maintained over a long period. The mean peak right adrenal aldosterone output at the end of the acetylcholine infusion in these calves was about 4 ng min^{-1} kg^{-1}, which corresponds to a dose of about 250 μg day^{-1} in 22 kg dogs, making allowance for the output from the left adrenal as well. This is well over the top of the range within which Young & Guyton (1977) found that I.V. infusions of aldosterone produced changes in plasma potassium concentration in dogs. In humans, I.V. infusions of aldosterone at a dose of about 8·0 ng min^{-1} kg^{-1} (precisely the level realized in the present experiments when both glands are taken into account) have been reported to produce a significant reduction in sodium excretion and urine volume within 4 h (Adamson & Jamieson, 1972). However, it is difficult to interpret changes in the rate of aldosterone secretion without a knowledge of the sodium status of the animals, which was not established in this study in which the finding was entirely incidental.

Adrenal medullary responses to intra-aortic infusions of acetylcholine included release of CRF, [Met^5]enkephalins and catecholamines. Each of these responses was reduced by between 70 and 81 % by pre-treatment with atropine (Table 1). It follows that the responses to this dose of acetylcholine were principally, though not entirely, dependent upon activation of muscarinic receptors and the fact that each response was reduced by almost the same amount after atropine could be interpreted to indicate a common source, presumably the chromaffin cells.

Unlike the glucocorticoid response, it seems most unlikely that these medullary responses to muscarinic stimulation could be secondary to an increase in blood flow. Total adrenal blood flow was well above the normal range before acetylcholine was administered because of the adrenal vasodilator action of the exogenous ACTH

which was infused continuously; furthermore it was only about 34% greater in the absence of atropine than it was following administration of the drug. In contrast, the differences in the outputs of adrenaline, noradrenaline, free and total [Met^5]-enkephalin and CRF in the presence and absence of atropine (and so attributable to muscarinic stimulation) were 38, 21, 10 and 74 ng min^{-1} kg^{-1} and 16·6 pg min^{-1} kg^{-1} respectively. These amounted to increases in the outputs of these agonists of 238, 263, 250, 435 and 346% respectively when the muscarinic receptors were susceptible to excitation over those obtained when those receptors were blocked. Furthermore, an increase in blood flow would not of itself result in an increase in any known stimulus to the release of these agonists, as it would in the case of cortisol, by increasing the presentation rate of ACTH. However, the possibility that muscarinic adrenal vasodilatation may have contributed to these responses cannot be excluded because the techniques provided no information about regional changes in adrenal blood flow.

Muscarinic stimulation of catecholamine release was first described by Feldberg, Minz & Tsudzimura (1934) in the cat and has since been amply confirmed both in this (Douglas & Poisner, 1965; Lee & Trendelenberg, 1967; Kirpekar, Prat & Schiavone, 1982) and other species, including the gerbil (Douglas, Kanno & Sampson, 1967), rat (Yoshikazi, 1975; Wakade & Wakade, 1983) and guinea-pig (Role & Perlman, 1983). In each of these, however, it has generally been supposed to have a trivial effect by comparison with the nicotinic mechanism. In other species release of catecholamines has been ascribed to activation of just one variety of cholinergic receptor; supposedly exclusively muscarinic in chick adrenal (Ledbetter & Kirschner, 1975; Knight & Baker, 1986) and nicotinic in the bovine adrenal (see for instance Ballesta, Borges, Garcia & Hidalgo, 1989). Thus, the present results implicating muscarinic receptors in the release of catecholamines from the adrenal medulla conflict with the results of numerous *in vitro* studies, both on freshly isolated (Schneider, Cline & Lemair, 1979; Oka, Isosaki & Watanabe, 1982) and cultured bovine chromaffin cells (Yanagihara, Isosaki, Ohuchi & Oka, 1979; Trifaró & Lee, 1980; Fisher, Holz & Agranoff, 1981). We suppose that the reason for such a pronounced muscarinic response in the present experiments was simply that the dose of acetylcholine employed sufficed to activate those receptors, without affecting the nicotinic receptors significantly. The vast majority of studies on the mechanism of catecholamine release from the adrenal medulla have involved splanchnic nerve stimulation at intensities far above anything which could occur naturally, or very high doses of acetylcholine, or cholinergic agonists, likely to favour activation of nicotinic receptors. *In vivo* studies have also almost invariably been carried out under general anaesthesia, which is known to modify catecholamine release substantially (Edwards *et al.* 1980) and may well have led to preferential activation of nicotinic receptors. In the conscious calf there is a substantial hypertensive response to splanchnic nerve stimulation after complete nicotinic blockade with hexamethonium, which is completely abolished by atropine and clearly mediated by muscarinic receptors (A. V. Edwards & C. T. Jones, unpublished observations). Furthermore, even in isolated bovine chromaffin cell preparations muscarinic receptors are known to be present (Ballesta *et al.* 1989) and their activation leads to biochemical responses other than catecholamine secretion which include increasing cyclic GMP levels (Yanagihara *et al.* 1979),

phospholipid turnover (Fisher *et al.* 1981) and intracellular inositol trisphosphate (Forsberg, Rojas & Pollard, 1986). Our finding in the present study that catecholamine secretion from the adrenal medulla of the conscious calf, in response to intra-aortic infusions of acetylcholine, is mainly attributable to muscarinic activation has been substantiated by the further finding that it is *not* significantly affected by full nicotinic blockade with hexamethonium (A. V. Edwards & C. T. Jones; unpublished observations).

Recent studies of the consequences of muscarinic activation in the adrenal medulla of the rat have shown that, unlike nicotinic activation which mobilizes extracellular calcium, it leads to an independent mobilization of intracellular calcium (Harish, Kao, Raffaniello, Wakade & Schneider, 1987; Wakade, Malhotra & Wakade, 1986) via inositol phosphate production and thereby causes catecholamine release (Malhotra, Wakade & Wakade, 1988). VIP, which is also released within the adrenal gland in response to splanchnic nerve stimulation (Bloom *et al.* 1988), and has been implicated in the control of adrenal catecholamine secretion (Wakade, 1988), acts in a precisely similar fashion (Malhotra *et al.* 1988) and so is likely to potentiate the response to muscarinic activation. Catecholamine output in response to stimulation of the adrenal innervation at high frequencies (10 Hz) rapidly fades whereas the response to low frequency stimulation (1 Hz) is well maintained (Wakade, 1988); nicotinic receptors rapidly desensitize during prolonged exposure to acetylcholine whereas the catecholamine output in response to both VIP and muscarinic activation is long-lasting (Malhotra *et al.* 1988). From the available evidence, it could be proposed that, at the rates at which these nerve fibres are likely to fire *in vivo* under normal conditions, the secretion of catecholamines (and probably other agonists such as CRF and enkephalins) may depend upon activation of muscarinic receptors and interaction with VIP and possibly other purinergic or peptidergic agents. The concentration of acetylcholine required to activate nicotinic receptors might then only be achieved *in extremis* when a maximal discharge is required for a relatively short period.

If the nerve fibres innervating the adrenal medulla normally act by releasing more than one chemical agonist one might anticipate differences between the spectrum of responses to nerve stimulation and that to one agonist in isolation, as with acetylcholine here. Close inspection of the medullary response shows that this is the case. Thus, a dose of acetylcholine which produced a much greater average mean output of enkephalins (free, $9{\cdot}3 \pm 0{\cdot}4$ ng min^{-1} kg^{-1}; total, 103 ± 5 ng min^{-1} kg^{-1}) than splanchnic nerve stimulation at 4 Hz in normal calves (free, $3{\cdot}1 \pm 0{\cdot}1$ ng min^{-1} kg^{-1}, $P < 0{\cdot}001$; total, $21{\cdot}8 \pm 1{\cdot}0$ ng min^{-1} kg^{-1}, $P < 0{\cdot}001$; Bloom *et al.* 1988) produced a completely different pattern of CRF release, rising more abruptly but to a significantly lower mean peak (Fig. 9; Edwards & Jones, 1988). Furthermore catecholamine output in response to acetylcholine at this dose was only about half that observed in response to splanchnic nerve stimulation at 4 Hz. Exogenous ACTH tended to reduce the output of catecholamines and of free [Met^5]enkephalin in response to acetylcholine just as it does in response to splanchnic nerve stimulation (Edwards & Jones, 1987). On the other hand, its effect on CRF was completely reversed and the output of a peptide that is reduced in the presence of ACTH in response to splanchnic nerve stimulation was significantly enhanced thereby during

acetylcholine infusions. These differences are consistent with the view that splanchnic nerve terminals contain agonists other than acetylcholine which are released with it and serve to modify the adrenal medullary responses.

Pancreatic endocrine responses to intra-arterial infusions of acetylcholine also differed from the corresponding responses to vagal stimulation in conscious calves that have been reported previously (Bloom & Edwards, 1981). Thus whereas glucose was found to potentiate insulin release, without affecting PP release, in response to both types of stimulation, it strongly inhibited release of glucagon in response to acetylcholine without affecting the response to vagal stimulation.

It is concluded that acetylcholine is capable of producing adrenal responses both directly and indirectly (secondary to changes in blood flow) and that there is an important muscarinic component in the case of medullary responses which is likely to be of considerable physiological significance and so is deserving of further attention.

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