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# Autonomic control of adrenal function

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ABSTRACT

Recent studies of adrenal function in conscious calves are reviewed. These have involved collecting the whole of the adrenal effluent blood from the right adrenal gland at intervals and, where necessary, prior functional hypophysectomy by destruction of the pituitary stalk under general halothane anaesthesia 3 d previously. The adrenal medulla was found to release numerous neuropeptides, in addition to catecholamines, in response to stimulation of the peripheral end of the right splanchnic nerve, which was carried out below behavioural threshold. Many of these responses were enhanced by stimulating intermittently at a relatively high frequency. Intra-aortic infusions of a relatively low dose of acetylcholine (4.5 nmol $min^{-1}$ $kg^{-1}$) elicited similar responses. In the adrenal cortex, agonists which either potentiated the steroidogenic response to ACTH or exerted a direct steroidogenic action included VIP, CGRP, CRF and ACh acting via muscarinic receptors. Stimulation of the peripheral end of the right splanchnic nerve strongly potentiated the steroidogenic response to ACTH and there is compelling evidence that the innervation normally plays an important part in cortisol secretion.

## INTRODUCTION

It has been widely accepted that the adrenal medulla secretes catecholamines in response to activity in exclusively preganglionic sympathetic fibres which fire tonically at low frequencies and release acetylcholine which activates the chromaffin cells via nicotinic receptors. It has also been accepted that the secretion of cortisol from the adrenal cortex is determined solely by the release of adrenocorticotrophin (ACTH) from the anterior pituitary. The object of this review is to assess the extent to which these views need to be reappraised in the light of recent work, and to focus principally on studies carried out on conscious animals (mainly calves) in the Physiological Laboratory at Cambridge.

Briefly, the adrenal medulla releases numerous peptides, in addition to catecholamines, in response to stimulation of the splanchnic sympathetic innervation, and these responses can be enhanced by stimulating intermittently at frequencies well above those at which sympathetic fibres have generally been supposed to fire naturally. Postganglionic neurons have been identified in both the medulla and cortex and intra-aortic infusions of small amounts of acetylcholine have been found to elicit adrenal medullary responses principally by activating muscarinic receptors, in marked contrast to many results of in vitro studies. Vasoactive intestinal peptide (VIP) is also present in nerve terminals in the gland and evokes release of catecholamines when administered intra-arterially. For the adrenal cortex agonists which either potentiate the steroidogenic response to ACTH or exert a direct steroidogenic action include VIP, calcitonin gene related peptide (CGRP), corticotrophin releasing factor (CRF) and acetylcholine, acting via muscarinic receptors. Finally, activation of the splanchnic sympathetic innervation strongly potentiates the steroidogenic action of ACTH and there is compelling evidence that the innervation normally plays an important part in the control of cortisol secretion.

## ADRENAL MEDULLARY FUNCTION

The 'adrenal clamp' technique involves removal of the right kidney, cannulation of the right renal vein and insertion of a specially designed clamp along the posterior vena cava in such a way that the jaws divert

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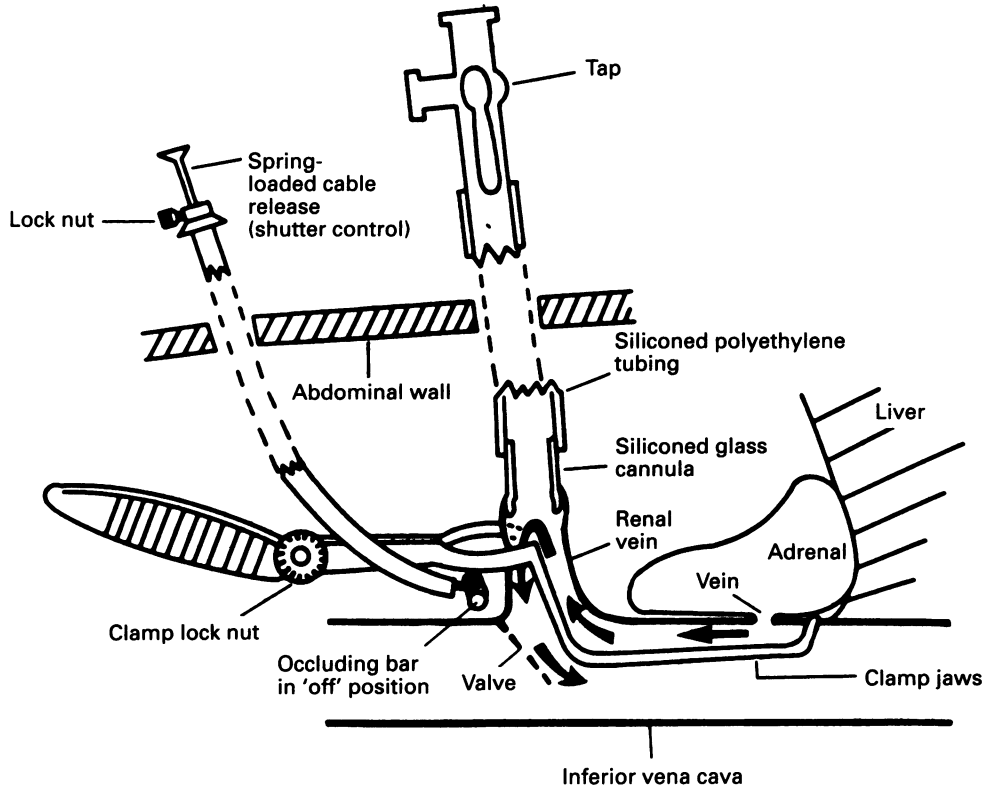


Fig. 1. Diagrammatic representation of the positioning of an 'adrenal clamp' in relation to the adrenal gland and renal vein. (From Edwards et al. 1974, by permission of the Physiological Society.)

the venous effluent adrenal blood back to enter the vena cava via the renal vein (Edwards et al. 1974) (Fig. 1). The operation is carried out under full general anaesthesia, during which appropriate intravascular catheters can be inserted and a fluid electrode attached to the peripheral end of the right splanchnic nerve. Following recovery from anaesthesia, the whole of the adrenal venous effluent can be collected at intervals simply by occluding the channel through the renal vein. Adrenal output can then be quantified precisely in the conscious animal, the bloodflow being estimated gravimetrically.

### *Adrenal medullary sensitivity*

The adrenal medulla was found to be remarkably resistant to a variety of stresses which readily produced an increase in the output of glucocorticoids from the adrenal cortex. Thus hypoxia failed to elicit a significant increase in catecholamine output unless the arterial $P_{O_2}$ fell below 15 mmHg (Bloom et al. 1976, 1977), hypercapnia failed to produce an adrenal medullary response unless the arterial $P_{CO_2}$ rose above 100 mmHg (Bloom et al. 1977) and insulin hypoglycaemia was only effective in this regard when the arterial plasma glucose concentration had fallen below 2 mmol $l^{-1}$ for a prolonged period (Bloom et al. 1975).

### *Responses to splanchnic nerve stimulation*

*Dopamine-β-monooxygenase.* The gland responds very readily to stimulation of the peripheral end of the splanchnic nerve, which can be accomplished below any discernible behavioural threshold in conscious animals by employing a fluid electrode that minimises spread of activity. The outputs of both adrenaline and noradrenaline are linearly related to stimulus frequency over the range 2–10 Hz, with peak outputs occurring at 15 Hz. Continuous stimulation at either 4 or 10 Hz produced a small but significant rise in the output of dopamine-β-monooxygenase activity (Edwards et al. 1980). The amounts released could not be related either to the frequency of stimulation or to the output of adrenaline or noradrenaline and so proved not to be a reliable index of exocytosis under these conditions, as had previously been suggested (Geffen et al. 1969).

*Enkephalins.* Opioid peptides are present in the adrenal medullae in many species in the form of a proenkephalin precursor possessing 6 copies of metenkephalin and 1 copy of leuenkephalin, the pro-

cessing of which is under neural control (Schultzberg et al. 1978; Costa et al. 1979; Stern et al. 1979; Viveros et al. 1979). They are localised both in the chromaffin granules of adrenergic, but not noradrenergic, cells (Livett et al. 1982; Pelto-Huikko et al. 1982; Roisin et al. 1983), where they are synthesised (Stern et al. 1979; Chang et al. 1982) and within 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 (Hexum et al. 1980; Govoni et al. 1981). In the conscious dog 10–20% haemorrhage results in the release of enkephalins (Engeland et al. 1986) and in the conscious calf enkephalins are released from the gland in response to splanchnic nerve stimulation almost entirely in the form of high molecular weight precursors (Edwards et al. 1986; Bloom et al. 1988). Scrutiny of the profiles of chromatograms of met$^5$-enkephalin-like immunoreactivity from extracts of the gland showed it was virtually all material with $M_4$ values above 4000. In contrast, about 30% of the total in adrenal effluent plasma was accounted for by molecules with $M_4$ values of about 4000 (Rossier et al. 1988). This peak might correspond to peptides I, E, B, F, BAM 22 or BAM 18, all of which have been isolated from bovine adrenal (Metters & Rossier, 1984).

The adrenal medulla contains numerous opioid binding sites (Castanas et al. 1985*a*, *b*), at least some of which are located postsynaptically on adrenal chromaffin cells (Kumakura et al. 1980), but their physiological functions and those of adrenal opioids have yet to be elucidated. It seems unlikely that endogenous adrenal opioids could act on adrenal chromaffin cells to modify their responses to prostaglandins (Marley et al. 1988) or the inositol phosphate second-messenger system (Bunn et al. 1988), or to affect their responses to histamine or angiotensin (Marley & Livett, 1986; Marley & Bunn, 1988). They have, however, been found to inhibit the adrenal catecholamine response to nicotine in isolated chromaffin cells (Marley et al. 1986; Marley & Livett, 1987) and the isolated, perfused adrenal gland of the rat (Chen & Dixon, 1990). In the conscious calf model, there is evidence that enkephalins released in response to stimulation of the peripheral end of the splanchnic nerve are autoinhibitory as their release was significantly enhanced by pretreatment with naloxone and the proportion of high molecular weight enkephalin molecules preferentially increased (Edwards & Jones, 1989*a*). Splanchnic nerve stimulation resulted in an abrupt rise in the output of both free and total met$^5$-enkephalin-like immunoreactivity from the adrenal gland in conscious calves which was substantially potentiated by stimulating in bursts (Bloom et al. 1988) (Fig. 2). This pattern of stimulation also increased the proportion released in high molecular weight form.

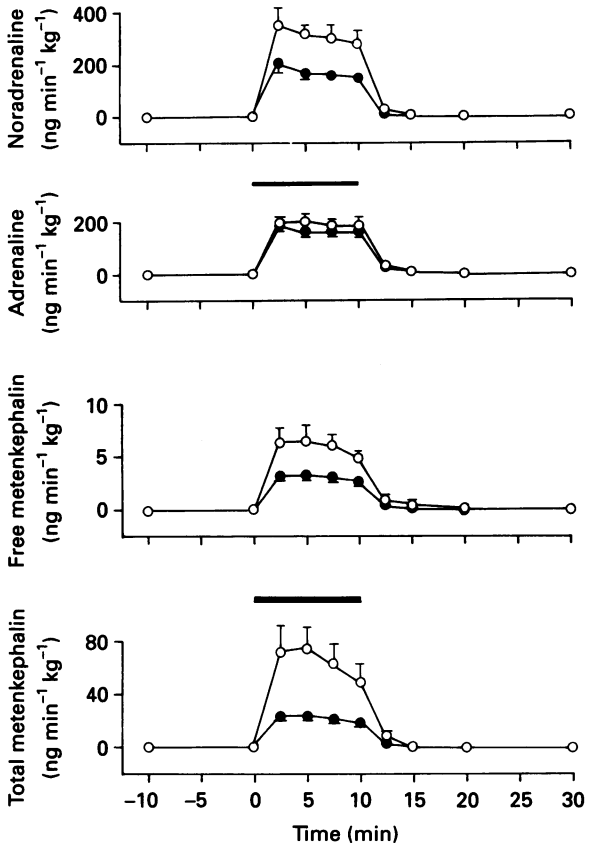


Fig. 2. Comparison of the output of adrenaline, noradrenaline, free and total met$^5$-enkephalin-like immunoreactivity from the right adrenal medulla in response to stimulation of the peripheral end of the right splanchnic nerve in 7 conscious calves at either 4 Hz continuously (●), or at 40 Hz for 1 s at 10 s intervals (○), for 10 min. Vertical bars, S.E. of each mean value where these exceed the size of the symbol. Horizontal bar, duration of stimulation. (Modified from Bloom et al. 1988, by permission of the Physiological Society.)

*Catecholamines.* Adrenal medullary responses to splanchnic nerve stimulation are critically dependent on the pattern of stimulation that is applied. Stimulation in bursts at relatively high frequencies significantly potentiates the output of catecholamines, and enkephalins (Bloom et al. 1988) (Fig. 2). Thus stimulation at 40 Hz for 1 s at 10 s intervals elicited a substantial and significant increase in the output of noradrenaline, above that which occurred in response to stimulation at 4 Hz continuously, in conscious calves. This effect far exceeded the rise in adrenaline output and so produced a significant shift in the noradrenaline : adrenaline ratio, in favour of noradrenaline.

In the dog several groups have reported an increase in the proportion of noradrenaline released from the adrenal gland in response to continuous splanchnic nerve stimulation as the stimulus frequency was increased (Rapela & Covian, 1954; Klepping, 1956; Rapela, 1956). However, the changes were comparatively small and interpretation of the results is complicated by the fact that experiments were carried out under anaesthesia. In certain species, notably the cat, the proportions of adrenaline and noradrenaline released from the adrenal have been found to vary with the particular type of stimulus employed (von Euler, 1956). Thus adrenaline is released preferentially in response to hypoglycaemia in cats (Dunér, 1954) and sheep (Crone, 1965). In the cat, von Euler & Folkow (1953) originally showed that carotid occlusion favours the release of noradrenaline whereas stimulation of either the brachial or sciatic plexus favours the release of adrenaline. The effect of carotid occlusion is in accord with more recent findings that in cats, but not dogs, reduction of the pressure in the carotid sinus preferentially releases noradrenaline whereas stimulation of the carotid chemoreceptors preferentially releases adrenaline (Anichkov et al. 1960; Critchley et al. 1980), as does asphyxia (Redgate & Gellhorn, 1953). This difference between the adrenal medullary responses of (anaesthetised) cats and dogs is further exemplified by the finding that the relative proportions of the 2 amines that are secreted in response to electrical stimulation of the hypothalamus vary in accordance with the position of the stimulating electrode in cats but not dogs (Redgate & Gellhorn, 1953; Folkow & von Euler, 1954; Goldfien & Ganong, 1962; Malmejac, 1964). Differential release of one or other of these catecholamines in response to such stimuli as hypoglycaemia or asphyxia could well be due to differential sensitivity of the chromaffin cells themselves, to a lack of glucose or oxygen, or to an excess of carbon dioxide. It is more difficult to understand how differential release in response to electrical stimulation of the hypothalamus could be mediated peripherally. However, the results obtained in the conscious calf show that this could be achieved simply by altering the pattern of efferent splanchnic nerve activity.

*Corticotrophin releasing factor.* The adrenal gland has also been reported to contain corticotrophin-releasing factor-like immunoreactivity (CRF) (Suda et al. 1984; Hashimoto et al. 1984) which is released from the gland in quite substantial amounts in response to haemorrhage in conscious dogs (Bruhn et al. 1987). In conscious calves, stimulation of the peripheral end of the right splanchnic nerve for 10 min increased the mean output of CRF progressively, so that it had risen about 20-fold, to a peak incremental value of $24 \pm 4$ pmol $min^{-1}$ $kg^{-1}$ at 10 min (Edwards & Jones, 1988). This response was significantly increased by stimulating in bursts at 40 Hz for 1 s at 10 s intervals, which raised the mean adrenal CRF output by $44 \pm 7$ pmol $min^{-1}$ $kg^{-1}$ at 10 min ($P < 0.05$). In hypophysectomised calves, administration of synthetic adrenocorticotrophic hormone ($ACTH_{1-24}$) at a dose of 5 ng $min^{-1}$ $kg^{-1}$ reduced the output of adrenal CRF in response to splanchnic nerve stimulation by about 50%. Release of this peptide from the adrenal gland thus appears to be susceptible to inhibition via the ACTH–adrenal cortical axis, just like its pituitary counterpart, to which it is also structurally closely related (Edwards & Jones, 1988).

*Responses to acetylcholine*

Muscarinic stimulation of catecholamine release was first described by Feldberg et al. (1934) in the cat and has since been amply confirmed both in this (Douglas & Poisner, 1965; Lee & Trendelenberg, 1967; Kirpekar et al. 1982) and other species, including the gerbil (Douglas et al. 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 type of cholinergic receptor, supposedly exclusively muscarinic in the chick adrenal (Ledbetter & Kirschner, 1975; Knight & Baker, 1986) and nicotinic in the bovine adrenal (see e.g. Ballesta et al. 1989). In the conscious calf, this question has been examined employing an infusion of acetylcholine at a dose of 4.5 nmol $min^{-1}$ $kg^{-1}$ into the aorta above the origin of the adrenal arterial supply, in functionally hypophysectomised animals to prevent fluctuations in endogenous ACTH. Under these conditions acetylcholine produced an abrupt rise in the outputs of adrenaline and noradrenaline which reached a peak at 2.5 min and declined steadily thereafter for the duration of the 10 min infusion (Jones et al. 1991) (Fig. 3). Unlike the response to splanchnic nerve stimulation, the output of adrenaline was substantially greater than that of noradrenaline. Both responses are greatly reduced following the administration of atropine (roughly in the same proportion) but were by no means abolished thereby (Fig. 3). There was an abrupt rise in the output of enkephalin peptides from the right adrenal gland in response to acetylcholine. Like the response to nerve stimulation, large mol-

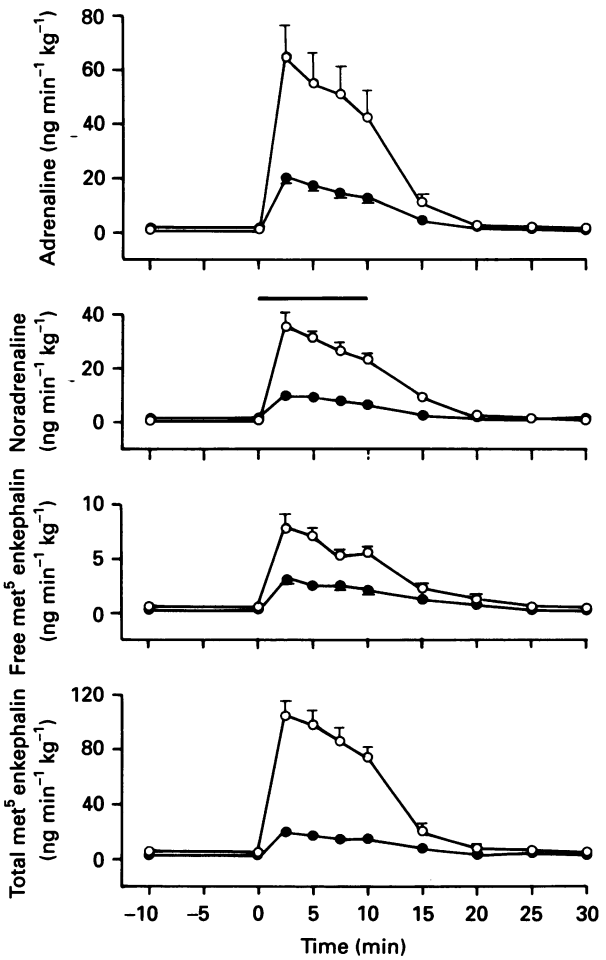


Fig. 3. Comparison of the outputs of adrenaline, noradrenaline, free and total enkephalin-like immunoreactivity from the right adrenal medulla in response to an intra-aortic infusion of acetylcholine (4.5 nmol $min^{-1}$ $kg^{-1}$ for 10 min), during an i.v. infusion of exogenous ACTH (2 ng $min^{-1}$ $kg^{-1}$) in 4 conscious functionally hypophysectomised calves, in the presence (●) and absence (○) of atropine (0.2 mg $kg^{-1}$). Vertical bars, S.E. of each mean value where these exceed the size of the symbol. Horizontal bar, duration of infusion. (Modified from Jones et al. 1991, by permission of the Physiological Society.)

ecular forms predominated and exogenous ACTH was found to inhibit the release of free, but not total, met⁵-enkephalin in response to acetylcholine (Jones et al. 1991) just as it does during splanchnic nerve stimulation (Edwards et al. 1986; Edwards & Jones, 1987*a*). The output of both forms of met⁵-enkephalin from the adrenal gland was substantially reduced, but not completely suppressed, by pretreatment with atropine (0.2 mg $kg^{-1}$), both in the presence (Fig. 3) and the absence of ACTH.

Acetylcholine also produced an abrupt increase in the output of CRF from the right adrenal gland, which rose from $0.8 \pm 0.1$ to a peak mean value of $15.2 \pm 1.4$ pg $min^{-1}$ $kg^{-1}$ at 2.5 min. This response was significantly potentiated in the presence of exogenous

Table *Average mean right adrenal outputs of catecholamines, 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 calves receiving exogenous ACTH (2 ng $min^{-1}$ $kg^{-1}$ i.v.)*

| | A | B | C | D |
|---|---|---|---|---|
| Adrenaline (ng $min^{-1}$ $kg^{-1}$) | 51.3±3.2 | 15.7±1.7 | 40.6±2.9 | 56.3 |
| Noradrenaline ng $min^{-1}$ $kg^{-1}$) | 30.0±0.2 | 8.0±0.9 | 25.4±2.0 | 33.4 |
| Free met⁵-enkephalin (ng $min^{-1}$ $kg^{-1}$) | 6.9±0.8 | 2.6±0.2 | 6.9±0.8 | 9.5 |
| Total met³-enkephalin (ng $min^{-1}$ $kg^{-1}$) | 87.6±7.1 | 16.6±1.3 | 76.3±6.9 | 92.9 |
| CRF (pg $min^{-1}$ $kg^{-1}$) | 22.0±1.5 | 4.8±0.4 | 22.2±0.5 | 27.0 |

A, without blockade; B, atropine (0.2 mg $kg^{-1}$); C, hexamethonium (> 10 mg $kg^{-1}$); D, B+C.
(From Jones & Edwards, 1992, by permission of the Physiological Society.)

ACTH 2 (ng $min^{-1}$ $kg^{-1}$, i.v.) and the values during the infusion were consistently higher under these latter conditions. It was also substantially reduced, but not entirely abolished, following the administration of atropine (0.2 mg $kg^{-1}$, i.v.).

These results, which strongly implicate muscarinic receptors in the release of catecholamines, among other agonists, from the adrenal medulla in the conscious calf conflict with the results of numerous in vitro studies, both on freshly isolated (Schneider et al. 1979; Oka et al. 1982) and on cultured bovine chromaffin cells (Yanagihara et al. 1979; Trifaro & Lee, 1980; Fisher et al. 1981). Presumably, the muscarinic responses to acetylcholine in the present experiments were particularly pronounced simply because the dose employed sufficed to activate muscarinic receptors without affecting the nicotinic receptors significantly. Most studies of the mechanism of catecholamine release from the adrenal medulla have involved splanchnic nerve stimulation at intensities far above anything which could conceivably 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 anaesthesia, which substantially modifies catecholamine release 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, following complete nicotinic blockade with hexamethonium, which is completely abolished by atropine and so clearly mediated by muscarinic receptors (A. V. Edwards & C. T. Jones, unpublished observations). Furthermore, even in isolated bovine chro-

maffin cell preparations muscarinic receptors are known to be present (Ballesta et al. 1989) and their activation leads to biochemical responses other than secretion of catecholamines, which include increasing levels of cGMP (Yanagihara et al. 1979), phospholipid turnover (Fisher et al. 1981) and intracellular inositol triphosphate (Forsberg et al. 1986). The finding that the adrenal medullary responses to intra-aortic infusions of acetylcholine, at a dose of 4.5 nmol $min^{-1}$ $kg^{-1}$, are due mainly to activation of muscarinic receptors has now been vindicated by the further finding that they are not greatly reduced following full nicotinic blockade with hexamethonium (Jones & Edwards, 1992*a*) (Table).

## ADRENAL CORTICAL FUNCTION

Control of adrenal glucocorticoid secretion in the short term has generally been supposed to be largely, if not entirely, determined by adrenocorticotrophin (ACTH) released from the anterior pituitary. However, there have been an increasing number of reports of situations in which adrenal glucocorticoid output and plasma ACTH concentration have been poorly correlated and in which adrenal cortical function must be influenced by other factors (Krieger, 1979). Searches for such other mechanisms have produced a body of, mainly indirect, evidence to implicate the splanchnic sympathetic adrenal innervation (Engeland et al. 1981; Wood et al. 1982*a*, *b*; Dempsher & Gann, 1983) which is entirely consistent with the concept that the innervation to the gland exerts long-term effects on adrenal cortical growth (Engeland & Dallman, 1976; Dallman et al. 1976; Kleitman & Holzwarth, 1985*a*). Diurnal rhythms in adrenal cortical sensitivity have been demonstrated (Srivasta & Meier, 1972; Ottenweller et al. 1978; Kaneko et al. 1981; Ottenweller & Meier, 1982) and presumably normally potentiate the effect of diurnal fluctuations in plasma ACTH concentration. Furthermore, rats maintained on a restricted water schedule exhibit a rapid and pronounced fall in plasma corticosterone concentration immediately after drinking, which cannot be ascribed to a fall in plasma ACTH concentration, or to an increase in the rate of steroid catabolism (Wilkinson et al. 1982).

The contention that adrenal cortical function is influenced by the autonomic innervation is strongly supported by morphological evidence of various types of nerve terminal that are present within the adrenal cortex in a number of different species (Holzwarth et al. 1987; Kesse et al. 1987; Charlton, 1990; Hinson, 1990). In man, it has recently been reported that nerve terminals come into close contact with endocrine cells in the zona fasciculata (Dorovini-Zis & Zis, 1991) and seem likely to be cholinergic (Charlton et al. 1991).

### *Physiological evidence of neural control of glucocorticoid secretion*

Stimulation of the peripheral end of the splanchnic nerve in the conscious, functionally hypophysectomised, calf at a moderate frequency (4 Hz) produces no significant increase in adrenal glucocorticoid output (Edwards & Jones, 1987*b*) (Fig. 4), in the absence of exogenous ACTH, although it produces the expected abrupt increase in the adrenal output of catecholamines and enkephalins. In contrast, splanchnic nerve stimulation at the same frequency, under precisely similar conditions, except that the animals were receiving an i.v. infusion of $ACTH_{1-24}$, resulted in a substantial increase in adrenal cortisol output, without affecting the concentration of ACTH in the arterial plasma (Edwards & Jones, 1987*a*). In these experiments the dose of exogenous ACTH raised cortisol output by about 50% of maximal (500 ng $min^{-1}$ $kg^{-1}$) and this was roughly doubled (increasing by a further 400 ng $min^{-1}$ $kg^{-1}$) during splanchnic nerve stimulation (Fig. 4). This finding has since been confirmed in hypophysectomised dogs, in which splanchnic nerve stimulation was shown to increase the conversion of 11-deoxycortisol to cortisol, suggesting a change in 11$\beta$-hydroxylase activity (Engeland & Gann, 1989) by a mechanism which has yet to be established.

Splanchnic nerve stimulation in the conscious, functionally hypophysectomised calf model also causes an increase in adrenal bloodflow. Accordingly, it might be supposed that the rise in cortisol output, in the presence of exogenous ACTH, was secondary to a rise in the rate at which ACTH was presented to the gland, even though there was no change in the concentration of the peptide in the arterial plasma, as Urquhart (1965) originally suggested. However, if it is assumed that changes in total adrenal bloodflow represent a reasonably accurate index of changes in cortical bloodflow, it is a simple matter to estimate ACTH presentation rate during these experiments. It can then be seen that there is a closely linear relation between adrenal ACTH presentation rate and adrenal cortisol output for each of the samples collected before and after splanchnic nerve stimulation. In contrast, cortisol output during splanchnic nerve stimulation was significantly greater than that which could be accounted for by the existing ACTH presentation rate (Fig. 5). The assumption that the

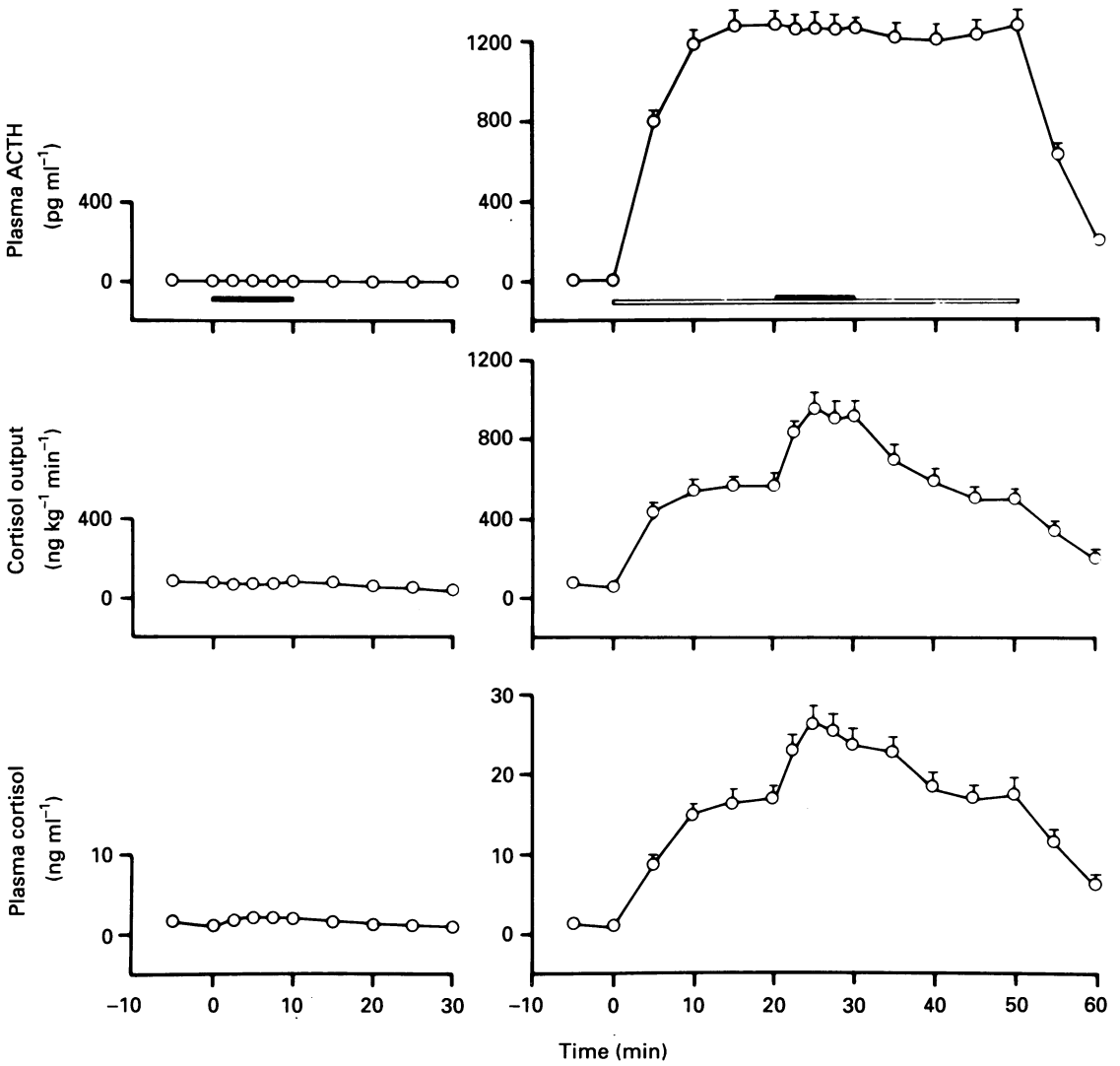


Fig. 4. Comparison of the changes in mean arterial plasma ACTH and cortisol concentration, and right adrenal cortisol output, in response to stimulation of the peripheral end of the right splanchnic nerve (4 Hz for 10 min) in 11 conscious functionally hypophysectomised calves in the presence and absence of exogenous $ACTH_{1-24}$ (5 ng $min^{-1}$ $kg^{-1}$). Open horizontal bar, duration of ACTH infusion. Filled horizontal bars: duration of splanchnic nerve stimulation. Vertical bars, S.E. of each mean value where these exceed the size of the symbol. (From Edwards & Jones 1987*a*, by permission of the Physiological Society.)

total adrenal bloodflow represents a reliable index of cortical bloodflow is supported by the finding that medullary bloodflow amounts to a mere 7% of the total (Sparrow & Coupland, 1987), at least in the rat. In the dog, Breslow and colleagues (Breslow et al. 1987) have shown that the rise in adrenal bloodflow which occurs during haemorrhage, and is mediated via the innervation to the gland, is largely confined to the medulla. Accordingly, it is even less plausible that the rise in adrenal cortisol output which occurs during splanchnic nerve stimulation, in the presence of exogenous ACTH, could be due solely to an increase in ACTH presentation. This is not to say that an increase in ACTH presentation, secondary to an increase in cortical bloodflow, could not have contributed to the increase in adrenal cortisol output. Changes in ACTH presentation in this particular model quite clearly result in appropriate changes in cortisol output as evidenced by the fall in adrenal cortisol which occurs when adrenal bloodflow is deliberately reduced, as with intra-aortic infusions of endothelin (Bloom et al. 1990). The conclusion is simply that the increase in cortisol output which occurs in response to splanchnic nerve stimulation, in the presence of exogenous ACTH, in the conscious functionally hypophysectomised calf cannot be accounted for by that mechanism.

### *Cortical responses to peptidergic agonists*

*Vasoactive intestinal peptide (VIP).* VIP-containing neurons are known to be present in the adrenal

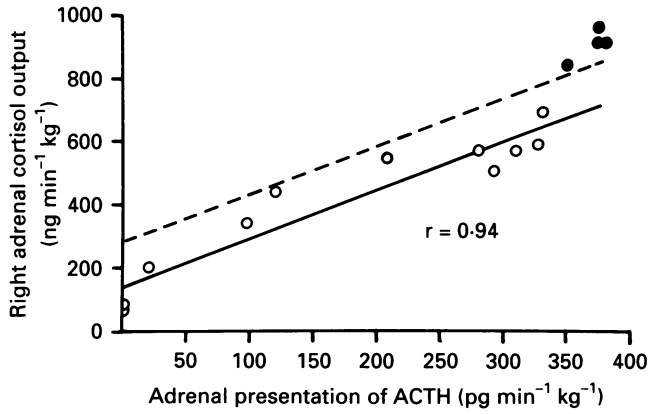


Fig. 5. Relation between mean right adrenal presentation rate of ACTH and mean right adrenal cortisol output in 11 conscious, functionally hypophysectomised, calves in which the peripheral end of the right splanchnic nerve was stimulated (4 Hz for 10 min) during an infusion of exogenous $ACTH_{1-24}$ (5 ng min$^{-1}$ kg$^{-1}$). ○, before and after splanchnic nerve stimulation. ●, during stimulation. Regression line calculated by the method of least squares applied to the values obtained before and after stimulation. Hatched line, regression line + 2 S.D. (From Edwards & Jones 1989*b*, by permission of International Universities Press.)

cortex (Hökfelt et al. 1981; Holzwarth, 1984; Cheung & Holzwarth, 1986; Cunningham & Holzwarth, 1989) and VIP has been shown to stimulate steroid secretion by adrenal cortical cells, albeit at a higher concentration than ACTH (Kowal et al. 1977; Morera et al. 1979; Leboulanger et al. 1983). Furthermore VIP is released from the gland in response to stimulation of the peripheral end of the splanchnic nerve in the conscious calf (Bloom et al. 1988). Accordingly, the possibility that the potentiation of the steroidogenic response to ACTH, which occurs during splanchnic nerve stimulation, might be mimicked by intra-aortic infusions of this peptide was investigated in the conscious hypophysectomised calf model. This involved monitoring the adrenal cortical responses to intra-aortic infusions of VIP at a dose of 50 pmol min$^{-1}$ kg$^{-1}$ in the presence and absence of exogenous ACTH. As with splanchnic nerve stimulation, VIP had no discernible effect on cortisol output in the absence of ACTH but produced quite a substantial rise in its presence, without affecting the concentration of ACTH in the arterial plasma (Bloom et al. 1987). The output of cortisol from the gland was consistently greater than that which could be accounted for by the presentation rate of ACTH alone throughout the VIP infusion. Furthermore, VIP faithfully mimicked the effect of splanchnic nerve stimulation on the adrenal vasculature causing an increase in adrenal bloodflow in the absence, but not in the presence, of exogenous ACTH. It was therefore concluded that release of this peptide from splanchnic nerve terminals in the adrenal cortex most probably accounts, at least in part, for the powerful adrenal cortical steroidogenic response to splanchnic nerve stimulation that occurs in the presence of submaximal doses of ACTH.

These results have recently been confirmed by Holst and his colleagues in the isolated, innervated, perfused adrenal gland of the pig (Erhardt-Bornstein et al. 1991), except that both VIP and splanchnic nerve stimulation were found to exert a steroidogenic action in both the presence and the absence of ACTH. This might be due to the fact that they employed higher stimulus frequencies and larger doses of VIP, possibly outside the physiological range. The amount of VIP it is practicable to infuse intra-aortically is limited by its hypotensive action in the conscious calf model whereas no such restraint is necessary in the perfused gland preparation.

*Calcitonin gene-related peptide.* CGRP is also present in the adrenal gland (Kuramoto et al. 1987; Kong et al. 1989) and was tested in functionally hypophysectomised calves in the same way as VIP (Bloom et al. 1989). The peptide was infused into the aorta above the origin of the adrenal arterial supply at a dose of 33 pmol min$^{-1}$ kg$^{-1}$ and, like VIP, caused a pronounced increase in adrenal blood flow in the absence of exogenous ACTH; this effect was reduced by not abolished in the presence of ACTH. Unlike VIP, CGRP stimulated the release of cortisol from the adrenal gland of these animals in the absence of exogenous ACTH. This effect was reduced during an i.v. infusion of exogenous ACTH at a low dose (2 ng min$^{-1}$ kg$^{-1}$) and completely suppressed by ACTH at a higher dose (5 ng min$^{-1}$ kg$^{-1}$). It exerted a direct steroidogenic action on the gland which in no way resembled the response to splanchnic nerve stimulation. CGRP has also been found to stimulate secretion of cortisol in the isolated, perfused in situ rat adrenal preparation (Hinson & Vinson, 1990). These authors attributed this latter effect to an increase in

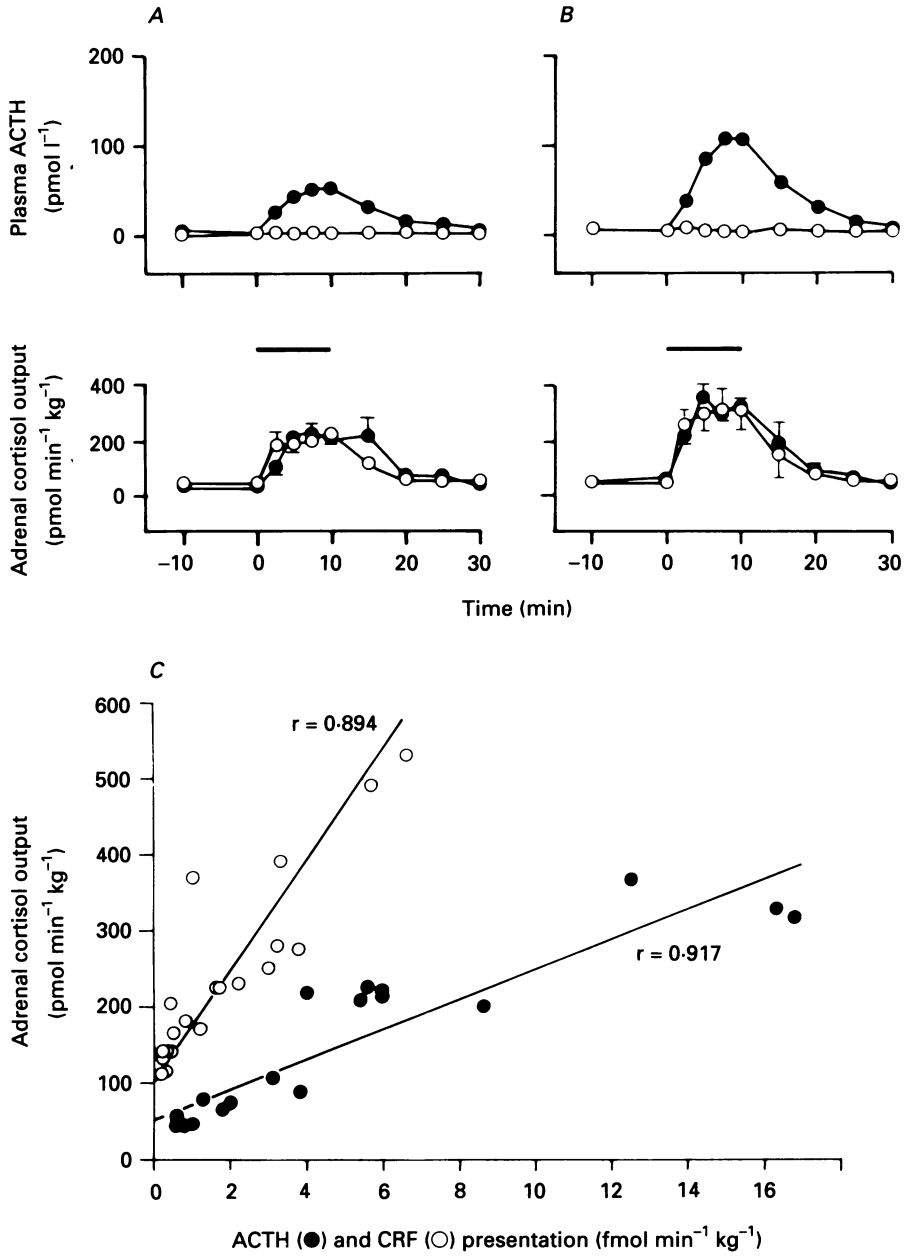


Fig. 6. (A) Comparison of the changes in mean plasma ACTH concentration and mean right adrenal cortisol output in conscious functionally hypophysectomised calves in response to intra-aortic infusion of CRF (○; $n = 5$) or $ACTH^{1-24}$ (●; $n = 5$) at 4 pmol $min^{-1}$ $kg^{-1}$; (B) 8 pmol $min^{-1}$ $kg^{-1}$. Vertical bars, S.E. of each mean value where these exceed the size of the symbol. Horizontal bars, duration of infusions. (C) Relation between mean estimated CRF (○) and ACTH (●) presentation rate to the right adrenal gland and mean right adrenal cortisol output in conscious functionally hypophysectomised calves in response to intra-aortic infusions of CRF and ACTH as in A and B. Regression lines calculated by the method of least squares applied to all mean values. (Modified from Jones & Edwards 1990*a*, by permission of the Physiological Society.)

adrenal bloodflow but it has yet to be established that an increase in bloodflow can cause an increase in cortisol output in vivo, above and beyond that attributable to the increase in ACTH presentation.

*Corticotrophin releasing factor (CRF).* Since CRF is also released from the adrenal gland in response to splanchnic nerve stimulation (see above) and it was suggested that it might have a direct effect on adrenal steroidogenesis, at least in the case of aldosterone production in the zona glomerulosa of the rat (Mazzochi et al. 1989), the possibility arose that this peptide might also be implicated in the steroidogenic response to splanchnic nerve stimulation. This was tested by infusing it intra-aortically in the functionally hypophysectomised calf model, as described for VIP and CGRP except that much lower doses were employed. CRF caused an increase in the output of cortisol from the adrenal gland, which was dose-related over the range 1.3–2.6 ng $min^{-1}$ $kg^{-1}$ and maximal at the higher of these doses (Jones &

Edwards, 1990*a*) (Fig. 6). This response was observed at a dose below that which caused a significant fall in adrenal vascular resistance (5.2 ng $min^{-1}$ $kg^{-1}$). Cortisol output was also linearly related to the rate at which CRF was presented to the gland during these infusions. In addition, infusions of CRF produced the release of small but readily detectable amounts of ACTH-like peptides from the gland, mainly in the form of $ACTH^{1-39}$ but with some proopiomelanocortin being released. These findings have recently been confirmed in the rat by Andreis et al. (1991), who further suggested that the hypothalamopituitary CRF/ACTH system may be replicated in the adrenal gland and operate to control glucocorticoid secretion.

In the functionally hypophysectomised calf model this would appear to be unlikely as roughly equimolar amounts of CRF and ACTH are released from the gland in response to splanchnic nerve stimulation (about 10 fmol $min^{-1}$ $kg^{-1}$ during splanchnic nerve stimulation at 40 Hz for 1 s at 10 s intervals; Edwards & Jones, 1988; Jones & Edwards, 1990*a*). This is more suggestive of corelease than of a cascade phenomenon. Furthermore, CRF has significantly greater steroidogenic potency than ACTH (Jones & Edwards, 1990*b*) (Fig. 6). It is difficult to visualise a biological justification for any hypothetical causal relation between the release of the 2 peptides in view of the fact that they are released in about the same amounts and that CRF is actually the more potent agonist. If both peptides are released together, either from chromaffin cells or nerve terminals within the adrenal medulla, there is reason to doubt that they could reach the adrenal cortex before entering the peripheral circulation; the cortex and medulla are now thought to be supplied by largely separate capillary beds, at least in the rat (Sparrow & Coupland, 1987) and, to the extent that it is not, flows from cortex to medulla (Vinson et al. 1985). Both peptides exert other actions within the gland, such as vasodilatation, and it is entirely possible that, when released within the gland, they normally act to modulate the bloodflow without affecting steroidogenesis at all.

It was also concluded that it was unlikely that CRF, released in response to splanchnic nerve stimulation, contributes to the potentiation of ACTH-induced steroidogenesis which then occurs. Splanchnic nerve stimulation exerts a potent steroidogenic effect in the presence of exogenous ACTH but has no detectable effect in the absence of ACTH (Fig. 4) whereas CRF clearly does (Fig. 6). Furthermore, the release of CRF from the adrenal gland which occurs in response to splanchnic nerve stimulation is significantly reduced during infusions of exogenous ACTH in these animals (Edwards & Jones, 1988). This conclusion has been corroborated more recently by the finding that a dose of a specific CRF-antagonist, which completely blocks the adrenal steroidogenic response to intra-aortic infusions of CRF, in the presence of exogenous ACTH, in functionally hypophysectomised calves, has absolutely no effect on the steroidogenic response to splanchnic nerve stimulation in calves given i.v. infusions of exogenous ACTH (Jones & Edwards, 1992*b*).

### *Cortical responses to nonpeptidergic agonists*

*Acetylcholine.* Acetylcholine was first shown to stimulate steroidogenesis by a direct action on adrenal cortical cells in isolated perfused calf adrenal glands by Rosenfeld (1955). This has since been confirmed in isolated bovine adrenal cortical cells, in which the effect was found to depend on activation of muscarinic receptors (Hadjian et al. 1982), the adrenal gland of the frog (Benyamina et al. 1987) and rat (Porter et al. 1988). The presence of cholinergic fibres in the cortex has been reported in a number of species (see Robinson et al. 1977, for references); in the human adrenal, acetylcholinesterase positive nerve plexuses traverse the zona fasciculata in radial trunks and ramify throughout the cortical parenchyma (Charlton et al. 1991).

In the functionally hypophysectomised calf model intra-aortic infusions of a low dose of acetylcholine (4.5 nmol $min^{-1}$ $kg^{-1}$ for 10 min) were tested in the presence and absence of exogenous ACTH (2 ng $min^{-1}$ $kg^{-1}$, i.v.). Very much like splanchnic nerve stimulation ACh elicited a significant increase in adrenal cortisol output, associated with an increase in peripheral plasma cortisol concentration, in the presence of ACTH, but not otherwise. However, unlike splanchnic nerve stimulation, the increase in cortisol output did not exceed that which would be predicted from the increase in ACTH presentation which occurred, secondary to acetylcholine-induced adrenal vasodilatation. However, when the study was extended to investigate the responses of animals pretreated with hexamethonium a direct steroidogenic response was revealed (Jones & Edwards, 1992*a*). In the absence of hexamethonium this effect is obscured by the greater change in ACTH presentation rate which occurs at the higher perfusion pressure which then obtains.

*Catecholamines.* Several groups have reported the fact that catecholamines are capable of exerting a steroidogenic effect on adrenocortical cells in vitro, leading to aldosterone production by cells of zona

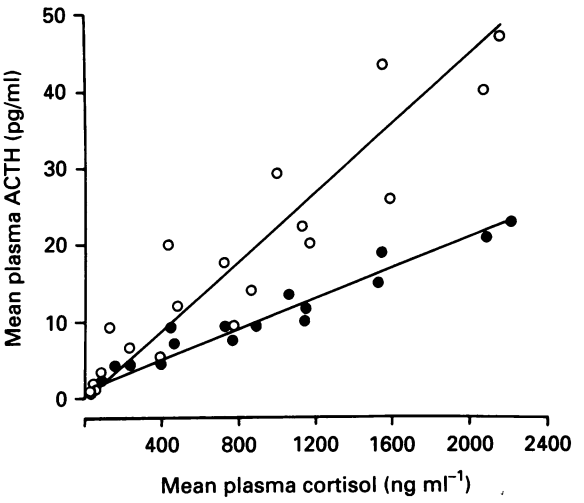


Fig. 7. Relations between mean plasma ACTH and cortisol concentration in conscious 2–6 wk calves given i.v. infusion of ACTH at a dose of either 5 or 10 ng min$^{-1}$ kg$^{-1}$. ○, Control animals ($n = 7$, $r = 0.93$). ●, Cut splanchnic nerves ($n = 7$, $r = 0.98$. Regression lines calculated by the method of least squares. (From Edwards & Jones 1987*b* by permission of the Physiological Society.)

glomerulosa origin (DeLéan et al. 1984; Pratt et al. 1985) and cortisol production by cells derived from the zona reticularis (Water et al. 1988). In each case the effect has been found to be mediated by β-adrenoceptors and, for glucocorticoid production, by receptors of the $\beta_1$ subclass (Lightly et al. 1990). Activation of β-adrenoceptors apparently stimulates cleavage of the side chain of cholesterol, by promoting accumulation of mRNA for the appropriate cytochrome (Erhardt-Bornstein et al. 1991), which happens to be the rate-limiting step in steroid synthesis (Stone & Hechter, 1954). Holst and colleagues have recently confirmed the finding that catecholamines stimulate the secretion of both aldosterone and cortisol in the intact gland, employing the isolated, perfused adrenal gland of the pig (Bornstein et al. 1990). The existence of catecholaminergic neurons in the cortex (Robinson et al. 1977; Kleitman & Holzwarth, 1985*b*), together with reports of chromaffin cells scattered within the cortex and in close association with cortical cells (Gallo-Payet et al. 1987; Bornstein et al. 1990, 1991) rather suggests that catecholamines act via neural or paracrine pathways within the gland (Bornstein et al. 1990; Hinson, 1990).

*Physiological significance*

The evidence reviewed above shows that stimulation of the splanchnic sympathetic innervation, at a frequency likely to fall within the physiological range, substantially potentiates the secretion of the adrenal cortex in response to ACTH in normal conscious animals, without detectable steroidogenic effect in the absence of ACTH. The question remains as to whether control of adrenal steroidogenesis via the sympathetic innervation is important under resting conditions in normal animals. It was addressed by comparing the steroidogenic responses of normal calves and lambs to ACTH with those of animals in which the splanchnic nerves, and consequently the sympathetic innervation to the adrenal gland, had been cut at least 7 d previously. In both species prior adrenal denervation significantly reduced adrenal steroidogenesis, assessed by the rise in arterial plasma cortisol concentration, in response to ACTH (Edwards et al. 1986; Edwards & Jones, 1987*a*). Mean plasma ACTH was linearly related to mean plasma cortisol concentration throughout each experiment and the slopes of the regression lines therefore provide an index of cortical sensitivity. The data from the calves are illustrated in Figure 7 which shows that adrenal cortical sensitivity to ACTH was reduced about twofold.

*Adrenal bloodflow*

The significance of changes in adrenal bloodflow which are associated with changes in adrenal cortical or medullary activity is controversial. For the medulla, Breslow and colleagues have shown that increased activity in response to hypoxia, haemorrhagic hypotension and splanchnic nerve stimulation, in anaesthetised dogs, is associated with 3–4 fold rises in bloodflow, which are restricted to the medulla (Breslow et al. 1987, 1989). However, prevention of the vascular response, by reducing the perfusion pressure, did not significantly influence the output of adrenaline or noradrenaline in response to supramaximal splanchnic nerve stimulation for a short period (20 Hz for 5 min) (Breslow et al. 1991*a*). This finding is in accord with expectation, in view of the large amounts of catecholamine stored in the chromaffin cells and the importance of this release mechanism during life-threatening episodes of hypotension. The further observation that glucocorticoid output was unaffected by reducing cortical bloodflow in these animals has been criticised on the grounds that cortical responsiveness could have been blunted by pretreatment with dexamethasone for 48 h; also that cortisol output was so low to start with that a further reduction might be below detection limits (Engeland, 1991).

The suggestion that adrenal cortical blood flow contributes to the control of glucocorticoid secretion dates from the mid 1960s when corticosterone output

in anaesthetised hypophysectomised rats, given a steady infusion of exogenous ACTH, rose with (whole gland) bloodflow for 30–40 min until a steady state was established (Porter & Klaiber, 1965). At about the same time the estimated rate of adrenal presentation of ACTH was found to correlate better with steady-state cortisol secretion than the concentration of ACTH in blood perfusing canine adrenal in situ (Urquhart, 1965). A little later it was reported that changes in adrenal bloodflow in conscious dogs pretreated with dexamethasone correlated well with adrenal cortisol output, so long as an exogenous ACTH infusion was below maximal levels (L'Age et al. 1970). Unfortunately this study can be criticised on the grounds that dexamethasone suppression of endogenous ACTH release is not absolute and the agents employed to vary adrenal bloodflow (histamine and methacholine) could well have had direct effects on the adrenal cortical cells.

Adrenal cortical bloodflow is inexorably linked to glucocorticoid secretion by virtue of the fact that ACTH exerts a specific vasodilator effect on the adrenal vasculature (above a certain concentration) which has been demonstrated in anaesthetised calves (Balfour, 1953), dogs (Hartman et al. 1955; Stark et al. 1965), rats (Holzbauer & Vogt, 1957; Sapirstein & Goldman, 1959) and human patients (Grant et al. 1957), conscious sheep (Wright, 1963), dogs (Stark et al. 1965; L'Age et al. 1970) and calves (Edwards et al. 1974, 1975) and the isolated perfused adrenal gland of the rat (Hinson et al. 1986). The vasodilatation is not secondary to increased steroidogenesis as it persists in the presence of cycloheximide at a dose which completely blocks steroidogenesis (Maier & Staehlin, 1968; Edwards et al. 1975) and the suggestion that it might be due to production of prostaglandins (Maier & Staehlin, 1968) has not been vindicated by subsequent studies. ACTH is thought to act mainly on capsular and subcapsular arterioles (Vinson et al. 1985), around which mast cells are congregated (Hinson et al. 1991*a*). Recent evidence, obtained in the in situ isolated perfused rat adrenal gland shows that degranulation of these cells with compound 48/80 mimics the vascular response to ACTH, while sodium cromoglycate, which prevents mast cell degranulation, inhibits adrenal vasodilatation in response to ACTH (Hinson et al. 1989, 1991*b*). This strongly suggests that mast cells are implicated in the adrenal vasodilator response to ACTH but this has yet to be confirmed in a whole animal model.

To the extent that adrenal vasodilatation increases the rate of ACTH presentation to adrenal cortical cells, it could be expected to lead to an increase in corticosteroidogenesis, although doubts have recently been expressed as to whether increased binding of ACTH to its receptor(s) can be achieved without increasing ACTH concentration (Breslow et al. 1991*b*). Of course it would be the concentration of ACTH in the adrenal extracellular fluid which would be determinative and this could well rise with an increase in plasma flow without any change in plasma ACTH concentration, depending upon the kinetics. The consensus is certainly that glucocorticoid output varies, up to a maximum, with ACTH presentation and the close linearity of the relations between these 2 variables illustrated in Figures 5 and 6B testifies to this. The question has been addressed directly in the conscious hypophysectomised calf model, in which the potent vasoconstrictor agonist endothelin was employed to vary adrenal blood flow during the course of ongoing i.v. infusions of exogenous ACTH (Jones et al. 1990). Under these conditions endothelin produced a fall in adrenal cortisol output which correlated precisely with that predicted from the fall in ACTH presentation. The possibility that this might be attributable to some other, nonvascular, action of endothelin was eliminated by adjusting the dose of exogenous ACTH during the infusion of endothelin, so as just to compensate for the fall in adrenal plasma flow and maintain a constant ACTH presentation rate. Cortisol output could still be related to ACTH presentation with precision. It is also important to note that these infusions of endothelin, which raised the concentration in the plasma by no more than 10 pmol $l^{-1}$, had no detectable effect of the output of catecholamines, enkephalins, aldosterone or CRF from the gland.

The adrenal vasculature is less sensitive to ACTH than the cells of the zona fasciculata and it is generally agreed that corticosteroidogenic responses occur at ACTH levels below those necessary to produce vasodilatation in the gland (Wright, 1963; L'Age et al. 1970; Edwards et al. 1975). Just recently, Breslow and colleagues have reported that in dexamethasone-pretreated, pentobarbital-fentanyl-anaesthetised ventilated dogs increased adrenal cortisol output, in response to exogenous ACTH, was associated with a significant increase in oxygen extraction from the blood with no significant increase in flow (Sakima et al. 1991). Our contention is merely that, when the flow does increase, it will necessarily produce an increase in glucocorticoid output by increasing ACTH presentation.

In the in situ isolated perfused rat adrenal gland it is reported that increases in flow which are not associated with any increase in ACTH presentation

also lead to increased corticosteroidogenesis (Hinson et al. 1989). It has further been suggested that the effect is mediated by release of endothelins within the gland since ACTH stimulates the release of detectable (statistically significant) amounts of endothelin from this preparation (Hinson et al. 1991 *c*) and endothelins elicit a dose-dependent and highly sensitive release of both glucocorticoids and aldosterone from dispersed preparations of adrenal cortical cells (Hinson et al. 1991 *a*, *c*). One difficulty in interpreting these data arises from the fact that the increase in steroidogenesis in the in situ perfused rat adrenal preparation in response to an increase in flow (with no increase in ACTH presentation) is limited to the glucocorticoids, whereas an increase in association with aldosterone production would be expected. Also it does not appear to apply in the conscious hypophysectomised calf model. This might be due to a species difference as it has yet to be established whether or not endothelins are released from the bovine adrenal gland in response to ACTH.

## CONCLUSIONS

Studies in conscious calves have revealed the fact that medullary responses to stimulation of the splanchnic sympathetic innervation are substantially potentiated by employing an intermittent pattern of stimulation. It has yet to be established what mechanisms underlie this phenomenon. It might be due to enhanced synaptic transmission due to increased mobilisation of $Ca^{2+}$, or of some other intracellular messenger. Alternatively, the probability of vesicle release might be increased by modulation of $K^+$ efflux and $Ca^{2+}$ influx across the chromaffin cell membrane, as apparently occurs in postganglionic sympathetic nerve terminals supplying the tail artery of the rat and determines the probability of vesicle release therefrom (Stjärne et al. 1991). Yet another possibility is suggested by the finding by Ip & Zigmond (1984) that, in the superior cervical ganglion of the rat, acute activation of tyrosine hydroxylase (which catalyses the rate-limiting step in noradrenaline synthesis) is mediated in part by acetylcholine, but also in part by a noncholinergic agonist. Release of the noncholinergic transmitter is potentiated by high frequency stimulation. In the bovine adrenal medulla, VIP is released in response to splanchnic nerve stimulation. The amounts appearing in the venous effluent plasma were too small to indicate whether release was potentiated by high-frequency stimulation, but this has been clearly established in other tissues such as the submandibular gland of the cat (Andersson et al. 1982). Furthermore the peptide has been shown to release adrenal catecholamines when infused in small amounts intra-aortically (Edwards & Jones 1993).

Secretion of cortisol from the zona fasciculata in response to ACTH is potentiated by stimulation of the splanchnic sympathetic innervation. It has yet to be established whether the effect can be potentiated by stimulating in bursts, but it occurs at a frequency (4 Hz) likely to fall within the physiological range. The contention that the effect is operative under normal resting conditions has been confirmed by the finding that the sensitivity of the adrenal cortex to ACTH is substantially reduced, within 7 d, following adrenal denervation. To the extent that activity of the innervation produces adrenal cortical vasodilatation it is likely to contribute to enhancement of corticosteroidogenesis by increasing the rate at which ACTH is presented to the cortical cells. It also appears to be due, at least in part, to VIP and possibly to acetylcholine or catecholamines. It does not appear to be due to release of CGRP, enkephalins or CRF.

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