ATRIAL NATRIURETIC PEPTIDE IS A PHYSIOLOGICAL INHIBITOR OF ACTH RELEASE: EVIDENCE FROM IMMUNONEUTRALIZATION *IN VIVO*

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ABSTRACT

The brain is thought to exert a predominantly stimulatory action on ACTH secretion mediated mainly by corticotrophin-releasing factor-41 (CRF-41) and arginine vasopressin (AVP). Several data, however, also point to the existence of an ACTH-inhibiting factor. Atrial natriuretic peptide (ANP), at concentrations found in hypophysial portal blood, inhibits ACTH release *in vitro*. The aim of the present studies was to use ANP immunoneutralization to determine whether ANP does in fact inhibit ACTH release *in vivo*. Intracerebroventricular infusion (l μl/min for 30 min) of sheep anti-ANP serum into male rats anaesthetized with sodium pentobarbitone had no significant effect on jugular venous plasma concentrations of ACTH or LH but did decrease significantly the plasma concentrations of prolactin. Intravenous infusion of 0.8 ml sheep anti-ANP serum but not control (non-immune) sheep serum, through an indwelling intra-atrial cannula in conscious male rats resulted in a marked and significant increase in plasma ACTH and corticosterone concentrations. The ACTH and corticosterone response to a 30-s ether stress was not significantly potentiated in the same conscious rats infused with anti-ANP serum. Intra-atrial infusion of anti-ANP did not significantly affect plasma prolactin, LH, glucose or sodium concentrations or plasma osmolality. These results show for the first time that ANP is a potent inhibitor of ACTH secretion in the conscious male rat and that, therefore, ANP is a hypothalamic neurohormone which is likely to play an important inhibitory role in the neural control of ACTH release.

INTRODUCTION

Adrenocorticotrophin (ACTH) secretion is stimulated by corticotrophin-releasing factor-41 (CRF-41), arginine vasopressin (AVP) and, under some circumstances, oxytocin (Antoni, 1986; Rivier & Plotsky, 1986; Fink, Robinson & Tannahill, 1988; Sheward & Fink, 1991; Tannahill, Sheward, Robinson & Fink, 1991). However, the increased secretion of ACTH or corticosterone which follows cerebral decortication in the dog (Egdahl, 1961, 1962), hypothalamic deafferentation in the rat (Halász, Vernikos-Danellis & Gorski, 1967) and pituitary isolation in the sheep (Engler, Pham, Fullerton *et al.* 1988) suggests the existence also of a control mechanism which inhibits ACTH secretion. While such an inhibitory mechanism could be mediated simply by reducing the release of CRF-41 and/or AVP into hypophysial portal blood, a specific ACTH-inhibitory factor might also be involved. Atrial natriuretic peptide (ANP) is a possible candidate for the ACTH-inhibitory factor because (i) perifusion of rat anterior pituitary cells with ANP (1-28) or ANP (5-28) at 0.1 to 100 nmol/l inhibited CRF-41 and AVP-induced secretion of ACTH (Dayanithi & Antoni, 1989) and (ii) ANP concentrations in hypophysial portal blood are 1-10 nmol/l and three to four times higher than ANP concentrations in peripheral blood (Lim, Sheward, Copolov *et al.* 1990). Here we have used the technique of immunoneutralization to determine whether ANP inhibits ACTH release *in vivo*.

MATERIALS AND METHODS

All experiments were carried out on adult male Wistar Cob rats of about 250 g body weight which were either purchased from Charles River UK Ltd (Margate, Kent) or bred in the Brain Metabolism Unit. The animals were maintained under controlled lighting (lights on 0500-1900 h) and temperature (22°C) and had free access to diet 41 B (Oxoid, Basingstoke, Hants, U.K.) and tap water.

Immunoneutralization of ANP was carried out with an anti-ANP serum, raised in sheep against human ANP (S117), which cross-reacts 100% with α rat ANP (Tsunoda, Mendelsohn, Sexton *et al.* 1988). Control (non-immune) sheep serum was obtained from the Scottish Antibody Production Unit, Carluke, Lanarkshire, U.K.

Two separate experiments were carried out. In the first experiment anti-ANP or control serum was infused into the third cerebral ventricle (intracerebroventricular infusion; I.C.V.) through a cannula (no. 27 gauge) previously implanted stereotoxically under tribromoethanol anaesthesia and held in place with small metal screws and dental cement. The animals were allowed 14-21 days to recover from the implantation of the cannula during which time they were given water containing 4 g chlortetracycline/l. The animals were anaesthetized with sodium pentobarbitone (36 mg/kg i.p.: Sagatal, RMB Animal Health Ltd, Dagenham, Essex, U.K.) and blood samples (0.4 ml) were withdrawn from the external jugular vein at 45 and 1 min before and 30 and 60 min after the infusion of anti-ANP or control serum. The sera were infused at a rate of 1 μl/min for 30 min beginning immediately after the second (1 min) blood sample. In the second experiment, the sera were infused into conscious rats through an intra-atrial cannula that had been implanted at least 2 to 3 days previously (Aiyer, Fink & Greig, 1974) under halothane anaesthesia. This experiment was designed to answer two questions. First, does ANP exert an inhibitory effect on basal ACTH secretion? Secondly, does ANP moderate the ACTH response to an ether stress? Blood samples (0.3 ml) were, therefore, withdrawn 30 min before and 15, 30 and 60 min after the intravenous infusion of 0.8 ml of either anti-ANP or control serum. The animals were then exposed to ether vapour for precisely 30-s after which they were replaced in their cages and 0.3 ml blood samples were taken at 15, 30 and 60 min after the ether stress. The stress used was relatively mild in order to enable us to detect potentiation of ACTH release should it occur. A total of eight animals were used in each group. In three animals of each group a further blood sample was taken 300 min after the infusion of the serum. These

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experiments were planned so that (i) the post-stress samples were all taken after the 'basal' samples had all been taken from each animal, and (ii) the same number of control and anti-ANP-treated animals were studied on the same day and time. The experiment was carried out in the same room in the animal house in which the animals had been kept after implantation of the intra-atrial cannula because previous studies (K Brown-Grant & G Fink, unpublished) had shown that in terms of plasma corticosterone concentrations the animals remain unstressed under these conditions.

The blood samples were kept on ice for only a brief period before centrifugation, and the plasma was stored at -40°C. The plasma samples were assayed for ACTH and corticosterone as described in detail by Fink *et al.* (1988). The antiserum used in the corticosterone radioimmunoassay cross-reacts with cortisol, the major glucocorticoid in sheep plasma, at less than 0.005%. Plasma luteinizing hormone (LH) and prolactin were also determined (as described in detail by Horn & Fink, 1985) because ANP has been implicated in the central control of prolactin and LH release (Samson & Bianchi, 1987; Samson, Aguila & Bianchi, 1988) and because the measurement of other pituitary hormones would serve as a control for the specificity of any action of the anti-ANP serum on ACTH release. All samples from each of the two experiments were assayed in one assay. Plasma glucose, osmolality and sodium were also determined, since alterations in these may affect ACTH secretion. The latter determinations, carried out by the Department of Clinical Chemistry, Royal Infirmary of Edinburgh, were made by the glucose oxidase peroxidase, freezing-point depression and flame-emission photometry methods respectively. The significance of differences was determined by Mann Whitney U test or paired t-test as appropriate.

RESULTS

Intracerebroventricular Infusion Table 1 shows that the infusion of control or anti-ANP serum had no significant effect on the plasma concentrations of LH or ACTH. However, plasma prolactin concentrations were significantly reduced by anti-ANP infusion.

Intra-atrial Infusion The infusion of anti-ANP, but not control serum, produced a significant approximately two fold increase in the plasma concentrations of ACTH (Fig. 1) and corticosterone (Fig. 2). The plasma ACTH concentrations 15 min after anti-ANP serum infusion were five times greater than the concentrations in control animals (compare Fig. 1a and b) and remained significantly higher throughout the first 60 min after infusion. The mean (± S.E.M.) maximal increment in ACTH (relative to the preinfusion value) in the ANP-treated group was 99 ± 31 pmol/l at 15 min after the infusion of anti-ANP serum ($p < 0.02$). The mean maximal increment in corticosterone (relative to the preinfusion value) was 390 ± 138 nmol/l at 15 min after the infusion of anti-ANP serum ($p < 0.05$). The ether stress produced a significant ($p < 0.01$) maximal increment (346 ± 69 nmol/l) in the plasma concentration of corticosterone in the control group relative to the 60 min sample taken immediately before exposure to ether. In the anti-ANP-treated animals, the ether stress also produced a slight but insignificant increase in the plasma concentrations of ACTH and corticosterone.

The increase in plasma ACTH and corticosterone

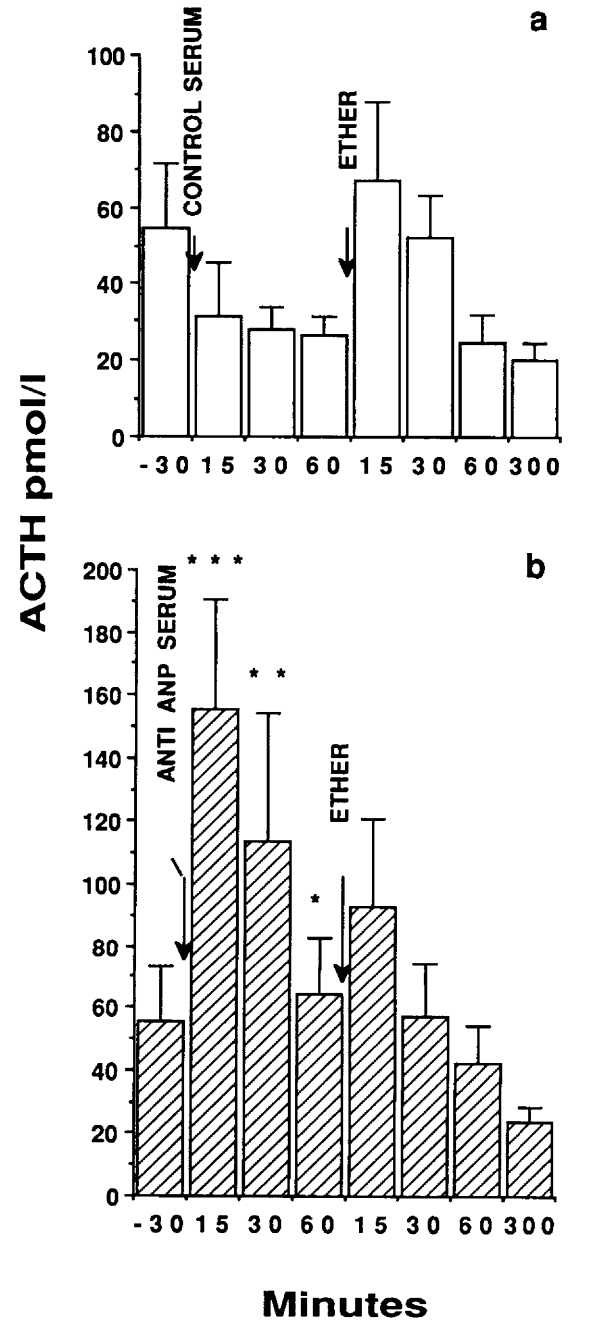

Figure 1

Mean ± S.E.M. plasma concentrations of ACTH in conscious male rats infused through an indwelling intra-atrial cannula with (*a*) control or (b) sheep anti-atrial natriuretic peptide (ANP) serum. Blood samples (0.3 ml) were withdrawn before and at intervals after the infusion of the sera and the exposure to ether for 30-s as shown (n = 8 per group). Significance of differences between (*a*) and (*b*): * $p < 0.05$; ** $p < 0.02$; *** $p < 0.002$ (Mann Whitney U test).

concentrations in the rats infused with anti-ANP or control serum could not be attributed to the ACTH or corticosterone concentrations of the infused sheep serum because the plasma concentration of ACTH in the sheep anti-ANP serum was 98 pmol/l which, assuming a highly conservative dilution of only about 30-fold in the total albumin space, could add only about 3 pmol/l to the rat plasma ACTH concentrations. Similarly, the concentration of ACTH in the infused control sheep serum, 72 pmol/l, would also not add significantly to the rat plasma ACTH values. The corticosterone concentrations of the anti-ANP serum was 94 nmol/l and could not be detected in the control serum. The differences in plasma ACTH and corticosterone concentrations could also not be attributed to differences in plasma glucose, sodium or osmolality which were similar in the two groups (8.57 ± 0.68 mmol/l, 161.7 ± 7.2 mmol/l and 277.0 ± 7.8 mOsmol/kg in control infused rats compared with 9.36 ± 0.70 mmol/l, 159.3 ± 5.2 mmol/l and 279.9 ± 9.1 mOsmol/kg in anti-ANP serum infused rats respectively). Previous studies show that intravenous infusion of anti-ANP serum increases blood pressure and cardiac output (Sasaki, Kida, Kato *et al.* 1987), but such changes would have either no effect or decrease rather than increase ACTH release. The plasma concentrations of prolactin and LH were similar in animals infused with anti-ANP compared with control serum. As expected, the ether stress resulted in increased concentrations of prolactin, from 2.3 ± 0.4 to 5.4 ± 1.2 ng and 1.8 ± 0.4 to 4.4 ± 1.9 ng NIDDK rat PRL-RP-3 in animals infused with control or anti-ANP serum respectively.

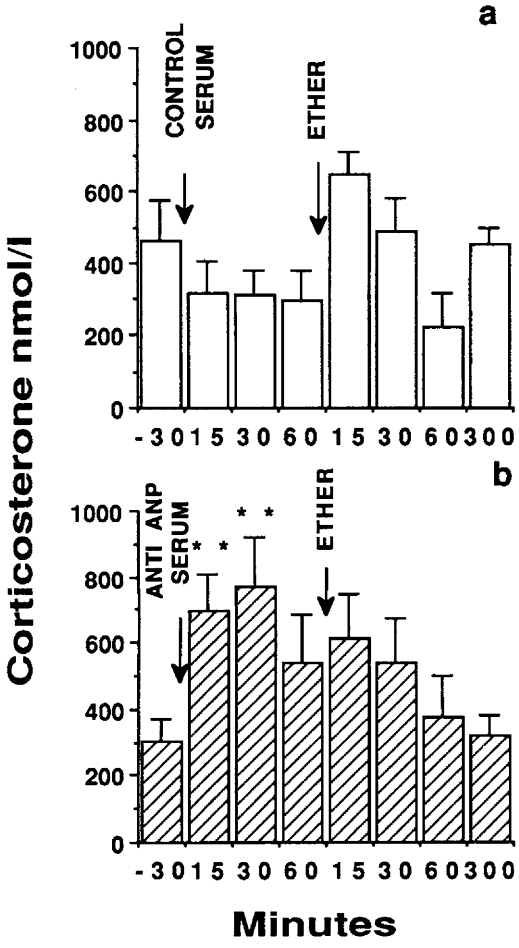

Figure 2

Mean ± SEM plasma concentrations of corticosterone in blood samples from animals in Fig. 1 infused with (*a*) control or (b) anti-ANP serum. Significance of differences between (*a*) and (*b*); ** $p < 0.02$ (Mann Whitney U test).

DISCUSSION

These results show, for the first time, that ANP exerts an inhibitory effect on ACTH release *in vivo*. Since i.c.v. infusion of the anti-ANP serum had no effect on ACTH release (Table 1), and since anti-ANP serum infused systemically is unlikely to cross the blood-brain barrier, the action of ANP is likely to be on the anterior pituitary gland. This is consonant with the finding that ANP significantly inhibits ACTH release from pituitary cells *in vitro* at concentrations which are in the range of those in hypophysial portal vessel blood (Dayanithi & Antoni, 1989; Lim, *et al.* 1990; Sheward, Lim, Alder *et al.* 1991). In both groups of animals (Figs. 1 and 2) the plasma corticosterone concentrations paralleled the plasma ACTH concentrations; this excludes the possibility that in the anti-ANP-infused rats the increase in plasma ACTH is due to possible inhibitory effects of the antiserum on corticosterone secretion which, by reducing glucocorticoid negative feedback, would result in increased ACTH secretion. Furthermore, in a separate study we found that in long-term adrenalectomized male rats anaesthetized with sodium pentobarbitone, i.v. infusion of 200 µl of anti-ANP, but not control serum, significantly increased plasma ACTH concentrations (G Fink & R C Dow, unpublished).

Immunoneutralization of ANP did not significantly potentiate the ACTH response to ether stress, as shown by the fact that at 15 min after the ether stress, the mean plasma ACTH concentration was less than the mean maximal increment of ACTH that occurred at 15 min after the infusion of anti-ANP serum (Fig. 1b). There are two possible explanations for this which require further investigation. First, ANP may not play a major role in moderating the ACTH response to a mild ether stress. Secondly, the ACTH released as a consequence of ANP immunoneutralization during the first hour reduced the responsiveness of the pituitary gland to a level at which further potentiation of the ACTH response to stress could not be elicited.

The fact that the anti-ANP serum significantly reduced prolactin secretion (Table 1) suggests that (i) the antiserum did diffuse sufficiently far in brain to exert a neuroendocrine effect, and (ii) ANP might exert a central stimulatory effect on prolactin release. Our i.c.v. data on LH and prolactin would appear to conflict with the reports that i.c.v. infusion of ANP reduced LH and prolactin secretion (Samson & Bianchi, 1987; Samson *et al.* 1988); however, in addition to other major experimental differences the latter data were obtained in long-term castrated rats.

The present data together with those of Dayanithi & Antoni (1989) and our findings of ANP release into portal vessel blood (Lim *et al.* 1990; Sheward *et al.* 1991) show that ANP must now be considered as a peptide that mediates the central nervous control of ACTH secretion.

TABLE 1

Mean (± SEM) plasma hormone concentrations before and after i.c.v. infusion of anti-atrial natriuretic peptide (ANP) or control serum at time 0 min.

Hormone	Serum Infused	Time (min) -45	-1	0	+30	+60
ACTH	Control	90 ± 10.1	61.7 ± 7.9		116.5 ± 30.6	117.6 ± 22.5
pmol/l	Anti-ANP	144.3 ± 38.3	86.6 ± 6.2		90.5 ± 17.2	85.7 ± 17.2
Prolactin	Control	8.4 ± 3.0	9.3 ± 4.0		24.1 ± 6.6	11.6 ± 3.2
(ng NIADDK Rat prolactin -RP-3/ml)	Anti-ANP	17.3 ± 11.4	3.4 ± 1.0		3.1 ± 0.6*	1.7 ± 0.2*
Luteinizing Hormone	Control	0.45 ± 0.02	0.41 ± 0.01		0.6 ± 0.06	0.43 ± 0.02
(ng NIH-LH-S18/ml)	Anti-ANP	0.45 ± 0.02	0.45 ± 0.01		0.45 ± 0.01	0.44 ± 0.02

*significantly different from control $p < 0.005$ (Mann Whitney U test). n = 10 per group.

How the three major stress neurohormones, CRF-41, AVP and ANP, all located in the paraventricular nuclei (Palkovits, Eskay & Antoni, 1987), interact to control ACTH release probably varies from one circumstance to another. Whether ANP acts simply to apply a 'steady brake' on ACTH release or whether it plays the role of a major regulatory signal in the stress response or glucocorticoid negative feedback remains to be determined. At a pituitary cellular level it would appear that ANP exerts its effects mainly through cyclic GMP (Dayanithi & Antoni, 1989) while CRF-41 activates cyclic AMP (Labrie, Gagné & Le Fèvre, 1982). This suggests that the inhibitory action of ANP on ACTH release is not due to an action on the CRF-41 cyclic AMP receptor complex. Whatever mechanism is involved, our data show importantly that as well as stimulating ACTH the central nervous system has a mechanism for inhibiting ACTH which is mediated by ANP.

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