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# BINDING AND EFFECT OF ATRIAL NATRIURETIC FACTOR ON CYCLIC GMP FORMATION AND ALPHA-MSH SECRETION OF INTERMEDIATE PITUITARY CELLS

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The present study shows for the first time that in proopiomelanocortin cells of the rat intermediate pituitary gland ANF binds to two receptor forms, with apparent molecular weights of 150K and 70K. Scatchard plots revealed specific and high affinity non-interacting sites, with a $K_D$ value of about 3 nM and a density of 7,000 sites/cell. The presence of these binding sites was further confirmed by autoradiographic studies. Activation of these receptors led to an increase in cellular content of cGMP, with half-maximal effect being elicited with about 5 nM ANF, while cAMP formation was unaltered. Alpha-MSH secretion of intermediate pituitary cells was unaffected by ANF, whether the cells were incubated in the absence or presence of corticotropin-releasing factor or bromocryptine. These data thus indicate the presence of multiple ANF receptor sites in the intermediate pituitary which are coupled to cell production of cGMP, but independent of alpha-MSH secretion.


Atrial natriuretic factors comprise a family of peptides initially isolated from the cardiac atrium and known to participate in the regulation of cardiovascular homeostasis (1). These peptides have also been detected in different brain regions, including the hypothalamus (2,3), thereby

Abbreviations: ANF, Atrial natriuretic factor; POMC, proopiomelanocortin; DMEM, Dulbecco's essential medium; CRF, corticotropin-releasing factor; SRIF, somatosatin; VIP, vasoactive intestinal peptide; ACTH, adrenocorticotropin hormone; DSS, dissuccinimidyl suberate; IBMX, 3-isobutyl-1-methyl-xanthine.

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suggesting a possible central control of pituitary function via the portal circulation. In fact, ANFs have been shown to enhance cGMP production in anterior pituitary gland (4,5) and to affect ACTH secretion (6). This seems to be consistent with the demonstration of ANF receptors in the anterior pituitary (7,8,9). Moreover ANF was also reported to stimulate cGMP formation in tumor POMC cells (AtT-20 cell line;4). The purpose of the present study was to examine non-tumoral POMC cells of the intermediate pituitary gland of the rat for both the presence and functional activity of ANF receptors.

## MATERIALS AND METHODS

Drugs.

Labeled rat($^{125}$I)-ANF (sp.act. 2200 Ci/mmol) was purchased from NEN Corp. Rat ANF (8-33), as well other peptides were from Peninsula Laboratories.

Cell cultures.

Neurointermediate pituitary glands from male Wistar rats were separated from the anterior lobe and intermediate pituitary carefully dissected from the neural lobe. Fragments of intermediate pituitary were then digested with a mixture of collagenase and trypsin as described (11). Isolated cells were purified by centrifugation (1,100 x g/10 min) over a layer of Ficoll (Histopaque-1119) and cultured for 1-3 days in DMEM containing 7.5% horse serum and 2.5% fetal calf serum.

Binding experiments.

1. A membrane fraction, obtained from an homogenate of 150 intermediate pituitary glands, was incubated at 18°C/60min with 0.25 nM rat ($^{125}$I)-ANF, with or without a 1,000-fold excess of unlabeled rANF in the presence of protease inhibitors (10). The tracer was cross-linked to its receptor with 1 mM DSS and membrane fractions were solubilized with Triton X-100 solution containing protease inhibitors. The detergent solubilized material was filtered through a column (1x50cm) of Ultrogel ACA 34 equilibrated and eluted with 50mM Tris-HCl buffer (pH 7.4), supplemented with 50mM NaCl, 5 mM $MgCl_2$, 1mM EDTA, 0.1% Triton X100 and 0.1% BSA.

2. Cells cultured in 12-well dishes were washed, equilibrated and incubated in Krebs-Ringer-Hepes buffer (pH 7.3) containing 0.2% BSA and protease inhibitors at 18°C/60min with 0.08 nM labeled ANF alone or together with increasing concentrations of unlabeled ANF(8-33) or a 1,000-fold excess of various peptides (9). After incubation, cells were washed 4-times with ice-cold buffer and dissolved in 1M NaOH for counting of radioactivity. Binding data were analysed with the aid of the "Ligand" program (Elsevier-Microsoft).

3. Autoradiographic localization of ANF binding sites in the pituitary gland was conducted essentially as previously reported (11). For light microscopic studies, radioactive tissue sections were post-fixed in 2% glutaraldehyde and coated with NTB-2 Kodak emulsion. After an exposure time of 21 days, slides were developed and silver grains were counted

in areas corresponding to 250 $\mu m^2$ and in 20 different areas on 4 tissue sections.

Cell production of cyclic nucleotides and alpha-MSH secretion.

This was performed as described (10). Briefly, cells (1 day cultures) were washed, equilibrated and incubated in DMEM containing 0.2% BSA, 0.2 mM IBMX and increasing concentrations of ANF(8-33). Tissue contents of cGMP and cAMP were extracted by sonication in 0.1 M HCl. Secretion experiments were conducted by incubating 3 day cultured cells for 3 h in the presence of increasing concentrations of ANF, alone or along with either 10 nM CRF or bromocryptine (incubation medium contained 0.1mM ascorbic acid).

Measurements of cAMP, cGMP and alpha-MSH.

Cyclic nucleotides and alpha-MSH were radioimmunoassayed as reported (12,13).

## RESULTS

Membrane fractions of intermediate pituitaries were labeled with ($^{125}$I)-ANF, which was further covalently linked to the receptor with DSS. Detergent solubilized material extracted from these membranes eluted on an Ultrogel column as 4 radioactive peaks: peak 1, which appeared in the void volume and was not competed by cold ANF, most probably corresponds to aggregates or uncompletely solubilized material; peak 4 represents unbound tracer; labelled peaks 2 and 3, in contrast, were inhibited by excess unlabeled ANF and eluted as two distinct entities with apparent molecular weights of about 150K and 70K (Fig.1).

Scatchard plot of ANF binding to cultured intermediate pituitary cells (Fig.1: inset) revealed a single type of high affinity sites, with $K_D$ and Bmax values of 3.4 ± 2.5 nM and 7,000 ± 2,500 sites/cell, respectively (mean ± SE of 3 experiments). In addition, the Hill coefficient was close to unity, indicating non-interaction between sites. Also, none of the various peptides tested, including VIP, SRIF, CRF and ACTH, were able to displace labeled ANF.

Finally, radioautographic studies also point to the presence in intermediate pituitary cells of binding sites for labeled ANF. The numbers of silver grains corresponding to total and non-specific binding (observed in the presence of $10^{-7}$ M unlabeled ANF) were counted to be 44.6 +/- 2.8 and 12.4 +/- 0.6 per 250 $\mu m^2$ (average of 20 different areas on 4 tissue sections).

The effect of ANF on cGMP and cAMP accumulation in 1 day cultured cells is shown in Fig.2. ANF produced a dose-

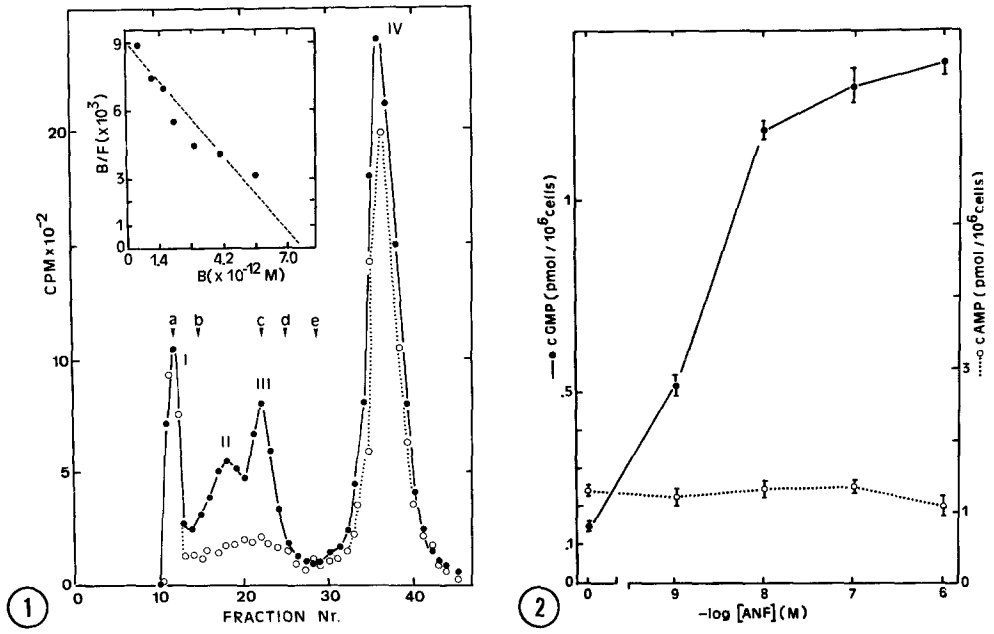


Fig.1. Elution profile of detergent extracts of intermediate pituitary membrane fractions labeled with ($^{125}$I)-ANF, in the presence (open symbols) and absence of excess cold ANF (dark symbols). Markers were: blue dextran (a); catalase (b); BSA (c); ovalbumin (d) and chymotrypsin (e). Inset: Scatchard plot of specific binding of ANF to cultured intermediate pituitary cells. B/F: ratio of bound to free ANF.

Fig.2. Effect of increasing concentrations of ANF on both cGMP and cAMP productions of 1 day cultured intermediate pituitary cells. Points are means +/- SE of 4-6 assays done in triplicate.

dependent increase in tissue content of cGMP, with the maximal effect (about 10-times the basal level) and half-maximal stimulation being elicited with about $10^{-7}$ M and $0.5x10^{-8}$ M ANF, respectively. In contrast, tissue content of cAMP remained unaffected by ANF.

Alpha-MSH secretion from cultured intermediate pituitary cells was unchanged by ANF at concentrations up to $10^{-6}$ M. Also, ANF failed to significantly alter either the stimulatory effect of CRF or the inhibitory influence of bromocryptine on peptide secretion (Fig.3A). In addition, Fig.3B shows that CRF-induced accumulation of cAMP in both cells and incubation media was also not affected by ANF.

## DISCUSSION

The present study provides evidence for the presence in POMC cells of the intermediate pituitary gland of two

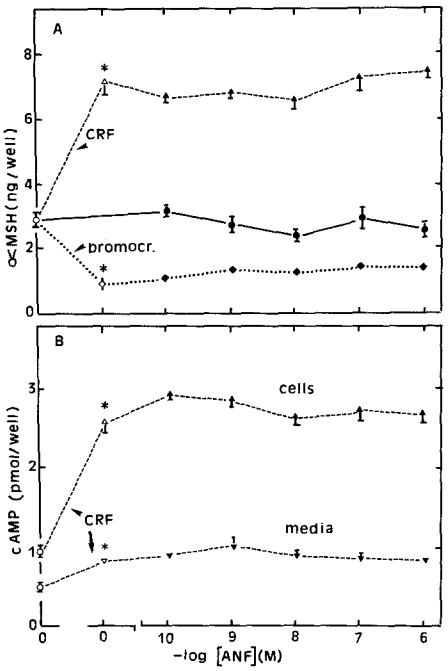


Fig.3. A: Effect of increasing concentrations of ANF on alpha-MSH secretion of 3 day cultured intermediate pituitary cells, incubated in the absence or presence of 10 nM CRF or bromocryptine. B: Stimulation of intra- and extracellular cAMP contents was unaffected by increasing concentrations of ANF. Points are means +/- SE of 6 assays done in duplicate (in some cases the error bar was comprised within the symbol's size). (+): $p<0.01$ vs.control, according to paired t-test.

receptor forms for ANF. Previous studies already demonstrated receptor heterogeneity in a number of tissues (review 14), including the anterior pituitary gland (9). The observation, however, that Scatchard plot of binding data generated a straight line, suggests that both receptor components have similar binding affinity. $K_D$ values were calculated to be in the nanomolar range and were comparable to those found in other cell types, while in contrast the density of sites was substantially lower (15-16). Binding specificity was apparent from the fact that none of the various peptides tested (VIP,CRF,SRIF,ACTH) were able to displace ($^{125}$I)-ANF from its binding sites. Moreover, the existence of ANF binding sites in intermediate pituitary cells was further confirmed by autoradiographic studies, which indicated that about 70% of total binding of labeled ANF corresponded to specific binding. These results, however, appear to be in contradiction with a similar autoradiographic study that actually failed to detect ANF binding in the intermediate

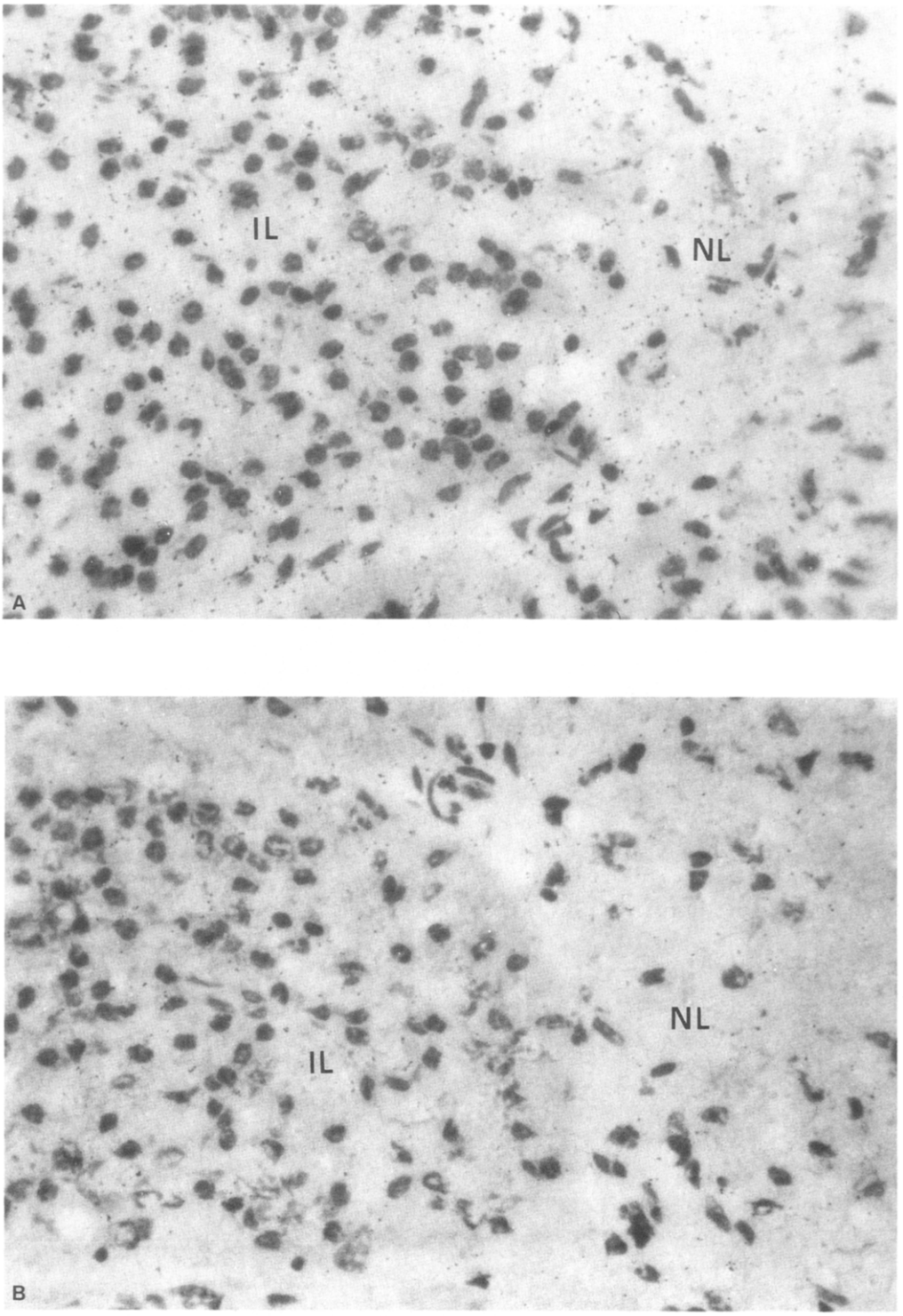


Fig.4. Autoradiographic localization of $^{125}$I-ANF binding to the intermediate lobe (IL) of the rat pituitary gland, which is boarded by the neural lobe (NL). A: total binding. B: non-specific binding (in the presence of an excess of unlabeled ANF). Magnification: 500 x.

pituitary (8). This discrepancy may be assumed to be due to differences in the techniques being used (see Fig. 4).

In cultured anterior pituitary cells, as well as in POMC tumor cells (AtT-20 cell line), ANF was reported to enhance tissue cGMP accumulation (4,5). We show here that non-tumoral POMC cells of the intermediate pituitary respond to ANF by increasing cGMP production as well, but that cAMP production was unaffected. While in cultured human ovarian cells ANF has been described to enhance both cGMP formation and progesterone secretion (17), in adrenal cells, on the contrary, ANF-induced cGMP production appeared to be associated with inhibition of aldosterone secretion stimulated by secretagogues (18). Also, in anterior pituitary cells, as well as in AtT-20 corticotrophs, ANF was reported to be without influence on either basal or stimulated ACTH secretion (4,5), thereby infirming previous evidence that seemed to favor an inhibitory role of ANF on ACTH secretion (6). Finally, both stimulatory and inhibitory effects of ANF on alpha-MSH secretion from perifused frog neurointermediate pituitary glands (19) and cultured rat intermediate pituitary cells (6), respectively, have been reported. The present study clearly indicates that in cultured rat intermediate pituitary cells ANF not only failed to alter basal secretion of alpha-MSH, but also was unable to modulate peptide secretion that was either stimulated by CRF or inhibited by a dopaminergic agonist. Consistent with these findings, ANF was found to be without influence on CRF-induced stimulation of cAMP formation as well.

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