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Release of immunoreactive atrial natriuretic factor from the isolated perfused rat lung

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The secretion of ANF by the rat lung was demonstrated in the present study. Four forms of immunoreactive ANF were detected in rat lung homogenates, including the 126 amino acid prohormone but only a low molecular mass peptide was released during lung perfusion. The released ANF inhibited forskolin-stimulated aldosterone secretion from rat zona glomerulosa cell suspensions, and this biological effect was comparable to that of the synthetic C-terminal part of the prohormone (Arg-101–Tyr-126).

Atrial natriuretic factor secretion; (Rat, Lung)

1. INTRODUCTION

Although recently discovered in mammalian cardiocytes, ANF [1,2] is not only a cardiac hormone. Its presence has been noted in various endocrine and neural rat tissues, such as different parts of the brain [3], the hypothalamus [4], the pituitary [5], ganglia of the autonomic nervous system [6] and the lungs [7,8]. In this investigation we provide direct evidence of the release of the biologically active form of ANF from isolated perfused rat lungs.

2. MATERIALS AND METHODS

Male Wistar rats (300 g) were anesthetized with an intraperitoneal injection of pentobarbital (1.6 mg/100 g body wt), after which they were killed and their lungs perfused according to the technique of Engineer et al. [9]. In brief, the heart and lungs were totally removed and rapidly perfused with oxygenated Krebs buffer via the pulmonary artery. The lungs were perfused at an initial rate of 10 ml/min and inflated several times to eliminate all traces of blood. After the cannula inside the pulmonary artery was tied, the heart was discarded. The lungs were again perfused, at a rate of 2 ml/min, and the trachea was clamped with the lungs inflated. Collection of the effluent was commenced after a 5-min stabilization period.

Three perfusates (30 ml each) were collected on ice during 15 min and immediately mixed with an equal volume of 0.1 M acetic acid containing the protease inhibitors to give a final concentration of 1 mg/ml EDTA, 10^{-5} M PMSF (phenylmethylsulfonyl fluoride), and 5 μM pepstatin-A. In another series of experiments, 2-ml aliquots of perfusate were collected separately every min on ice and mixed with an equal volume of 0.1 M acetic acid containing EDTA, PMSF and pepstatin-A in the same concentration mentioned above. Immunoreactive ANF (IR-ANF) in the perfusate was measured by specific radioimmunoassay (RIA) after extraction with Sep-Pak cartridges (Waters Associates, Milford, MA), as described [10]. The recovery of [I^{125}]ANF was 75–85%.

Three other lung perfusates (30 ml each) obtained during a 15-min perfusion period were pool-

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ed, and after Sep-Pak extraction and lyophilization, they were purified on a C_{18} μBondapak column, using an LKB system for reverse-phase HPLC (RP-HPLC). The samples were applied to the HPLC column in 0.1% trifluoroacetic acid (TFA). Elution was performed with a linear gradient of 15–45% acetonitrile in 0.1% TFA. 2-ml fractions were collected, and ANF was measured in 100-μl aliquots (in duplicate) by direct RIA [11]. Three fractions containing IR-ANF were lyophilized, and its biological activity was assessed by studying its effect on forskolin-stimulated aldosterone secretion in rat zona glomerulosa cell suspensions. After dissolving in medium, the amount of isolated ANF in the HPLC fractions of rat lung perfusates tested for biological activity was determined by RIA.

In additional experiments, the lungs of decapitated and exsanguinated rats were washed in saline and homogenized in 5 ml of 0.1 M acetic acid with proteases inhibitors. The supernatant, extracted with Sep-Pak after centrifugation at 20000 rpm, was purified by RP-HPLC under the conditions described above. The ANF content was determined in all HPLC fractions by RIA [11].

Zona glomerulosa cell suspensions of the adrenals were prepared as outlined elsewhere [12]. After the adrenals were freed from pericapsular fat, decapsulation was accomplished by bisecting the gland and gently squeezing out the inner portion. The capsular tissue was incubated in Krebs-Ringer bicarbonate buffer (pH 7.4) with 0.2% glucose (KRGB), 4% bovine serum albumin, 2 mg/ml collagenase and 0.2 mg/ml DNase in a water bath at 37°C with continuous shaking for two 10-min periods, followed by mechanical dispersion of the cells. After centrifugation and double washing with KRBG (4% BSA), the cells were re-suspended in KRBG containing 0.5% BSA, and passed through a nylon filter (pore size 50 μm). The number and viability of cells were checked under a light microscope after staining with trypan blue solution. The suspensions contained 3×10^5 cells in a volume of 1 ml incubation medium and, for each dilution of IR-ANF purified from rat lung perfusate, 4 replicates were done. Corresponding fractions of an HPLC 'blank' run were employed as controls. 100 μM forskolin (Calbiochem, La Jolla, CA) was used for the stimulation of aldosterone secretion. The cells were incubated at 37°C in water-saturated air containing 5% CO_2. At the end of the incubation period (1 h), the cells were centrifuged and the supernatant frozen at −20°C until radioimmunoassayed for aldosterone by a technique developed in our laboratory. Aldosterone was measured directly in the incubation medium without prior extraction, using antibody generously provided by Dr P. Vecsei (Dept of Pharmacology, University of Heidelberg). The 200 μl of medium diluted 1:4 (control) or 1:10 (experimental) were assayed in duplicates.

The statistical significance of the difference between forskolin-stimulated aldosterone (mean ± SE) in the absence (control) or presence of various concentrations of IR-ANF isolated from rat lung perfusate or synthetic ANF (Arg-101–Tyr-126) was analysed by Student's *t*-test for unpaired observations. For calculation of the rat lung IR-ANF molar concentration, a molecular mass of 3063 Da for the 28 amino acid peptide ANF (Ser-99–Tyr-126) was used.

3. RESULTS

Fig.1 (panel A) shows the RP-HPLC elution pattern of rat lung homogenates, which yielded four immunoreactive peaks. A similar pattern was observed when the homogenates were boiled for 5 min in 0.1 M acetic acid containing trasylol (10000 units/ml) and leupeptin (100 μM). RP-HPLC of rat lung perfusates (fig.1, panel B) revealed the presence of one immunoreactive peak which had the same elution profile as synthetic ANF (Ser-99–Tyr-126). The immunoreactive peak which eluted at a higher acetonitrile concentration had the same elution pattern as the 126 amino acid prohormone ANF (Asn-1–Tyr-126). Fig.2 illustrates time-related release of IR-ANF during perfusion. IR-ANF was determined in each fifth 2-ml sample. Its secretion was diminished with time but even after a 40-min perfusion, about 50 pg/ml was found in the perfusate. After extraction and RP-HPLC purification, a 30-min perfusion of 3 rat lungs yielded 44 ng of IR-ANF.

Fig.3 reveals that the isolated IR-ANF was biologically active. It markedly inhibited forskolin-stimulated aldosterone secretion from rat zona glomerulosa cells in a manner comparable

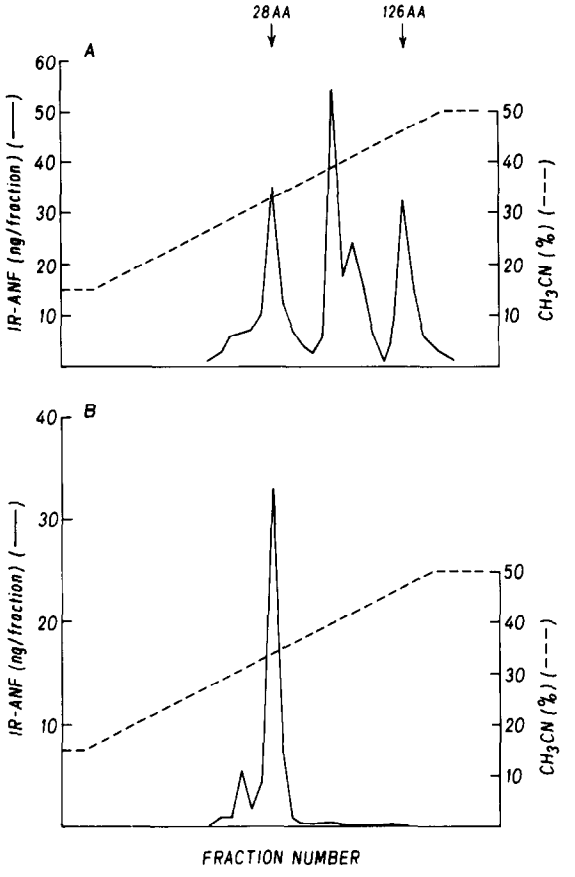

Fig.1. Reverse-phase HPLC of extracts of rat lung homogenates (A) and perfusates (B). Arrows indicate the elution position of synthetic 28 amino acid ANF (Ser-99–Tyr-126) and 126 amino acid ANF prohormone (Asn-1–Tyr-126).

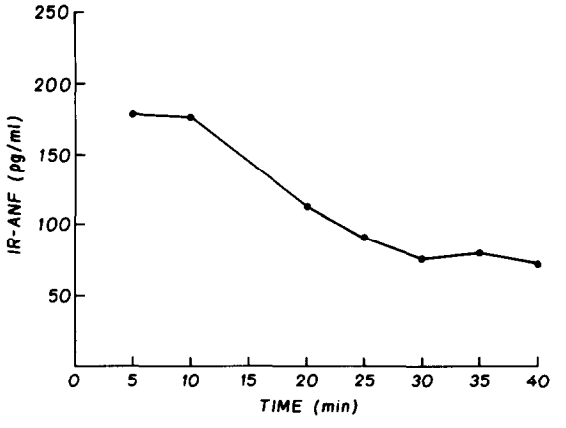

Fig.2. Time response of IR-ANF during a 40-min perfusion of isolated rat lung.

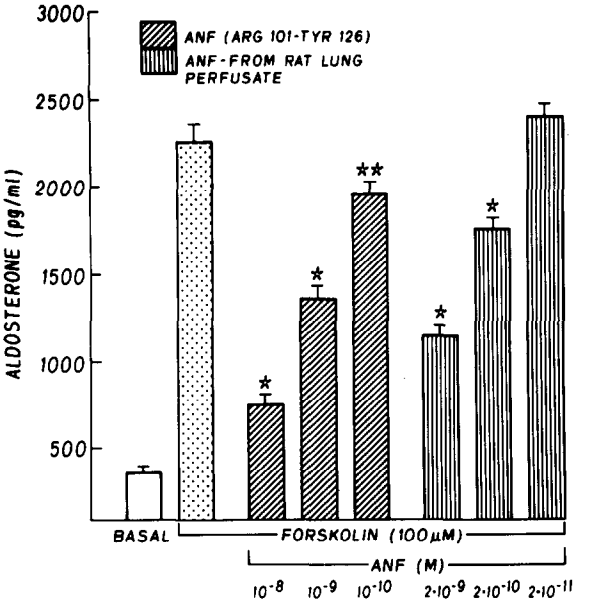

Fig.3. The effect of various concentrations of IR-ANF isolated for rat lung perfusates and synthetic ANF (Arg-101–Tyr-126) on forskolin (stippled column)-stimulated aldosterone secretion. The open column represents aldosterone under basal conditions. The bars represent the mean values ± SE in two experiments of 4 replicates each. * $p < 0.001$, ** $p < 0.05$.

to the effect of synthetic ANF (Arg-101–Tyr-126). Aldosterone secretion was similarly suppressed by 10^{-9} M synthetic ANF (Arg-101–Tyr-126) (51%) as well as by 2×10^{-9} M IR-ANF (59.9%) isolated from rat lung perfusates. No immunoreactive material was noted in corresponding fractions, of 'blank' runs on RP-HPLC, which exerted no inhibitory influence on aldosterone secretion.

4. DISCUSSION

Our results demonstrate the presence of multiple forms of ANF in rat lung extracts, including the 126 amino acid ANF prohormone (Asn-1–Tyr-126) but only a low molecular mass C-terminal peptide, most probably a 28 amino acid peptide, is secreted. However, the structure of the secreted IR-ANF has yet to be determined by amino acid analysis. Furthermore, the secreted IR-ANF was biologically active and its potency was similar to that of synthetic ANF (Arg-101–Tyr-126) [13].

There are a few possible explanations for the presence of IR-ANF in the lungs, the most likely being that it is synthesized in this organ. This hypothesis is supported by a recent demonstration of ANF messenger RNA in rat lungs [14] and by detection of the 126 amino acid prohormone (fig.1, immunoreactive peak (126 AA)) in rat lung homogenates. We have recently purified the 126 amino acid rat ANF prohormone and have compared its RP-HPLC pattern with that of IR-ANF found in peak 126 AA [15]. So far, this form of ANF has not been noted in plasma, but only in cardiocyte granules.

Since ANF receptors have been discovered in the lungs through binding studies and autoradiography [16], plasma ANF could be bound to the lung tissue and released during perfusion. However, this is unlikely as perfusion was performed with Krebs buffer (pH 7.4), in which binding between ligands and receptors is stable, and a considerable quantity of ANF is secreted (about 14.3 ng/lung).

ANF is another peptide in a large number of peptide hormones and neurotransmitters that have been discovered in normal and pathological lung tissue [17]. ANF may have a regulatory role in certain pulmonary and cardiovascular disorders, such as pulmonary edema and pulmonary hypertension, during which higher ANF plasma levels have been found [18]. The contribution of pulmonary ANF to the circulating ANF pool and its place in physiology and lung diseases remain to be explored.

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