Biochemical Pharmacology, Vol. 51, pp. 855–858, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
SSDI 0006-2952(95)02219-8

SHORT COMMUNICATION

Receptors for Atrial Natriuretic Peptide in Adrenal Chromaffin Cells

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ABSTRACT. Receptors for atrial natriuretic peptide (ANP) in isolated bovine adrenal chromaffin cells were characterized. ^{125}I-ANP specifically bound to the cells with a K_d of 103 pM and a B_{max} of 5.6 fmol/10^6 cells (16.4 fmol/mg of cell protein). C-ANF, a highly selective ligand for ANP-C receptors of natriuretic peptides, did not compete for ^{125}I-ANP binding at concentrations up to 10 nM. Chemical cross-linking of ^{125}I-ANP to the cells showed a single molecular size of the 120 kDa binding site on SDS gel electrophoresis under reducing conditions. CNP, a specific peptide for the ANP-B receptor, was much less potent than ANP in inhibiting ^{125}I-ANP binding and in displacing ^{125}I-ANP from the 120 kDa band. These results suggest that ANP specifically binds to the ANP-A receptor of 120 kDa and that there is no ANP-C receptor in bovine adrenal chromaffin cells. BIOCHEM PHARMACOL 51;6:855–858, 1996.

KEY WORDS. natriuretic peptides; receptor; binding; cross-linking; cGMP; adrenal medulla

A family of natriuretic peptides has been shown to consist of ANP†, BNP, and CNP [1–3]. Three types of receptors for these natriuretic peptides have been cloned: (1) ANPR-A containing a guanylyl cyclase domain that preferentially recognizes ANP and BNP, (2) ANPR-B containing a guanylyl cyclase domain that is fairly selectively activated by CNP, and (3) ANPR-C, which lacks a guanylyl cyclase domain that interacts with these three peptides with similar affinities [4–6]. In various tissues, ANPR-A and ANPR-B exist as a single polypeptide of 120–180 kDa, and ANPR-C is a disulfide-linked homodimer consisting of 60–70 kDa subunits [5, 7, 8]. The different distribution and density of natriuretic peptide receptor subtypes among various tissues and during development suggest that the physiological functions of these receptors are different [6, 9].

In bovine adrenal chromaffin cells, ANP [10], BNP [11] and CNP [12] are cosecreted with catecholamines following acetylcholine receptor stimulation. Of all the natriuretic peptides, ANP is the most highly concentrated in the adrenal medulla [13]. ANP stimulates the synthesis of catecholamines [14, 15] *via* the cGMP-dependent phosphorylation and activation of tyrosine hydroxylase [15], the enzyme catalyzing the rate-limiting step in catecholamine synthesis. Although the existence of ANP receptors in adrenal medulla has been reported [16], the properties of the receptors have not been fully analyzed.

To characterize ANP receptors in the adrenal medulla, we examined ^{125}I-ANP binding and the effects of natriuretic peptides on cGMP synthesis in isolated bovine adrenal chromaffin cells. The binding sites were further characterized by cross linking of ^{125}I-ANP to the receptors.

MATERIALS AND METHODS

Chemicals

Human ANP-[1–28] (ANP) and human CNP-[1–22] (CNP) were obtained from the Peptide Institute, Osaka, Japan. Des-[Gln18, Ser19, Gly20, Leu21, Gly22]rat ANP-[4–23]-NH_2 (C-ANF) was from Peninsula Laboratories, Belmont, CA. IBMX and disuccinimidyl suberate were from Sigma Chemical Co., St. Louis, MO. ^{125}I-Human ANP (2000 Ci/mmol) was from Amersham Japan, Tokyo, Japan. The cGMP radioimmunoassay kit was from Yamasa, Choshi, Japan.

Binding to Isolated Bovine Adrenal Chromaffin Cells

Bovine adrenal chromaffin cells (1×10^6) were isolated as reported previously [14, 15]. The cells were incubated with 0.01–0.5 nM of ^{125}I-ANP in 1 mL of KRP buffer (mM) (NaCl, 154; KCl, 5.6; $MgSO_4$, 1.1; $CaCl_2$, 2.2; NaH_2PO_4, 0.85; Na_2HPO_4, 2.15; glucose, 10; pH 7.4) containing 0.1% BSA in the absence or presence of the ANP analogues. The incubation was carried out in polypropylene centrifuge tubes at 4°C for 180 min. After centrifugation at 2700 g for 5 min at 4°C, the supernatant was aspirated and the tip of the tube cut for

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† *Abbreviations:* ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; C-ANF, des-[Gln^{18}, Ser^{19}, Gly^{20}, Leu^{21}, Gly^{22}]-ANF-[4-23]-NH_2; ANPR-A, ANP-A receptor; ANPR-B, ANP-B receptor; ANPR-C, ANP-C receptor; cGMP, guanosine 3′:5′-cyclic monophosphate; IBMX, isobutylmethylxanthine; and KRP, Krebs-Ringer phosphate.

Received 17 July 1995; accepted 20 October 1995.

the measurement of radioactivity by multigamma counter (Aloka, Tokyo, Japan). Specific binding was defined as total binding minus nonspecific binding, determined in the presence of 100 nM unlabeled ANP.

Affinity Cross-linking

The cells (5×10^5) were incubated with 4.2 nM ^{125}I-ANP for 180 min at 4°C in 100 μL KRP buffer in the absence or presence of 1 μM ANP, C-ANF, and CNP, and centrifuged at 2700 g for 5 min. The resultant cell pellet was suspended in 100 μL KRP buffer, and incubated with 0.2 mM disuccinimidyl suberate at 4°C for 60 min. After centrifugation at 10,000 g for 5 min, the cell pellet was solubilized in 50 μL SDS sample buffer (62.5 mM Tris-HCl, pH 6.8; 10% glycerol; 5% 2-mercaptoethanol; 2% SDS), and boiled for 5 min. The solubilized sample (40 μL) was electrophoresed on 10% polyacrylamide gel containing SDS (SDS-PAGE). After drying, the gel was exposed to a phosphor imaging plate for 72 hr, and the plate was scanned with phosphorimager (BAS 2,000, FUJIX, Tokyo, Japan).

cGMP Measurement

The cells (2×10^6) were preincubated for 10 min at 37°C with 0.3 mM IBMX in 1 mL KRP buffer containing 0.5% BSA and were further incubated with various concentrations of ANP or CNP for 10 min in the presence of IBMX. After addition of 100 μL 1 M HCl, the reaction mixture was centrifuged and the supernatant was subjected to the radioimmunoassay of cGMP as reported previously [14, 15].

RESULTS

When the adrenal chromaffin cells were incubated with various concentrations of ^{125}I-ANP, ^{125}I-ANP bound specifically to the cells. The binding was of high affinity and saturable (Fig. 1A). A Scatchard plot of the data revealed that the binding sites were composed of a single class of sites with a K_d of 103 ± 13 pM and a B_{max} of 5.6 ± 0.9 fmol/10^6 cells (16.4 ± 2.6 fmol/mg of cell protein) (N = 3) (Fig. 1B).

Unlabeled ANP, at 10–1000 pM, inhibited ^{125}I-ANP binding with an IC_{50} of 80 pM (Fig. 2A) and increased cGMP level in a dose-dependent manner (Fig. 2B). CNP raised cGMP to a greater extent than ANP, but did not compete effectively for ^{125}I-ANP binding. C-ANF, a synthetic ligand that binds selectively to ANPR-C [6], also failed to inhibit ^{125}I-ANP binding, except at high concentrations (>10 nM).

Affinity cross-linking of cells with ^{125}I-ANP followed by SDS-PAGE under reducing conditions showed that the receptor for ^{125}I-ANP migrated as a single band at a position of 120 kDa (Fig. 3). The addition of an excess concentration (1 μM) of unlabeled ANP totally displaced ^{125}I-ANP from the 120 kDa band, whereas CNP and C-ANF, even at 1 μM, had considerably less effect.

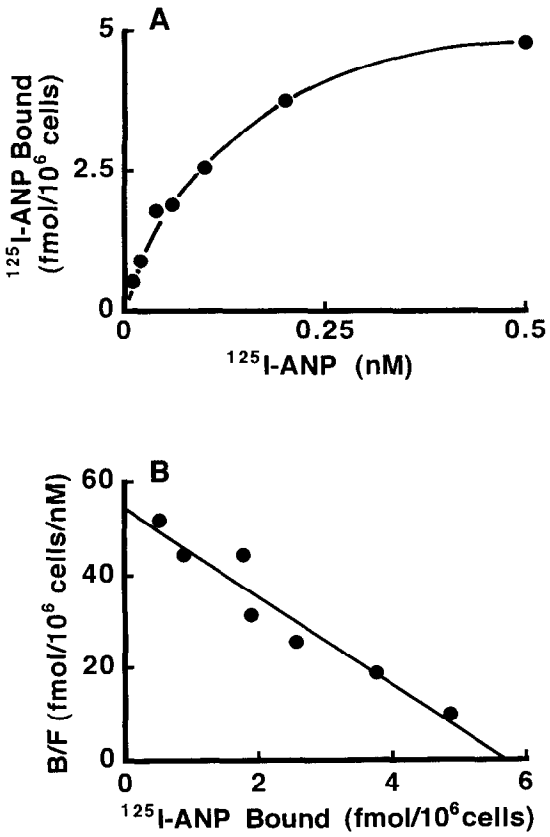

FIG. 1. Specific binding of ^{125}I-ANP to isolated adrenal chromaffin cells. (A) Cells (1×10^6) were incubated with 0.01–0.5 nM of ^{125}I-ANP at 4°C for 180 min in the absence or presence of 100 nM unlabeled ANP. Specific binding was calculated as the difference between total binding and nonspecific binding, and represented 74% of total binding at 0.1 nM ^{125}I-ANP. Data are the mean of 3 separate experiments, each performed in duplicate. (B) Scatchard plot.

DISCUSSION

In the present study, we have characterized ^{125}I-ANP binding sites in bovine adrenal chromaffin cells. Although autoradiographic studies with ^{125}I-ANP did not detect ANPR-A in adrenal medullary tissue of six different species [17, 18], the existence of ^{125}I-ANP binding sites was reported in membranes of bovine adrenal medulla [16]. The characterization of ^{125}I-ANP binding sites in the present report provides good evidence for the existence of ANP receptors in bovine adrenal chromaffin cells. The binding sites were saturable and of high affinity. They were displaced by low concentrations of unlabeled ANP but not by CNP nor C-ANF. The molecular weight of ^{125}I-ANP binding sites was 120 kDa on SDS-PAGE. These results suggest that the receptors recognized by ^{125}I-ANP in the bovine adrenal chromaffin cells can be classified as ANPR-A, but not as ANPR-B nor ANPR-C.

The molecular weight of 120 kDa for the ANP receptor in the adrenal medulla is comparable with that of ANP receptors coupled to guanylyl cyclase in the brain [19]. ^{125}I-ANP binding sites were not visualized at the position of 60–70 kDa

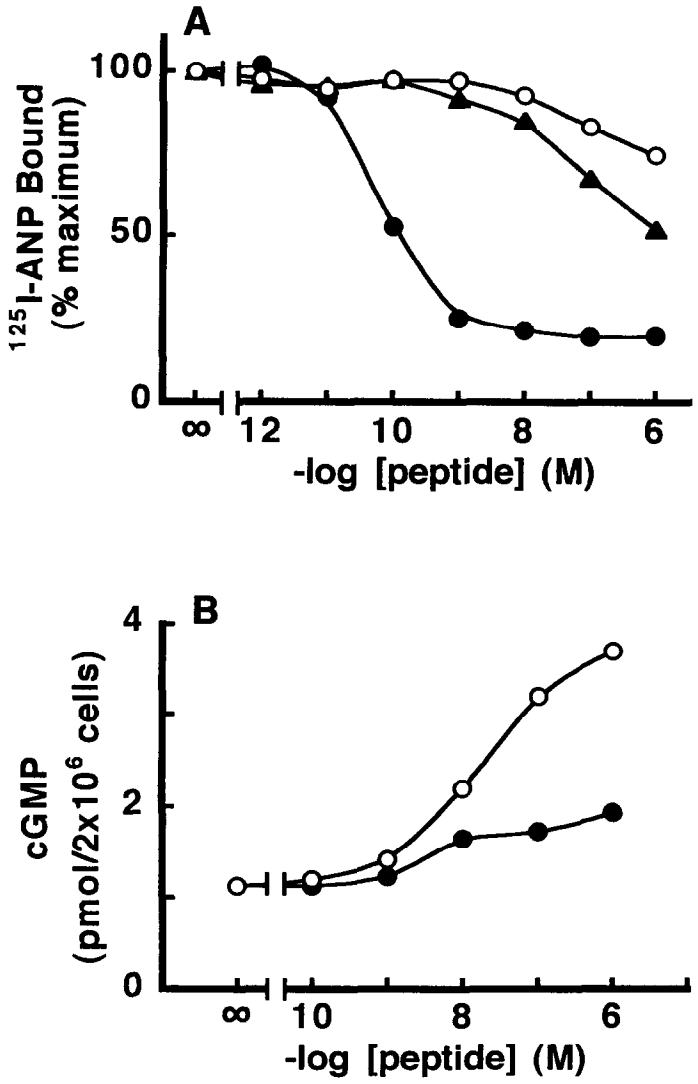

FIG. 2. Effects of peptides on ^{125}I-ANP binding and cGMP production in isolated adrenal chromaffin cells. (A) Cells were incubated with 6 pM of ^{125}I-ANP at 4°C for 180 min in the absence or presence of various concentrations of ANP (●), CNP (○) or C-ANF (▲). Each point is the mean of 3 separate experiments, each carried out in duplicate. (B) Cells (2 × 10^6) were preincubated with 0.3 mM IBMX in KRP buffer at 37°C for 10 min, and then incubated for an additional 10 min with or without various concentrations of ANP (●) or CNP (○) in the presence of IBMX. Each point is the mean of 2 separate experiments, each carried out in duplicate.

corresponding to subunits derived from homodimeric disulfide-linked ANPR-C. The absence of ANPR-C in the bovine adrenal medulla is consistent with an *in situ* hybridization study in monkey adrenal medulla showing that mRNA encoding ANPR-C is absent in adrenal chromaffin cells [20]. Of the target tissues of ANP, the adrenal medulla is one shown to be lacking ANPR-C, as in some regions of the brain [21]. In conclusion, ANP interacts with ANPR-A in adrenal chromaffin cells, and ANPR-A plays a role in the regulation of cell functions, such as catecholamine synthesis, in response to ANP secreted from these same cells [15].

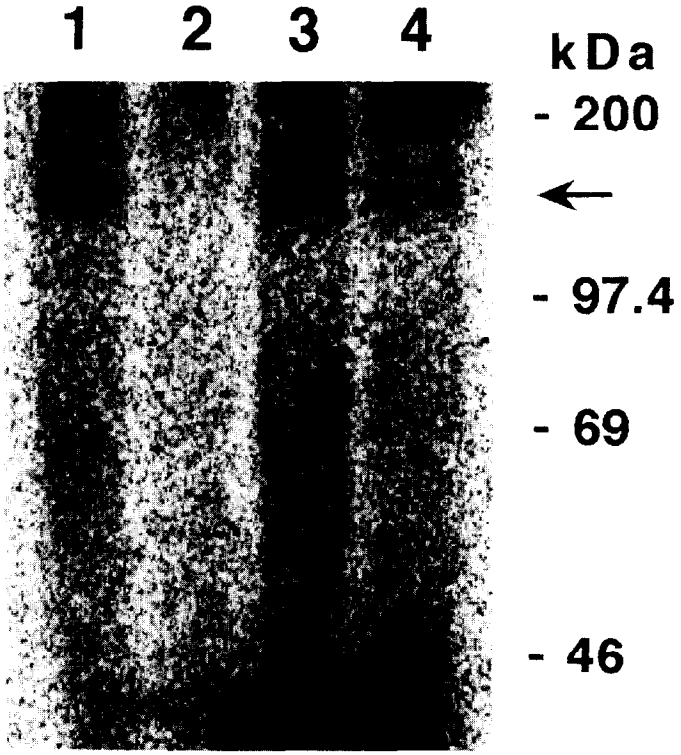

FIG. 3. Cross linking of ^{125}I-ANP to adrenal chromaffin cells. Cells (5 × 10^5) were incubated with 4.2 nM ^{125}I-ANP in the absence (lane 1) or presence of 1 μM ANP (lane 2), 1 μM C-ANF (lane 3) and 1 μM CNP (lane 4) at 4°C for 180 min, then cross-linked by 0.2 mM disuccinimidyl suberate for 60 min. The cells were solubilized with SDS sample buffer containing 2-mercaptoethanol and subjected to SDS-PAGE, and the dried gel was exposed to a phosphor imaging plate for 3 days. Arrow indicates 120 kDa band.

We would like to thank Keiko Kawabata and Keizo Masumoto for their expert assistance.

References

1. de Bold AJ, Atrial natriuretic factor: a hormone produced by the heart. *Science* **230:** 767–770, 1985.
2. Sudo T, Kangawa K, Minamino N and Matsuo H, A new natriuretic peptide in porcine brain. *Nature* **332:** 78–81, 1987.
3. Sudo T, Minamino N, Kangawa K and Matsuo H, C-Type natriuretic peptide (CNP): a new member of natriuretic peptide family identified in porcine brain. *Biochem Biophys Res Commun* **168:** 863–870, 1990.
4. Koller KJ, Lowe DG, Bennet GL, Minamino N, Kangawa K, Matsuo H and Goeddel DV, Selective activation of the B natriuretic peptide receptor by C-type natriuretic peptide (CNP). *Science* **252:** 120–123, 1991.
5. Maack T, Receptors of atrial natriuretic factor. *Ann Rev Physiol* **54:** 11–27, 1992.
6. Suga S, Nakao K, Hosoda K, Mukoyama M, Ogawa Y, Shirakami G, Arai H, Saito Y, Kambayashi Y, Inoue K and Imura H, Receptor selectivity of natriuretic peptide family, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide. *Endocrinology* **130:** 229–239, 1992.
7. Rosenzweig A and Seidman CE, Atrial natriuretic factor and related peptide hormones. *Ann Rev Biochem* **60:** 229–255, 1991.
8. Yandle TG, Biochemistry of natriuretic peptides. *J Intern Med* **235:** 561–576, 1994.

9. Levin ER, Natriuretic peptide C-receptor: more than a clearance receptor. *Am J Physiol* **264:** E483–E489, 1993.
10. Okazaki M, Yanagihara N, Izumi F, Nakashima Y and Kuroiwa A, Carbachol-induced cosecretion of immunoreactive atrial natriuretic peptides with catecholamines from cultured bovine adrenal medullary cells. *J Neurochem* **52:** 222–228, 1989.
11. Nguyen TT, Babinski K, Ong H and De Léan A, Differential regulation of natriuretic peptide biosynthesis in bovine adrenal chromaffin cells, *Peptides* **11:** 973–978, 1990.
12. Babinski K, Féthière J, Roy M, De Léan A and Ong H, C-type natriuretic peptide in bovine chromaffin cells. *FEBS Lett* **313:** 300–302, 1992.
13. Ueda S, Minamino N, Aburaya M, Kangawa K, Matsukura S and Matsuo H, Distribution and characterization of immunoreactive porcine C-type natriuretic peptide. *Biochem Biophys Res Commun* **175:** 759–767, 1991.
14. Tsutsui M, Yanagihara N, Minami K, Kobayashi H, Nakashima Y, Kuroiwa A and Izumi F, C-type natriuretic peptide stimulates catecholamine synthesis through the accumulation of cyclic GMP in cultured bovine adrenal medullary cells. *J Pharmacol Exp Ther* **268:** 584–589, 1994.
15. Yanagihara N, Okazaki M, Terao T, Uezono Y, Wada A and Izumi F, Stimulatory effects of brain natriuretic peptide in cyclic GMP accumulation and tyrosine hydroxylase activity in cultured bovine adrenal medullary cells. *Naunyn-Schmiedeberg's Arch Pharmacol* **343:** 289–295, 1991.
16. Heisler S and Morrier E, Bovine adrenal medullary cells contain functional atrial natriuretic peptide receptors. *Biochem Biophys Res Commun* **150:** 781–787, 1988.
17. Chai SY, Sexton PM, Allen AM, Figdor R and Mendelsohn FAO, In vivo autoradiographic localization of ANP receptors in rat kidney and adrenal gland. *Am J Physiol* **250:** F753–F757, 1986.
18. Maurer R and Reubi J-C, Distribution and coregulation of three peptide receptors in adrenals. *Eur J Pharmacol* **125:** 241–247, 1986.
19. Konrad EM, Thibault G, Schiffrin EL and Cantin M, Atrial natriuretic factor receptor subtypes in the rat central nervous system. *Hypertension* **17:** 1144–1151, 1991.
20. Wilcox JN, Augustine A, Goeddel DV and Lowe DG, Differential regional expression of three natriuretic peptide receptor genes within primate tissues. *Mol Cell Biol* **11:** 3454–3462, 1991.
21. Brown J and Czarnecki A, Distribution of atrial natriuretic peptide receptor subtypes in rat brain. *Am J Physiol* **258:** R1078–R1083, 1990.