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SPECIFIC BINDING ACTIVITIES AND CYCLIC GMP RESPONSES BY ATRIAL NATRIURETIC POLYPEPTIDE IN KIDNEY EPITHELIAL CELL LINE (LLC-PK_1)

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Receptor binding activities and cyclic GMP responses by α-human atrial natriuretic polypeptide (α-hANP) and its fragments were studied in a kidney epithelial cell line (LLC-PK_1). Binding of ^{125}I-α-hANP to the cells at 0°C was saturable, time-dependent and reversible, indicating the presence of a single class of binding sites. α-hANP(7-23)NH_2 fragment inhibited most effectively the specific binding of ^{125}I-α-hANP to the LLC-PK_1 cells, followed by α-hANP(17-28) and α-hANP(8-22), while α-hANP(1-6) and α-hANP(24-28) did not. α-hANP stimulated the formation of cyclic GMP in the LLC-PK_1 cells dose-dependently. Although no fragments of α-hANP used were effective for cyclic GMP formation in the LLC-PK_1 cells, α-hANP(7-23)NH_2 antagonized the action of α-hANP on cyclic GMP formation. These data suggest that the LLC-PK_1 cells retain specific receptors for atrial natriuretic polypeptide (ANP) and respond to ANP by stimulating cyclic GMP formation, and therefore this cell line may be useful for studying the mechanism of action for ANP in renal tubular cells.

Although atrial natriuretic polypeptide (ANP) possesses potent natriuretic and diuretic activities, in addition to vasorelaxant activity, the site of action and molecular mechanisms by which ANP elicits natriuresis remain unknown (1-3). Napier et al. (4) showed

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Abbreviations: α-hANP, α-human atrial natriuretic polypeptide; ANP, atrial natriuretic polypeptide; BSA, bovine serum albumin; PBS buffer, phosphate-buffered saline; EGTA, ethylene glycol bis(ß-aminoethyl ether)-N,N´-tetraacetic acid.

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the existence of specific membrane receptors for ANP in the renal cortex, and we have also demonstrated the presence of specific receptors for α-human atrial natriuretic polypeptide (α-hANP) on basolateral membranes isolated from the rat renal cortex (5). While examining for the mechanism of action by ANP, Hamet et al. (6) noted a specific elevation of cyclic GMP levels induced by ANP in kidney slices and primary cultures of renal tubular cells.

The kidney is composed of heterogeneous nephron segments and many cell types, including vascular smooth muscle. The use of cell culture techniques has permitted the study of a relatively homogeneous population of renal epithelial cells under carefully controlled conditions and has offered advantages for the study of a variety of renal cell functions (7). Therefore, renal cells in culture should also be suitable for the study of ANP-receptor interactions and the mechanisms of action of ANP. The present study was designed to investigate receptor binding activities and cyclic GMP responses by α-hANP and its fragments using the LLC-PK_1 kidney epithelial cell line.

MATERIALS AND METHODS

Materials: The following reagents were used: α-hANP (Peptide Institute, Inc., Osaka, Japan), bovine serum albumin (BSA, Poviet Producten B.V., Amsterdam, Holland), methylisobutylxanthine (Nakarai Chemicals, Kyoto, Japan). Cyclic GMP assay kit was purchased from Yamasa Shoyu Co., Ltd., (Chiba, Japan). The fragments of α-hANP were synthesized by a conventional solution method (8). All other chemicals used for the experiments were of the highest purity available.

Iodination of α-hANP: α-hANP was radioiodinated by the chloramine T method as described previously (5,9).

Cell Culture: The LLC-PK_1 cells obtained from the American Type Culture Collection (A.T.C.C. CRL-1392) were grown on 100 mm diameter plastic dishes (Corning) in medium 199 (Flow Laboratories) supplemented with 10% (v/v) fetal calf serum (Microbiological Associates) without antibiotics, in an atmosphere of 5% CO_2/95% air at 37°C (10-12). In most experiments, after the inoculation of $1x10^6$ cells in 10 ml of culture medium, the cells were given fresh medium on day 4 and were harvested on day 7. The cells (passages 240-255) were removed from dishes using 5 mM EGTA in phosphate-buffered saline without Ca^{2+} and Mg^{2+}, and washed twice with phosphate-buffered saline (PBS buffer, 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$), and

centrifuged at 200 g for 10 min. The cells were suspended in PBS buffer containing 0.2% BSA (buffer A).

Binding of ^{125}I-α-hANP to LLC-PK$_1$ Cells: Binding studies were performed at 0°C, except where specified otherwise, by rapid filtration technique (5). In the regular assay, the binding reaction was initiated by adding 100 µl of buffer A containing ^{125}I-α-hANP (approx. 60,000 cpm), with or without the competing ligands, to 100 µl of cell suspension ($5x10^5$ cells) in buffer A. At the stated times, the incubation was stopped by diluting the reaction sample with 1 ml of ice-cold PBS buffer. The tube contents were immediately poured onto Millipore filters (EHWP, 0.5 µm, 2.5 cm diameter) and washed once with 5 ml of ice-cold PBS buffer. The radioactivity of filters was determined by an automatic gamma counter. In degradation study for α-hANP, the reaction mixture (200 µl) was combined with 1 M acetic acid (100 µl), bolied for 5 min and assayed by high performance gel permeation chromatography (5). Trypan blue exclusion studies were carried out at the end of the experiments, and no significant change in cell viability (>95%) was found.

Determination of Cyclic GMP Contents: The cells ($2-3x10^6$ cells) in suspension were incubated at 37°C with or without α-hANP and its fragments in 1 ml of buffer A containing 0.5 mM methylisobutylxanthine. At the stated times, incubation was terminated with the addition of an equal volume of ice-cold 12% trichloroacetic acid. After centrifugation, the precipitate was treated with 1 ml of ice-cold 6% trichloroacetic acid. The combined supernatant (3 ml) was extracted three times with 6 ml of ether and was used for the determination of cyclic GMP according to Yamasa cyclic GMP assay kit technical guide.

RESULTS

The time course of ^{125}I-α-hANP binding to the LLC-PK$_1$ cells in suspension was measured at 0°C or 25°C in the presence or absence of 10^{-6} M unlabeled α-hANP (Fig. 1). At 0°C, the binding of ^{125}I-α-hANP to the LLC-PK$_1$ cells was time-dependent, and the specific binding reached a steady state at 60 min. Rapid dissociation of ^{125}I-α-hANP from the cells was observed after the addition of 10^{-6} M α-hANP. On the other hand, the binding of ^{125}I-α-hANP at 25°C was maximal at 5 min, and then rapidly decreased.

In order to examine the stability of ^{125}I-α-hANP under the incubation conditions, the incubation mixture was assayed by high performance gel permeation chromatography. In the incubation mixture for 60 min at 0°C and 25°C, the recovery in the intact form was 84% and 13% of the total radioactivity, respectively.

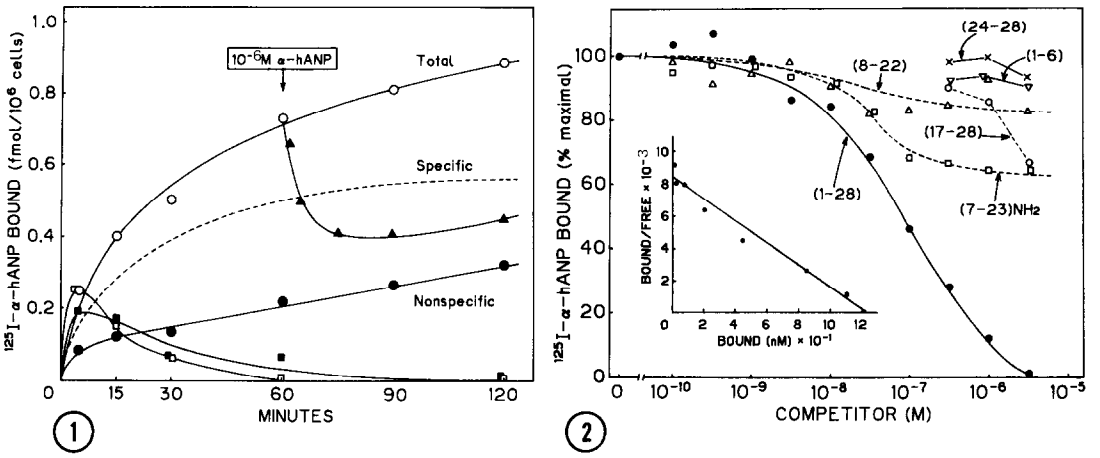
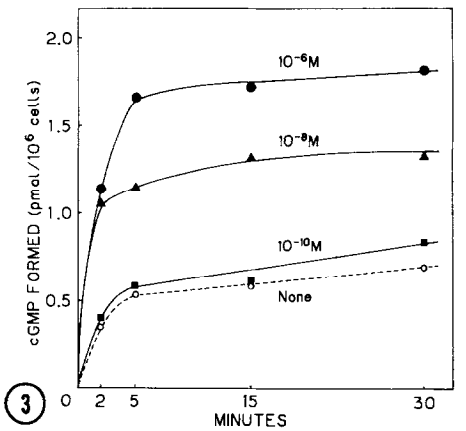


Fig. 1 Time course of ^{125}I-α-hANP binding to LLC-PK$_1$ cells. The cells (5×10^5 cells/tube) were incubated at 0°C (○,●) or 25°C (□,■) with ^{125}I-α-hANP (0.15 nM, 63,690 cpm) in PBS buffer containing 0.2% BSA (buffer A) in the presence (●,■) or absence (○,□) of 10^{-6} M unlabeled α-hANP. To determine reversibility of ^{125}I-α-hANP binding, unlabeled α-hANP was added after 60 min (10^{-6} M), and then incubation was continued for another 60 min at 0°C (▲). Dashed line shows specific binding of ^{125}I-α-hANP to the cells, defined as total binding minus nonspecific binding in the presence of 10^{-6} M unlabeled α-hANP. Each point represents the mean of three determinations.

Fig. 2 Effect of unlabeled α-hANP and its fragments on ^{125}I-α-hANP binding to LLC-PK$_1$ cells. The cells (5×10^5 cells/tube) were incubated at 0°C for 60 min with ^{125}I-α-hANP (0.17 nM, 72,860 cpm) in the presence or absence of unlabeled α-hANP and its fragments in concentrations shown in the abscissa. The bindings of ^{125}I-α-hANP in the presence and absence of 3.3×10^{-6} M unlabeled α-hANP were 0.5% and 1.5% of the total radioactivity added, respectively. Each point represents the mean of three determinations. Inset: Scatchard plot. Analysis of the binding data was performed by non-linear least squares regression test (23).

The binding profile for ^{125}I-α-hANP to the LLC-PK$_1$ cells is illustrated in Fig. 2. The binding of ^{125}I-α-hANP was competitively inhibited by increasing concentrations of unlabeled α-hANP. Scatchard analysis of the data revealed the presence of a single class of binding sites. The apparent dissociation constant (Kd) was 110 nM and the binding capacity was 240,000 sites/cell (mean of three experiments). Among the fragments of α-hANP used, α-hANP(7-23)NH_2 inhibited most effectively the specific binding of ^{125}I-α-hANP to the LLC-PK$_1$ cells, followed by α-hANP(17-28) and α-hANP(8-22), while α-hANP(1-6) and α-hANP(24-28) did not.



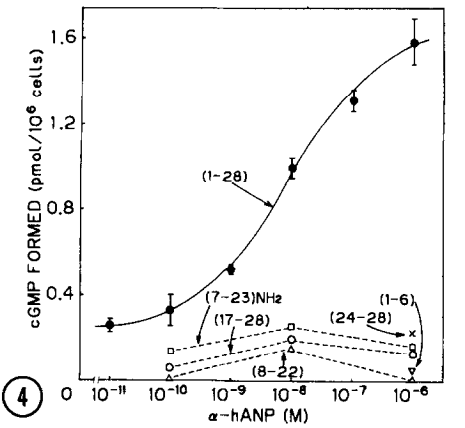


Fig. 3 Time course of α-hANP effect on cyclic GMP formation in LLC-PK_1 cells. The cells ($3x10^6$ cells/tube) were incubated at 37°C with (■, 10^{-10} M;▲, 10^{-8} M;●, 10^{-6} M) or without (○) α-hANP in buffer A containing 0.5 mM methylisobutylxanthine. At the stated times, cyclic GMP was determined by radioimmunoassay as described in the text. Each point represents the mean of two determinations.

Fig. 4 Effect of unlabeled α-hANP and its fragments on cyclic GMP formation in LLC-PK_1 cells. The cells ($3x10^6$ cells/tube) were incubated at 37°C for 15 min in buffer A containing 0.5 mM methylisobutylxanthine in the presence or absence of α-hANP and its fragments in concentrations shown in the abscissa. Each point represents the mean±S.E. of three determinations.

The effect of α-hANP on the formation of cyclic GMP in the LLC-PK_1 cells is shown in Fig. 3. α-hANP stimulated the formation of cyclic GMP in a dose-dependent manner and the stimulation was almost plateau between 5 and 30 min.

As is evident from Fig. 4, the concentration of half-maximal stimulation (ED_{50}) on cyclic GMP formation by α-hANP was approximately 10 nM, whereas all of the α-hANP fragments used had no effect.

Furthermore, each fragment was also tested for its ability to inhibit α-hANP-stimulated cyclic GMP formation in the LLC-PK_1 cells (Table I). At a concentration 100-fold that of α-hANP, only α-hANP(7-23)NH_2 showed a significant inhibitory effect on α-hANP-stimulated cyclic GMP formation.

Table I. Effect of α-hANP Fragments on Stimulation of Cyclic GMP Formation by α-hANP in LLC-PK_1 Cells

Additions	Cyclic GMP formed (pmol/10^6 cells)	%
α-hANP alone	0.51 ± 0.02	100
+ α-hANP(7-23)NH_2	0.39 ± 0.02[a]	76
+ α-hANP(8-22)	0.49 ± 0.04	96
+ α-hANP(24-28)	0.49 ± 0.05	96
+ α-hANP(17-28)	0.51 ± 0.05	100
+ α-hANP(1-6)	0.54 ± 0.04	106

The cells ($2x10^6$ cells/tube) were preincubated at 37°C for 5 min in the presence or absence of α-hANP fragments (10^{-6} M), and then incubated at 37°C for 15 min with α-hANP (10^{-8} M) in the presence or absence of α-hANP fragments (10^{-6} M). Data were corrected by subtraction of basal cyclic GMP level. Each value represents the mean±S.E. of five determinations. [a]$P<0.001$, significant difference from α-hANP alone using a two-tailed t test.

DISCUSSION

The LLC-PK_1 cells have been characterized as a model system for the analysis of epithelial functions in the proximal tubules (12-15) and distal tubules (16). The present study demonstrates that specific binding sites for α-hANP are present in the LLC-PK_1 cells, and α-hANP stimulates the formation of cyclic GMP in these cells. These results are compatible with previous reports that described the presence of specific receptors for α-hANP on the plasma membranes isolated from the renal cortex (4,5), and the stimulation of cyclic GMP formation by ANP in the renal tissues (6).

In the present study, the Kd value for α-hANP in the LLC-PK_1 cells was relatively high compared to our previous data in basolateral membranes from the rat renal cortex (5). The reason for this difference remains to be solved.

α-hANP(7-23)NH_2 markedly inhibited the binding of ^{125}I-α-hANP, but α-hANP(8-22) had only a slight inhibitory effect. This is consistent with the finding that the common ring structure formed by the disulfide bond is essential for receptor binding (17-19). How-

ever, α-hANP(7-23)NH_2 failed to stimulate cyclic GMP formation in the LLC-PK_1 cells. Present data are comparable with those of previous papers describing that C-terminal residues may play an important role in modulating the binding of ANP and/or its biological actions (17,20-22).

Furthermore, it is interesting to note that α-hANP(7-23)NH_2 antagonizes the action of α-hANP on cyclic GMP formation. The ability of α-hANP(7-23)NH_2 to suppress the action of α-hANP may be accounted for by its ability to inhibit α-hANP binding to membrane receptors. This finding suggests that α-hANP-stimulated cyclic GMP formation is coupled to α-hANP-receptor interactions.

In conclusion, the present results suggest that the LLC-PK_1 cells express specific receptors for α-hANP and respond to α-hANP by stimulating cyclic GMP formation. These cells may be useful as a model system for studying the direct effect of ANP on renal tubular cells.

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