Vol. 168, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 16, 1990 Pages 148-154

RECEPTORS FOR ATRIAL NATRIURETIC PEPTIDE (ANP) AND CYCLIC GMP RESPONSES IN HELA CELLS

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Received February 7, 1990

SUMMARY: Cultured HeLa cells display specific binding sites for atrial natriuretic peptide (ANP). Studies with [^{125}I]-(1-28)-rat ANP revealed the presence of a single class of high affinity binding sites on HeLa cells: The apparent dissociation constant (K_d) was 5 nM and the maximal number was 29,000 sites/cell as derived from Scatchard analysis. Furthermore, ANP elevates levels of cGMP in a dose dependent manner with an EC_{50} close to the K_d for [^{125}I]ANP binding. ANP-mediated elevation of cellular cGMP leads to a significant reduction of bumetanide-sensitive $Na^+,K^+,2Cl^-$-cotransport in HeLa cells, studied by $^{22}Na^+$ and $^{86}Rb^+$ influx measurements. These data indicate (1) that distinct functionally active receptors for ANP are present on HeLa cells and (2) ANP influences the activity of $Na^+,K^+,2Cl^-$-cotransport in these cells.

A group of peptides synthesized in mammalian atria, collectively called atrial natriuretic peptides (ANP), is implicated in diuresis, suppression of the renin-angiotensin-aldosterone axis and vasorelaxation of smooth muscle (1-3). ANP binding to functionally active receptors on target cells leads to an activation of membrane-bound guanylate cyclase thereby increasing the level of cellular cGMP (4, 5).

It was suggested that ANP-stimulated elevation of cGMP modulates the activity of distinct transport proteins as the $Na^+,K^+,2Cl^-$-cotransport system (6, 7) and the Na^+/H^+-exchanger (8, 9). Furthermore, there is evidence that the cotransporter is regulated by phosphorylation via cGMP-dependent protein kinases (10). ANP stimulates the $Na^+,K^+,2Cl^-$-cotransport system in vascular smooth muscle cells (11) and inhibits the cotransporter in winter flounder intestine tissue (6).

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0006-291X/90 $1.50

Recently, we have characterized a $Na^+,K^+,2Cl^-$-cotransport system in HeLa cells (12) and here we report an inhibitory effect of ANP on $^{22}Na^+/^{86}Rb^+$ influx via the cotransporter.

MATERIALS AND METHODS

Cells and Culture. HeLa cells were derived from a monolayer strain which is routinely used in our laboratory. Cells were maintained as described previously (12) in Joklik's minimal essential medium (MEM) supplemented with 5% (w/v) fetal calf serum (FCS), Penicillin and Streptomycin (50 IU and 50 µg/ml respectively, Seromed, Munich, FRG). Subconfluent monolayers plated in 24 well tissue culture plates (Nunc, Denmark) at a density of $5 \cdot 10^5$ cells/well were used for the studies described here.

[^{125}I]ANP Binding Assay. ANP Binding was performed in 24 well plates. Prior to incubation with [^{125}I]ANP, cell monolayers were washed two times with 500 µl/well of binding medium (MEM containing 0.05% w/v bovine serum albumin (BSA), pH 7.4, at 37°C). Various concentrations (0.5 nM - 100 nM) of [^{125}I]ANP (specific activity 2000 Ci/mM, Amersham-Buchler KG, Braunschweig, FRG) in binding medium were added to the wells and the plates were placed in a CO_2 humidified incubator at 37 °C for 30 minutes (equillibrium conditions). Nonspecific binding was determined in parallel samples in the presence of an excess of 1 µM unlabeled ANP. Incubation was terminated by aspiration of the binding medium and washing the cells rapidly 5 times (500 µl/well each) with ice-cold wash buffer (20 mM Na_2HPO_4, 5 mM NaH_2PO_4, 5 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.05% w/v BSA). Cells were solubilized in 0.2% w/v sodium dodecylsulphate (SDS) and radioactivity was counted on a Packard Gamma CP 153 counter. Alternately, 1 ml/well of 0.1% (w/v) trypsin in modified phosphate-buffered saline (PBS without Ca^{2+} and Mg^{2+}) with the addition of 5 mM EDTA was added to dissociate cells for counting on a Neubauer-chamber. Specific binding of [^{125}I]ANP was calculated as described by Oettling and Moeller (13). All experiments (n= 5) were done in duplicate and the maximal number of binding sites/cell was derived from a Scatchard plot (14).

Determination of Cyclic GMP. Cell monolayers in 24 well dishes were washed twice with binding medium (see above) and 0.5 mM isobutylmethylxanthine (IBMX, Sigma, Deisenhofen, FRG). Then, 250 µl/well binding medium plus 0.5 mM IBMX, containing various concentrations of ANP, was added to the cells. The reaction was stopped by addition of 750 µl/well 80% ethanol (w/v). After sonification the suspension of solubilized cells was centrifuged at 10,000 rpm in an Eppendorf centrifuge for 10 min. The supernatant was stored at -20 °C and allowed to evaporate to dryness overnight. Residues were dissolved in Na^+ acetate buffer (50 mM Na^+ acetate, pH 6.2) and cGMP levels were quantitated by radioimmunoassay, described by Schumacher et al. (15).

$^{22}Na^+/^{86}Rb^+$ Influx Studies. Initial uptake of $^{22}Na^+$ (4 min) and $^{86}Rb^+$ (10 min) into HeLa cells in 24 well plates was measured in phosphate-buffered saline (PBS) as described previously (12). Uptake mediated by the $Na^+,K^+,2Cl^-$-cotransport system was defined as the bumetanide-sensitive uptake fraction and was calculated as the difference between uptake values in the presence of 1 mM ouabain (plus 0.1 mM amiloride for determination of $^{22}Na^+$ uptake)

and uptake in the presence of 1 mM ouabain plus 50 μM bumetanide (plus 0.1 mM amiloride for determination of $^{22}Na^+$ uptake). Briefly, cells were incubated for 30 minutes in PBS +/- 100 nM ANP. The buffer was replaced by PBS containing radioactive tracer (1-2 μCi/ml $^{22}Na^+$ or 2 μCi/ml $^{86}Rb^+$) for Na^+ and K^+ movements, 1 mM ouabain, +/-50 μM bumetanide (plus 0.1 mM amiloride for determination of $^{22}Na^+$ uptake) and +/-100 nM ANP. Uptake was terminated and radioactivity was determined according to Kort and Koch (12).

Determination of Protein. In all experiments described above protein concentrations of cell monolayers were determined in parallel samples by the tryptophan-fluorescence method described by Avruch and Wallach (16). Cell monolayers in 24 well plates were solubilized by adding 1 ml/well 0.2% (w/v) SDS. Solubilized cells were aspirated and heat-denaturated at 70°C. Protein content was assayed by using a Perkin-Elmer fluorescence spectrophotometer 650-40 with slit widths 4/10 nm, excitation wavelength 284 nm and emission wavelength 325 nm. A standard curve using purified BSA was used for calibration. The mean ± standard error was 105 ± 17 μg protein/10^6 cells (n= 6).

RESULTS

Binding of ANP Binding of [^{125}I]ANP to intact HeLa cells was time dependent and saturable. Steady-state values were reached within 30 minutes and remained constant for 1 hr or more. The nonspecific binding determined in the presence of excess unlabeled ANP (1 μM) amounted to 25-35% of specific binding (Fig. 1). A plateau of specific binding of [^{125}I]ANP was achieved between 20 and 50 nM of radioligand (Fig. 2A). Scatchard analysis revealed a straight line, indicating the presence of a single class of binding sites

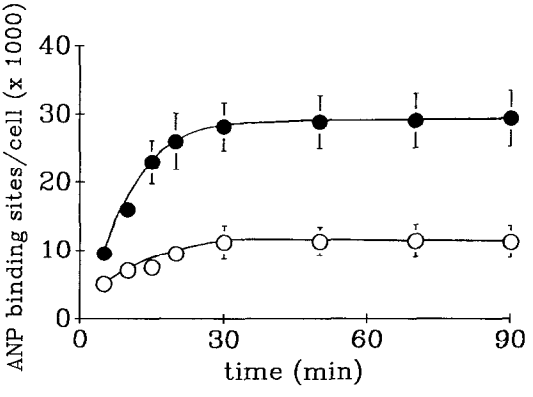

Fig. 1. Time dependence of [^{125}I]ANP binding to HeLA cells. HeLa cells were incubated at 37 °C in a CO_2 humidified incubator with 50 nM [^{125}I]ANP +/- an excess of 1 μM unlabeled ANP for the indicated times. Specific binding (●) was obtained by substracting nonspecific binding (○), which was measured in the presence of an excess of 1 μM unlabeled ANP from total binding. Each point represents the mean ± S.E. of 4 separate experiments.

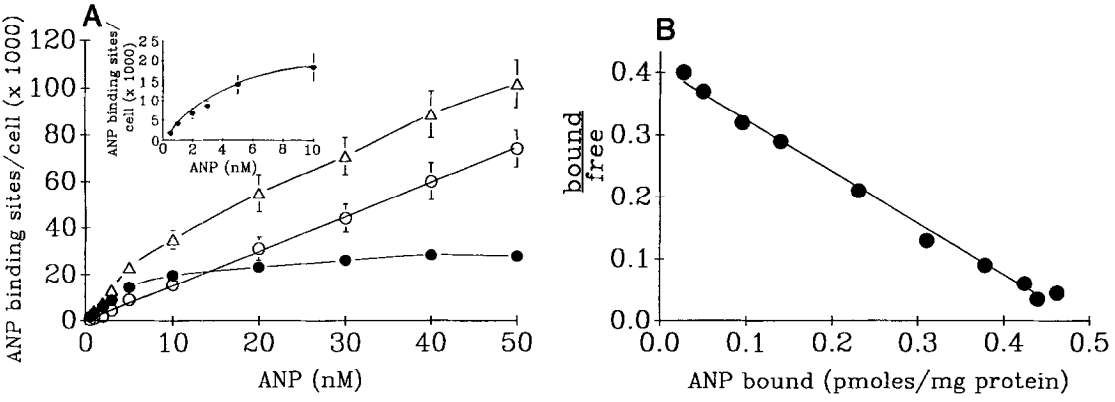

Fig. 2. (A) Saturable [^{125}I]ANP binding to HeLa cells. Cell monolayers were incubated at 37 °C for 30 minutes with the indicated concentrations of [^{125}I]ANP. Specific (●) and nonspecific (○) binding data were determined as described in "Material and Methods". The data are the mean values ± S.E. of duplicate determinations from 5 separate experiments. Insert shows specific binding in the range of 0.5-10 nM ANP. **(B) Scatchard analysis of [^{125}I]ANP binding to HeLa cells.** The ratio of bound to free ANP is plotted against the concentration of bound ANP. The dissociation constant (K_d) was 5 ± 0.8 nM (m ± S.E.) and receptor concentration (B_{max}) was 0.47 ± 0.07 pmol/mg protein.

(Fig. 2B). The apparent dissociation constant, K_d was 5 nM and the maximal number of binding sites, B_{max} was 29,000 sites/cell (=0.47 pmol/mg protein), as derived from Scatchard analysis.

cGMP stimulation of ANP To show functional coupling of the putative ANP receptor to intracellular events, intracellular levels of cGMP were determined in the presence of increasing concentations of ANP. Fig. 3 shows that ANP increases formation of

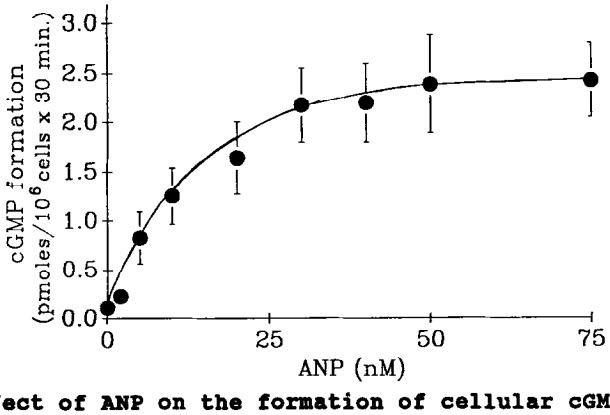

Fig. 3. Effect of ANP on the formation of cellular cGMP in HeLa cells. Cells in 24-well plates were incubated at 37 °C for 30 min. in MEM containing 0.5 mM IBMX and the indicated concentrations of ANP. cGMP accumulation was determined as described in "Materials and Methods". Each point represents the mean ± S.E. of duplicate determinations from 4 separate experiments.

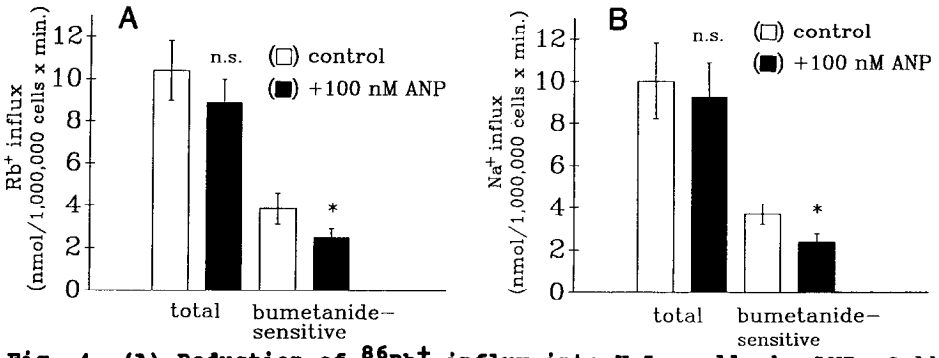

Fig. 4. (A) Reduction of $^{86}Rb^+$ influx into HeLa cells by ANP. Cell monolayers in 24-well dishes were preincubated for 30 minutes at 37 °C in PBS, with (solid bars) or without (open bars) 100 nM ANP. Then, $^{86}Rb^+$ influx was measured for 10 minutes as described in "Materials and Methods". Total $^{86}Rb^+$ uptake was obtained without any further addition and bumetanide-sensitive $^{86}Rb^+$ uptake was calculated as the difference by substracting the uptake value in the presence of 1 mM ouabain plus 50 μM bumetanide from the uptake value in the presence of 1 mM ouabain. **(B) Reduction of $^{22}Na^+$ influx into HeLa cells by ANP.** Cells were preincubated as described in (A), with (solid bars) or without (open bars) the addition of 100 nM ANP and $^{22}Na^+$ uptake was determined for a period of 4 minutes as described previously (12). Total uptake was measured with the addition of 1 mM ouabain and bumetanide-sensitive uptake was defined as the difference between uptake value in the presence of 1 mM ouabain plus 0.1 mM amiloride and uptake in the presence of 1 mM ouabain plus 0.1 mM amiloride plus 50 μM bumetanide. Bars in (A) and (B) represent the mean ± S.E. of duplicate determinations from 5 separate experiments. Significance was calculated by a two-tailed student's t-test. *p< 0,01. n.s.= data are not significantly different.

cGMP in HeLa cells at a concentration which is 20-fold larger than the respective K_d for ANP binding to these cells. A marked 250 fold elevation of cGMP levels was observed in response to exposure to 100 nM ANP (2.5 pmol/10^6 cells^{-1}·30 min^{-1}) when compared to basal cGMP levels in the absence of ANP (0.01 pmol/10^6 cells^{-1}·30 min^{-1}) . The EC_{50} value for cGMP stimulation is 9 nM which is approximately twice as high as the respective K_d for ANP binding.

Effect of ANP on $Na^+,K^+,2Cl^-$-cotransport The influence of ANP on function of the $Na^+,K^+,2Cl^-$-cotransport system was investigated by measurements of bumetanide-sensitive $^{22}Na^+$ and $^{86}Rb^+$ uptake in the presence of ANP. Incubation with 100 nM ANP (solid bars in figures 4A and 4B) for 30 minutes leads to a 40% reduction of bumetanide-sensitive uptake of both, $^{22}Na^+$ and $^{86}Rb^+$ uptake when compared to uptake in the absence of ANP (open bars in figures 4A and 4B).

DISCUSSION

HeLa cells have been extensively employed as a model system for the analysis of function and regulation of different ion-

transport proteins, including investigations on the Na^+,K^+-ATPase (17, 18), K^+ channels (19) and the $Na^+,K^+,2Cl^-$-cotransport system (12, 20). Since little is known about the hormonal regulation of the cotransporter in HeLa cells the present study was designed to investigate the effect of atrial natriuretic peptide on the function of the $Na^+,K^+,2Cl^-$-cotransport system.

This study confirmed previous work (21) that specific binding sites for ANP are present on HeLa cells. Results from saturable binding studies, using [^{125}I]ANP, revealed a single class of high affinity binding sites. The apparent K_d of 5 nM for [^{125}I]ANP binding was found to be in the same range as reported previously for HeLa cells (21) and smooth muscle cells (22). A remarkable 250-fold increase in cellular cyclic GMP upon incubation with nanomolar amounts of ANP indicates functionally active ANP receptors on HeLa cells. Comparable stimulation of cGMP by ANP was observed in vascular smooth muscle cells (23), Leydig cells (24) and renal cells (4). The stimulatory effect of ANP in these cells on levels of cellular cGMP was dose-dependent, with an EC_{50} value of 9 nM. Presently, we have no explanation for the discrepancy between the K_d for [^{125}I]ANP binding and the EC_{50} value for cGMP elevation in HeLa cells. In comparison to the study of Watt and Yip (21) our EC_{50} value for ANP-stimulated elevation of cGMP is approximately two-fold lower than reported by them.

ANP-mediated elevation of cellular cGMP leads to a significant 40% decrease in bumetanide-sensitive $^{22}Na^+$ and $^{86}Rb^+$ uptake. Furthermore, a similar reduction of $Na^+,K^+,2Cl^-$-cotransport also occurs in the presence of 8-Br-cGMP (synthetic analogue of cGMP; manuscript in preparation). An ANP-mediated inhibition of $Na^+,K^+,2Cl^-$-cotransport by ANP is also described for flounder intestine tissue (6), while the opposite effect, a stimulation of the $Na^+,K^+,2Cl^-$-cotransport system by ANP acting via cGMP elevation, is reported for vascular smooth muscle cells (25).

In conclusion, our results suggest that HeLa cells display specific receptors for ANP and respond to binding of ANP by elevation of cellular cGMP. Intracellular events, following ANP binding lead to a decrease in $Na^+,K^+,2Cl^-$-cotransport in these cells, which may be useful as a model system for studying other mechanisms induced by ANP.

ACKNOWLEDGMENT

We thank Dr. A. K. Mukhopadhyay for his kind assistance in the determinations of cellular cGMP.

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