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Natriuretic Peptide Receptors on Rat Thymocytes: Inhibition of Proliferation by Atrial Natriuretic Peptide*

ANGELIKA M. VOLLMAR, KARIN-NICOLE SCHMIDT, AND RÜDIGER SCHULZ

Institute of Pharmacology, Toxicology and Pharmacy, University of Munich, Königinstrasse 16, D-80539 Munich, Germany

ABSTRACT

Because the thymus expresses the natriuretic peptides (NP) as well as their respective receptors, an involvement of NP in the physiology of this organ has been suggested. To evaluate functional aspects of NP in the thymus, we looked for thymic cells bearing NP receptors (Npr). Furthermore, the regulation of Npr expression by activation of cells and the influence of NP on the proliferation of thymocytes was studied. Expression of receptor messenger RNAs (mRNAs) was examined by PCR and Northern blot. Existence of functional Npr was confirmed by measurement of cGMP, the second messenger of NP. Proliferation of thymocytes upon concanavalin A (Con A) stimulation was analyzed by incorporation of [^{3}H]thymidine. We report here that thymocytes express mRNAs for the three Npr, namely Npra, Nprb, and Nprc and that activation of Npra and Nprb increases cGMP levels. Stimulation of thymocytes with Con A (1 μg/ml, 48 h) resulted in an increase of mRNA coding for Npra, the receptor specific for atrial natriuretic peptide (ANP) and brain natriuretic peptide. Nprb and Nprc receptor expression was not altered under these conditions. In agreement with these data only ANP, but not the C-type natriuretic peptide, elicited increased cGMP response in Con A-stimulated cells. ANP inhibited also the proliferation of Con A stimulated thymocytes, whereas C-type natriuretic peptide did not show this effect. These results suggest that ANP affects the complex mechanisms of thymocyte proliferation and differentiation. (*Endocrinology* **137:**1706–1713, 1996)

HORMONES and neuropeptides are potent immunomodulators acting on various immune competent cells (for review see Refs. 1, 2). It is well documented that the thymus, where bone marrow-derived precursor cells proliferate and differentiate to mature T-lymphocytes, is under endocrine control (3, 4). The regulation of these processes appears to be extremely complex (5). In addition to circulating hormones affecting these functions, complete neuropeptide systems exist involving synthesis of peptides and expression of their respective receptors by thymic cells (3). For example, GH, PRL, oxcytocin (OT), arginine-vasopressin (AVP), or β-endorphin are likely to control thymus physiology via paracrine and autocrine mechanisms in addition to the classical endocrine pathway (3, 6–9).

Our previous work as well as data from others (10–13) implicate the natriuretic peptides (NP) in thymus regulation. This peptide family consists of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) and affects regulation of cardiovascular homeostasis (14–16). ANP and BNP are produced predominantly by the heart atrium and ventricle and act mainly as circulating hormones (14–16). In contrast, CNP is the major NP in the central nervous system (15, 16). However, it is also synthesized by endothelial cells and seems to function as a local vascular regulator (15, 16, 17). Most of the biological actions of NP are thought to be mediated by two guanylyl cyclase-linked receptor subtypes (Npra and Nprb) with different ligand selectivities (18). The Npra receptor is activated by ANP and BNP, whereas CNP is considered to be the specific ligand of the Nprb receptor (16, 18, 19). cGMP is thought to act as the second messenger (18, 19). All three NP bind to the C-type receptor (Nprc). This type of receptor lacks guanylate cyclase activity and elicits clearance function of the NP (19, 20).

We showed recently that all three NP are produced by the rat thymus (11). Expression of the thymic NP is regulated by different mechanisms because involution of this organ caused by dexamethasone or x-ray increases ANP but not BNP and CNP production (11, 21, 22). The detection of messenger RNA (mRNA) transcripts for all three NP receptors in the thymus (11) suggests the existence of a local NP system that may intrathymically modulate immune functions.

The aim of the present study was to investigate NP receptor expression on thymic cells and functional aspects of NP/NP-receptor interaction. Thymocytes have been examined for the corresponding Npr messenger RNAs (mRNAs) as well as for their guanylate cyclase response upon exposure to NP. In a second set of experiments, alterations of Npr expression by immunological stimuli as the mitogen Concavalin A (Con A) was evaluated.

Materials and Methods

Cell preparation and culture condition

Rats (Sprague Dawley, male, 100 g) were decapitated, and the thymi quickly removed and either employed for RNA extraction or for isolation of thymocytes. Thymocytes were isolated as described (10) and purified by means of Ficoll (Pharmacia, Freiburg, Germany) gradient centrifugation. Identity and homogeneity of the isolated cell population were assessed by staining cells with a monoclonal mouse antirat thymocyte antigen Thy 1.1 antibody (Serotec Camon, Heidelberg, Germany) and a secondary fluorescein isothiocyanate-labeled antimouse IgG (Becton Dickinson, Heidelberg, Germany). Cells were then submit-

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Address all correspondence and requests for reprints to: Dr. Angelika Vollmar, Institute of Pharmacology, Toxicology and Pharmacy, University of Munich, Koniginstrasse 16, Munich, Germany D-80539.
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ted to flow cytometry (FACScan, Becton Dickinson), and data were expressed in a logarithmic mode based on an accumulation of 10,000 cells.

As positive control for receptor mRNA expression as well as cGMP response, C-6 rat glioma cells transfected with either the complementary DNA (cDNA) of Npra or Nprb (cells have been provided by Dr. Gerzer, Köln, Germany) have been employed in the same manner as thymocytes.

For some experiments, thymocytes have been cultured. Thymocytes (10^6 cells/ml) were suspended in RPMI 1640 medium containing 10% FCS, L-glutamine (2×10^{-3} M) and penicillin (100 U/ml)/streptomycin (100 μg/ml) (all from Gibco, Eggenstein, Germany) and cultured in flasks (175 cm^2, Greiner, Solingen, Germany) for 48 h (37C, 5% CO_2). Con A (1 μg/ml, Boehringer Mannheim, Mannheim, Germany) stimulation was conducted for 48 h.

Before subjecting cells to any experiment, their viability was examined by either trypan blue exclusion or FACS analysis of cells stained with propidium iodide (Sigma, Deisenhofen, Germany). Only batches showing a viability of greater than 90% were included in the study.

Analysis of mRNA coding for Npr

mRNA extraction. Extraction of mRNA from either thymocytes, C-6 cells, or whole thymus tissue was performed as previously described in detail (23). Briefly, total RNA was isolated by the guanidinium thiocyanate/cesium chloride method, and mRNA was purified by means of poly-dT adsorption (PolyATract kit, Promega, Germany).

cDNA synthesis. mRNA (1 μg) was transcribed in cDNA as described before (23). Special attention was paid to evaluate the efficiency of the reverse transcription, *i.e.* the quantitation of cDNA synthesized. For this purpose, 1 μCi of (α-^{32}P)desoxycytidine triphosphate (3000 Ci/mmol, Hartman Analytic, Hannover, Germany) was added to an aliquot of the reaction mix. Incorporated radioactivity was determined after separation of free-labeled dCTP using glass fiber filters (GF/B, Whatman, Maidstone, UK). cDNA concentrations varied up to 100%. Therefore, cDNA concentrations of samples have been adjusted to 10 ng/μl before PCR analysis.

PCR analysis. PCR was performed as described before (23). The following primer pairs were employed according to (24): *Npra* (25) sense primer (third exon) 5′-AAGAGCCTGATAATCCTGAGTACT-3′; antisense primer (sixth exon) 5′-TTGCAGGCTGGGTCCTCATTGTCA-3′. *Nprb* (26) sense primer (third exon) 5′-AACGGGCGCATTGTGTATATCT-GCGGC-3′, antisense primer (sixth exon): 5′-TTATCACAGGATGG-GTCGTCCAAGTCA-3′; *Nprc* (27) sense primer (end of first exon): 5′-ATCGTGCGCCACATCCAGGCCAGT-3′: antisense primer (fifth and sixth exon): 5′-TCCAAAGTAATCACCAATCTCCTGGGTACCCGC-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) according to (28): sense primer 5′-TCCCTCAAGATTGTCAGCAA-3′; antisense 5′-AGATCCACAACGGATACATT-3′.

To each PCR reaction mixture 1 μCi (α-^{32}P)dCTP was added for quantitation of PCR products by measuring incorporated radioactivity. Receptor transcripts were amplified in 30 cycles of 1 min (Npra) or 0.4 min (Nprb, Nprc), denaturation at 93C, annealing at 60C (1 min) for Npra, 61C (0.5 min) for Nprb and 55C (0.5 min) for Nprc and extension at 73C for 2 min (Npra) or 1 min (Nprb, Nprc). Aliquots (6 μl) of the PCR products were submitted to PAGE (7.5% polyacrylamide) and further identified by silver nitrate staining followed by exposure to x-ray films.

Relative quantitation of PCR products. First, conditions for linear amplification were established for the Npra, Nprb, and Nprc as well as for GAPDH mRNA, respectively. Increasing amounts of initial cDNA template were amplified in 30 cycles employing the pairs of primers described above. Furthermore, increasing number of cycles (25–40) were run with constant amounts of cDNA.

For comparison of mRNA expression of the Npr, three different amounts of cDNA that had been proven to be amplified within the linear range (see above) were subjected to PCR. Equal cDNA content of samples was demonstrated by running a PCR for mRNA amplification of the housekeeping gene GAPDH.

After PAGE, the corresponding bands were cut into vials containing 30% H_2O_2 and counted in a β-counter in the presence of scintillation fluid. In all PCR experiments, the presence of possible contaminants was checked by control reactions in which either cDNA was omitted or mRNA was added instead of cDNA. PCR experiments were performed employing three independent RNA preparations of the thymocytes. Two independent RNA preparations were employed from the thymus and one from the transfected C-6 cells. Samples were subjected to PCR in triplicates. Values are expressed as means ± SD. Curves obtained from blotting radioactivity incorporated in the PCR products as a function of the initial amount of cDNA template were evaluated by means of linear regression analysis (95% confidence limits).

Northern blot analysis

mRNA corresponding to about 100 μg total RNA extracted from thymocytes was electrophoresed on a 1% agarose gel and transferred to nylon membranes (Nytran N; Schleicher & Schüll, Dassel, Germany). As a positive control, RNA from rat lung tissue was processed in the same manner. The blots were hybridized with a full length human Nprc cDNA (gift from Dr. Porter, Scios Nova, Inc., Mountain View, CA), and with 1.2-kb fragments from the 5′ end of the rat Npra as well as of the rat Nprb cDNA (gift from Drs. Schulz and Garbers, University of Texas, Dallas, TX). Linearized probes were labeled with [^{32}P]α-uridinetriphosphate (100 μCi) using *in vitro* transcription technique (kit from Boehringer Mannheim). Hybridizations were performed overnight at 63C as described elsewhere (10). Blots were washed with 0.1 × SSC in the presence of 0.1% SDS at room temperature (30 min) and at 63C (45 min) and exposed to x-ray films (−70C) for 6–12 days.

Measurement of cGMP levels

Thymocytes (10^6/tube) either freshly isolated, cultured, or Con A-activated, and C-6 cells (positive control) were incubated in 200 μl RPMI-1640 medium containing 0.5 mM isobutyl-1-methylxanthine (Sigma, Deisenhofen, Germany) for 5 min. This was followed by administration of increasing concentrations (10^{-11}–10^{-6} M) of either rat ANP 99–126 (NovaBiochem, Bad Soden, Germany), rat CNP 1–22 (Peninsula, Heidelberg, Germany) or rat (des-Gln^{18}, Ser^{19}, Gly^{20}, Leu^{21}, Gly^{22})-ANF 4–23-NH_2 (C-ANF, Sigma, Deisenhofen, Germany) for 1 h at 37C, 5% CO_2. Cells were spun down (300 × *g*, 10 min, 4C), the supernatant was collected, boiled for 3 min, and centrifuged (12,000 × *g*, 10 min). cGMP concentrations of supernatants were measured directly by an RIA (29).

Cell proliferation assay

Proliferation of thymocytes was determined by measuring [^{3}H]thymidine incorporation into the acid insoluble fraction of the cells. In brief, cells were seeded at a density of 10^6 cells/ml in 96-well plates (200 μl RPMI medium containing 10% FCS, Gibco, Eggenstein, Germany), and incubated (37C, 5% CO_2) with Con A (0.1–10 μg/ml, 48 h) in the presence or absence of increasing concentrations of the NP (ANP, CNP, C-ANF, 10^{-11}–10^{-6} M) (n = 6–12 each). Cells were pulsed with [^{3}H]thymidine (1 μCi/well, specific activity 25 Ci/mmol, Amersham, Braunschweig, Germany) for another 15 h. Following centrifugation (1200 rpm, 10 min), the supernatant (150 μl) was removed, and cells were incubated with 50 μl 20% trichloroacetic acid at 4C for 30 min before harvest on a glass fiber filter (ICN Biochemical, Meckenheim, Germany) with an autoharvester (Scatron, Norway). Radioactivity incorporated was measured in the presence of scintillation fluid. Experiments have been repeated at least four times.

Measurement of NP degradation

Stability of peptides was assayed by incubating thymocytes with ^{125}I-labeled ANP and CNP (50,000 cpm, specific activity 1000 Ci/mmol, Peninsula, Heidelberg, Germany), respectively, and the corresponding unlabeled peptides (10 nM). Thymocytes were cultured in the presence or absence of Con A as described above. Over the time course of the experiment, supernatants (n = 3) were collected (0 h, 1 h, 2 h, 18 h, 24 h, and 48 h) and analyzed by reverse phase-HPLC (23). Degradation of peptides during culture was estimated by counting radioactivity of HPLC fractions and comparing the profile and amount of eluted radioactivity with that of intact radiolabeled peptide. Stability of C-ANF (4–23) was assayed (n = 2) by measuring a given concentration of the peptide (1 nM) in the medium after 2 h, 24 h and 48 h incubation.

Following HPLC separation, C-ANF (4–23) positive fractions were quantified by a RIA previously described for ANP (23).

Results

Characterization and purity of thymocytes

Special care was taken regarding isolation and purification of thymocytes. A high degree of purity of cells is mandatory because receptor mRNA expression of cells was planned to be examined by the sensitive technique of PCR. Small amounts of nonthymocytes could be responsible for false PCR results. Therefore, identity and homogeneity of each batch of thymocytes were always assessed by labeling cells with a monoclonal mouse antirat thymocyte antigen Thy 1.1 antibody and subsequent analysis by flow cytometry. Figure 1 shows a representative FACS histograph indicating that more than 98% of cells stained for the thymocyte marker.

Expression of mRNA coding for NP receptors in rat thymocytes

RT-PCR and Northern blot analysis were employed to detect NP receptor gene expression in thymocytes. The PCR amplification products were size-fractionated by PAGE electrophoresis and gels exposed to x-ray films. A representative autoradiography (Fig. 2A) shows that thymocytes express all three NP receptors: single bands of the expected size (451 bp Npra; 692 bp Nprb; 573 bp Nprc) were found. Transcripts of the same size were obtained by amplification of cDNA from kidney known to all three NP receptors (14, 15, 19, 30). cDNA from cells transfected with either the Npra or Nprb sequence served as another positive control. For comparison, cDNA from whole thymus tissue was also employed to PCR confirming previous data. To control for contamination, mRNA of cells without RT was amplified and did not yield any PCR products.

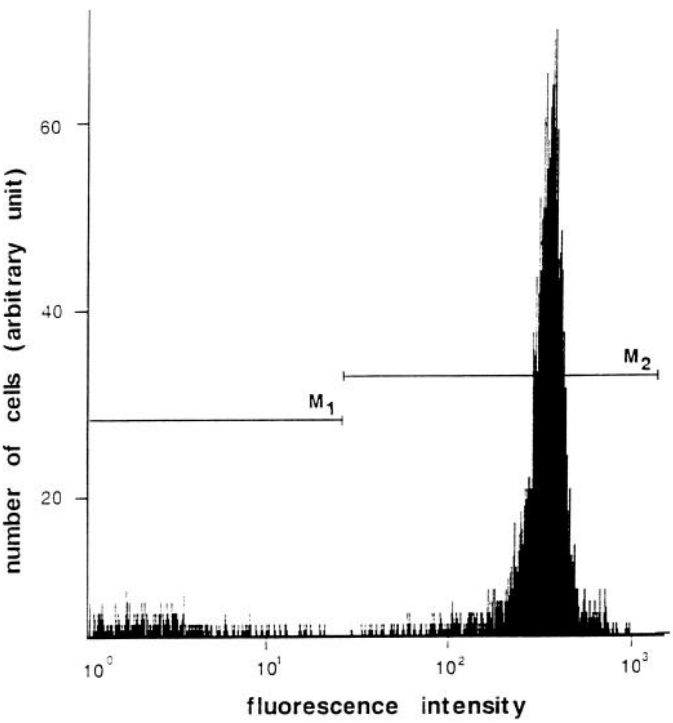

FIG. 1. Analysis of purified thymocytes by flow cytometry using an antibody against rat thymocyte antigen Thy 1.1. A histogram of fluorescence intensity *vs.* counts for cells stained with the antibody is shown. Marker set around stained (M2) and unstained cells (M1) allow determination of the number of thymocytes. For evaluation, the Lysis II FACScan software (Becton Dickinson) was used.

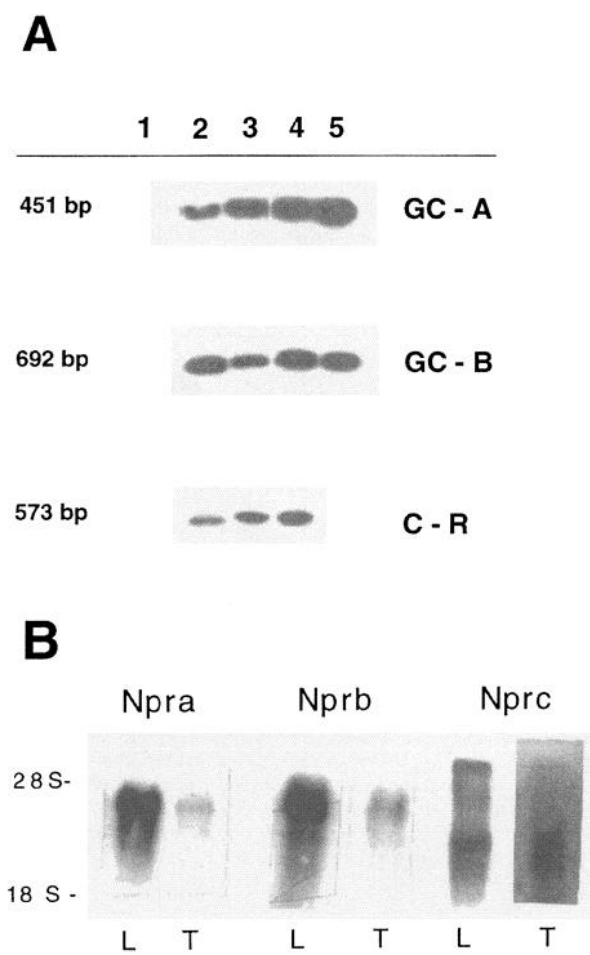

FIG. 2. A, Representative PCR of cDNA transcripts of NP-receptors in thymocytes. Corresponding amplification products were size fractionated by PAGE and exposed to x-ray films. Npra transcripts (451 bp): lane 1, mRNA from thymocytes (10 ng); lane 2, thymocyte cDNA (10 ng); lane 3, thymus cDNA (10 ng); lane 4, kidney cDNA (1 ng); lane 5, Npra transfected C-6 cells cDNA (0.5 ng). Nprb transcripts (692 bp): lane 1, mRNA from thymocytes (10 ng); lane 2, thymocytes cDNA (2 ng); lane 3, thymus cDNA (10 ng); lane 4, kidney cDNA (1 ng); lane 5, Nprb transfected C-6 cells cDNA (0.5 ng). Nprc (573 bp): lane 1, mRNA from thymocytes (20 ng); lane 2, thymocytes cDNA (20 ng); lane 3, thymus cDNA (20 ng); lane 4, kidney cDNA (5 ng). B, Autoradiograph of representative Northern blot of poly(A^+) RNA from thymocytes (T, corresponding to 100 μg total RNA) and lung (L, 30 μg total RNA). RNA was loaded on a 1% agarose gel, transferred to a nylon membrane, and hybridized separately with [^{32}P]α-UTP-labeled cRNA probes for the Npra, Nprb, or Nprc. Films have been developed after 6 days of exposure time except for the last lane at the right side, which has been exposed for 12 days.

PCR data were confirmed by Northern blot analysis of purified mRNA from thymocytes. Hybridization of thymocytes mRNA with radiolabeled Npr cRNA probes yielded a faint hybridization band of approximately 4.5-kb for the

Npra and Nprb mRNA, which comigrates with control mRNA extracted from lung. The Nprc transcript in lung tissue shows considerable size heterogenity. Two major bands at approximately 8 kb and 3.5 kb were detected. Thymocyte mRNA elicits the corresponding hybridization bands at a very low level (Fig. 2B).

Stimulation of cGMP in rat thymocytes by ANP and CNP

To look for the presence of functional receptor proteins on thymocytes, NP-stimulated cGMP accumulation was assayed. As shown in Fig. 3, exposure of cells to ANP significantly increased cGMP concentration up to 3-fold at concentrations of 10^{-7} M and 10^{-6} M. CNP (10^{-6} M) elicited an about 2-fold stimulation of cGMP production. C-ANF (4–23) at concentrations of 10^{-9}–10^{-7} M did not alter cGMP concentration; however, 10^{-6} M of C-ANF (4–23) elicited a tendency to increase cGMP accumulation (up to 15%). C-6 glioma cells transfected with cDNA of Npra and Nprb, respectively, served as positive controls. ANP stimulated cGMP accumulation in these cells at concentrations as low as 1 nM, whereas 100 nM of ANP is necessary to see significant effects on thymocytes. This could be explained by a lower number of binding sites on thymocytes as compared with the transfected cells. Subsequently, the magnitude of increase in cGMP is lower in thymocytes as compared to the transfected cells. Thus, ANP at 1 nM concentration might not elicit a sufficient cGMP response to exceed the threshold of basal cGMP production.

To prove that cGMP production derives from activation of particulate guanylate cyclase, *i.e.* the NP receptors, enzyme activity was also determined in thymocyte membrane preparations showing similar results (data not shown).

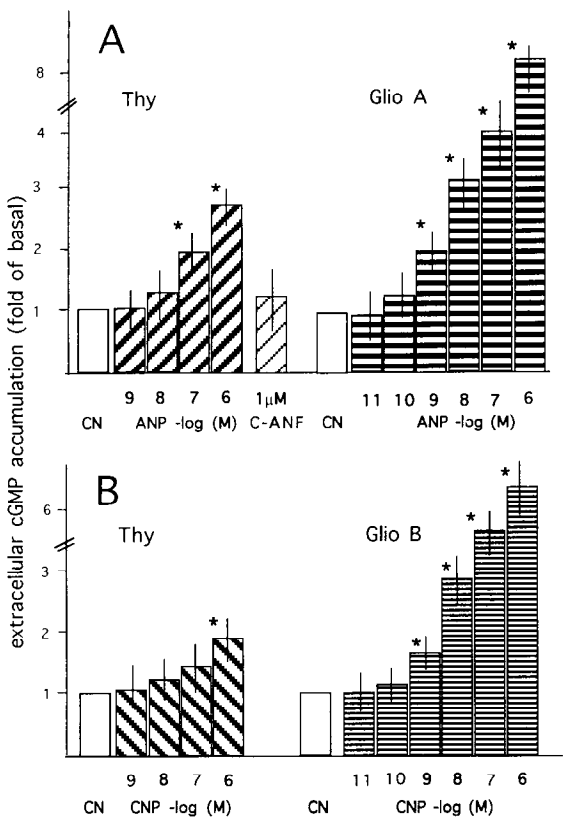

FIG. 3. cGMP responses to ANP (A) or CNP (B) in thymocytes. Freshly isolated thymocytes (Thy) were treated with increasing amounts of either ANP or CNP for 1 h in the presence of the phosphodiesterase inhibitor IBMX (0.5 mM). In addition, C-ANF (10^{-6}M) was tested. As positive control, C-6 glioma cells transfected either with Npra cDNA (panel A, Glio A) or with Nprb cDNA (panel B, Glio B) were treated in the same manner. cGMP concentration in the culture medium was determined by RIA. Data are mean ± SD, n = 3–4; *, $P < 0.05$; *t* test.

Effect of Con A stimulation of thymocytes on Npr-mRNA expression

mRNA of either freshly isolated cells, of cells that had been cultured for 48 h, or of cells exposed to Con A (48 h) were used for PCR-analysis of Npr specific transcripts. In order to compare corresponding mRNA levels, PCR conditions that allow relative quantitation of PCR products had to be established: 1) efficiency of the RT was determined by incorporation of radioactive labeled dCTP and was shown to vary considerably (up to 100%). Consecutively, cDNA contents of samples were equalized. Furthermore, PCR amplification of mRNA coding for the house keeping gene GAPDH was performed with each cDNA preparation in order to note differences in cDNA concentrations (Fig. 4). 2) Each PCR reaction has been checked to remain in the exponential phase determining the appropriate range of initial amount of template at a constant number of cycles (*i.e.* 30). Equal efficiencies of amplification of NP transcripts in the three different cell preparations were demonstrated by parallelity of dose curves (as seen in Fig. 5).

Figure 4 shows a representative experiment performed with three different amounts of initial cDNA subjected in triplicates to PCR for the Npra, Nprb, and Nprc. Bands were cut and incorporated radioactivity was blotted as a function of initial amount of cDNA.

Figure 5 summarizes the results of three independent experiments. Interestingly, stimulation of thymocytes with the lectin Con A (1 μg/ml) leads to an about 4-fold increase of mRNA expression of the ANP specific NP receptor (Npra), whereas Nprb and Nprc expression was not markedly affected by incubation of cells with the mitogen. Apparently, keeping thymocyte in culture itself results in a somewhat higher Npra mRNA concentration (1.5-fold) as compared with freshly isolated cells.

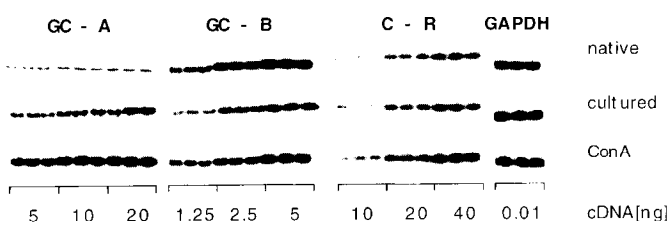

FIG. 4. Representative autoradiographs of PCR transcripts for the Npra, Nprb, and Nprc in freshly isolated thymocytes (native), in thymocytes cultured for 48 h (cultured) and in cells stimulated with Con A (1 μg/ml) (Con A) for 48 h. Three different concentrations of initial cDNA, each in triplicates, were subjected to the corresponding PCR. PCR of GAPDH transcripts was performed to control for similar initial cDNA content of samples. Amplification products were separated by PAGE, and incorporated radioactivity was counted.

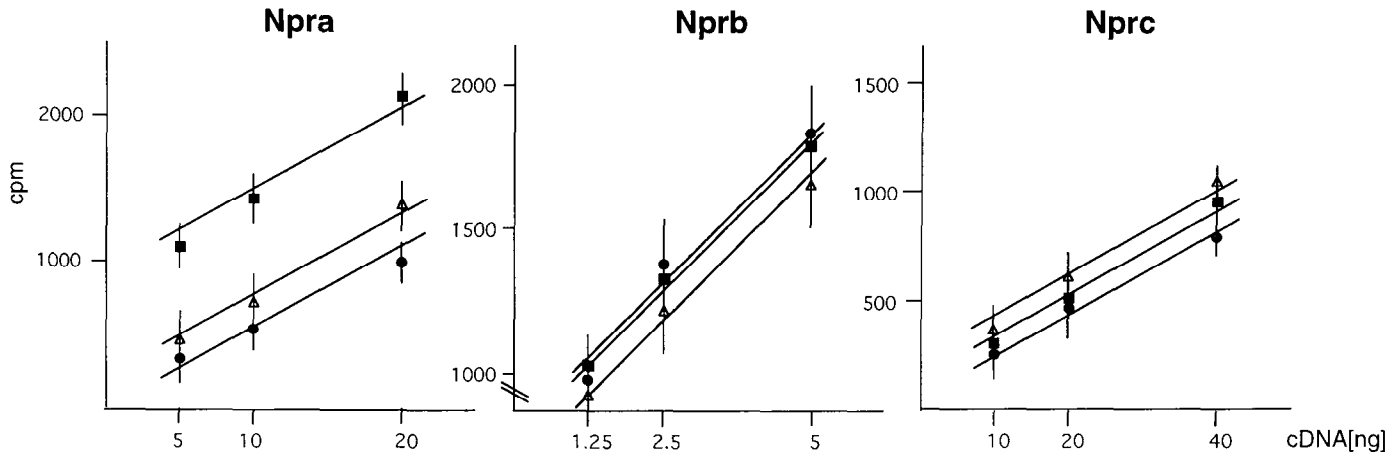

FIG. 5. Graphic line of incorporated radioactivity (cpm) of PCR amplification products blotted against the amount of initial cDNA. Levels of Npra, Nprb, and Nprc transcripts in native (●), cultured (△), and Con A (■) stimulated thymocytes are indicated by the amount of incorporated radioactivity (cpm) as described in *Materials and Methods*. Each value represents the mean of three independent experiments (±SD) run in triplicates.

NP-induced cGMP production of thymocytes upon cultivation and lectin exposure

A 2-day culture of thymocytes with Con A (1 μg/ml) resulted in an about 3-fold augmented cGMP accumulation upon treatment of cells with ANP (10^{-6} M) in comparison with native cells exposed to the same concentration of ANP. As shown in Fig. 6A, the activation is dose dependent ($P < 0.05$; 10^{-8} M–10^{-6} M). Basal cGMP production was slightly higher in cultured thymocytes as well as in stimulated cells than in freshly isolated thymocytes. Thus, when related to cGMP production of cells kept in culture, the ANP-induced guanylate cyclase activity of thymocytes treated with Con A was increased about 2-fold.

In contrast, no significant increase in cGMP production was seen in cultured nor in stimulated thymocytes after exposure to CNP (Fig. 6B). Stability of ANP and CNP over 1-h incubation was evaluated by adding radiolabeled peptides and was shown to be approximately the same (*i.e.* less than 5% degradation).

Effect of NP on [^{3}H]thymidine incorporation of thymocytes

Apparently, stimulation of cell proliferation causes altered receptor expression for NP. Therefore, we examined whether the NP regulate proliferation of thymocytes by determining [^{3}H]thymidine incorporation. Thymocytes were stimulated with 1 μg/ml Con A, a concentration which has been previously shown to elicit submaximal proliferation of cells (data not shown). As shown in Fig. 7A, addition of ANP at concentrations of 10^{-9} M–10^{-6} M, significantly inhibited mitogen-stimulated thymidine incorporation. Proliferation of cells that had not been exposed to Con A was not altered by ANP (data not shown). ANP (10^{-6} M) inhibited Con A induced cell growth by a mean of 50%, as calculated from six independent experiments set up with n = 12 samples for each treatment. CNP, however, did not share the inhibitory properties of ANP. C-ANF, a specific ligand of the Nprc receptor, was also not able to mimic the effect of ANP, suggesting that the suppression was mediated via the Npra receptor. Stability of the three peptides over the time course of the experiments was evaluated as described in *Materials and Methods*. The rates of degradation of the peptides are in a comparable range, although CNP seems to be less stable than ANP after an incubation time of 18 h and more. Degradation (expressed as % of intact NP) after 18 h amounts to about 30%

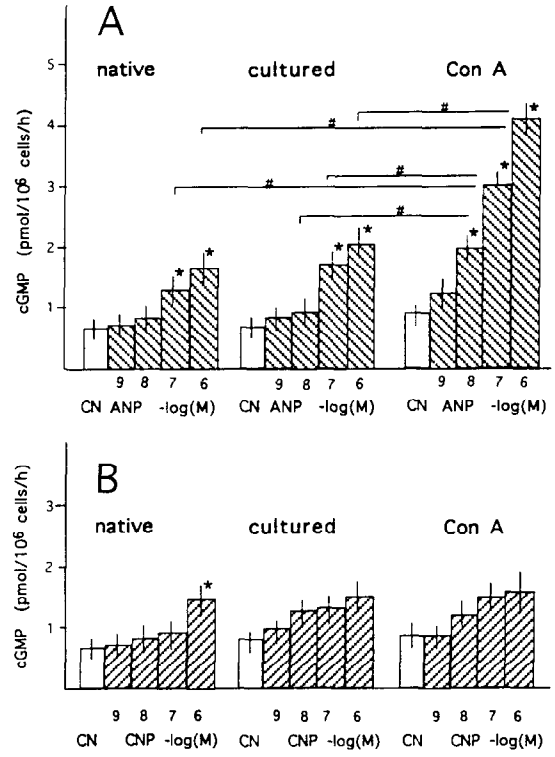

FIG. 6. cGMP production of native thymocytes, cultured (48 h), and Con A-stimulated cells. Each batch of cells was exposed to increasing concentration of either ANP (A) or CNP (B) for 1 h in the presence of 0.5 mM IBMX. cGMP concentration in the medium was determined by RIA. CN means basal cGMP production. Values represent mean ± SD, n = 3–4. *, $P < 0.05$ compared with the corresponding basal value (CN), #, $P < 0.05$ compared with the corresponding treatment of cells, *t* test.

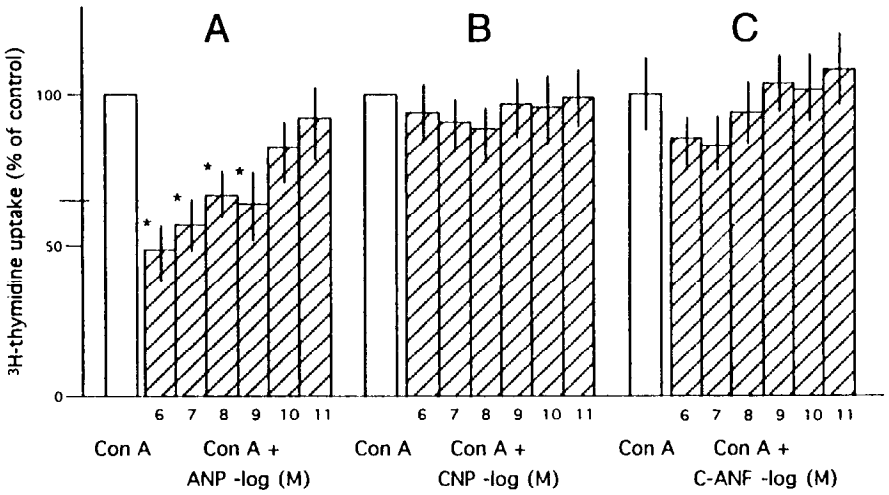

FIG. 7. Effects of NP on Con A-stimulated incorporation of [^{3}H]thymidine in thymocytes. Cells (2×10^5/well) were treated with Con A (1 μg/ml, 48 h) with or without decreasing concentrations (10^{-6} M–10^{-11} M) of ANP (panel A), CNP (panel B), and C-ANF (panel C). Thymocytes were then pulsed with [^{3}H]thymidine (1 μCi/well) for another 15 h. Values are normalized to levels found in Con A-stimulated cells (100%) and are given as means ± SD, n = 6–12. *, $P < 0.05$, t test. Each experiment was performed at least four times.

for ANP and 46% for CNP. After 24 h of incubation, 50% of ANP, 69% of CNP, and 57% of C-ANF were found to be degraded. Finally 63% of ANP, 81% of CNP, and 75% of C-ANF had been lost after a 48-h exposure to the cells.

Discussion

We have previously shown that ANP, BNP, and CNP, peptides mainly known for their distinct effects in the cardiovascular system, are coexpressed in rat thymus (10, 11). The fact that the three NP receptors, namely Npra, Nprb, and Nprc, seemed also to be present in the thymus prompted the question of a function for this local thymic natriuretic peptide system (11). The present study represents a step-by-step approach towards this question. We found that:

1) Thymocytes have been identified as a possible population of target cells for NP. mRNAs of the three NP receptors were detected in isolated thymocytes by RT-PCR as well as by Northern blot and presence of functional Npra and Nprb receptors was demonstrated by increased cGMP production upon exposure of cells to NP.
2) Expression of NP receptors is differentially modulated by cell activation. Thymocytes stimulated with Con A express higher concentrations of mRNA coding for Npra as well as increased guanylate cyclase activity upon ANP exposure. Nprb and Nprc seemed not to be affected by Con A.
3) ANP inhibits cell proliferation of Con A-activated thymocytes, whereas CNP and C-ANF do not show this effect.

This is the first study describing an effect for endogenous ANP on the thymus. Binding sites for ANP in thymus and thymocytes, however, have been reported before (31, and J. Gutkowska, Montreal, personal communication). However, at that time the existence of different NP receptor subtypes as well as the existence of two other NP had not been elucidated yet.

NP receptors have been reported on bone marrow derived stromal cells (32), and the authors discussed that ANP receptors might be also localized on thymic stromal cells. Thus, in addition to thymocytes, other thymic cells could express receptors for NP. Therefore, we paid special attention to the degree of purity of cells subjected to mRNA analysis by RT-PCR.

With regard to the relative distribution of the three NP receptors, the Nprb seems to be preferentially expressed on thymocytes. This assumption is based on the low amounts of thymocyte cDNA necessary to obtain a PCR signal in comparison with whole thymus tissue. Furthermore, Nprb expression may also exceed that of Npra and Nprc on thymocytes. An exact analysis of degree of Npr expression, however, requires absolute quantification of PCR products, which cannot be achieved using our PCR protocol.

This observation at the mRNA level is not reflected by the functional data of Npr expression, *i.e.* stimulation of cGMP production: ANP was more effective than CNP in stimulating guanylate cyclase-activity of thymocytes. Similar discrepancies have been reported by others (33–36). It is very difficult to speculate about the relationship between the amount of mRNA level and the number and activity of receptors present on the cell surface. Translational processes may be different between Npra and Nprb receptors, *i.e.* the rate of translation as well as the stability of the protein, may be lower in the case of the Nprb. Alternatively, the catalytic activity of the two receptors might differ as was previously suggested (33–36). Finally, CNP might not be the most potent ligand for the Nprb or thymocytes express a Nprb subtype with different ligand specificity. Moreover, the two receptors may mediate different functions (34). This latter notion is supported by the observations reported here, namely that the expression of Npr underlies different regulatory mechanism. The Npra, but not the Nprb and Nprc, are up-regulated in response to activation of cells with a mitogen. Subjecting cells to *in vitro* culture itself increased Npra expression. The increase, however, was modest in comparison with that induced by Con A. In this context, subtype switching of ANP receptors has been recently described for chondrocytes and aortic smooth muscle cells during culture (37, 38). The mechanism underlying such alteration of receptor distribution remains to be elucidated.

One may speculate that an increased expression of receptors represents a mechanism rendering the cells more sen-

sitive for an effect of the corresponding ligand. The fact that exposure of cells to the mitogen Con A increases Npra receptor mRNA expression supports a possible relationship between ANP/Npra mediated interaction and thymocyte activation. Indeed, ANP was shown to inhibit thymocyte proliferation induced by Con A. However, there is an apparent lack of correlation between the dose response curve for ANP stimulation of cGMP production and growth inhibition. A concentration of 10–100 nM ANP was necessary to elicit a significant increase of cGMP accumulation. These concentrations are in the range of those reported for bone marrow-derived stromal cells, aortic smooth muscle cells, or uterus tissue (32, 33, 35, 39). In contrast, ANP at concentrations as low as 1 nM significantly blocked proliferation of Con A-stimulated thymocytes. This kind of discrepancy has previously been reported for vascular smooth muscle cells (33). As mentioned by Porter *et al.* (33), submaximal levels of cGMP may be sufficient to inhibit growth.

NP have been shown before to be able to interfere with cell growth: an antimitogenic function of the peptides has been reported for other cell types such as cardiac fibroblasts (40), glia cells (41), endothelial (42), and vascular smooth muscle cells (33, 39, 43). The observation that CNP did not show any antigrowth effect on thymocytes in contrast to other cells (33, 39–41) was surprising. Degradation of CNP over the time of the experiment was 20–30% higher than that of ANP. However, this difference in stability is unlikely to account for the lack of effect on thymocyte proliferation by CNP because ANP is active over a three-log range of concentrations. The lack of response may be rather explained by the missing stimulation of Nprb, the specific receptor of CNP, by Con A.

Regarding the NP-receptor type that mediates the antiproliferative effect, both the guanylate cyclase coupled and the Nprc receptors have been reported to be responsible (39–43). Increased Npra upon Con A incubation and, moreover, the lack of effect of C-ANF, the specific Nprc-receptor ligand on thymocyte thymidine incorporation, suggest the guanylate cyclase linked receptor to promote this effect.

Primary thymocytes represent a highly heterogeneous cell population (44). In this context, it is worth mentioning that Con A at a concentration of 1 μg/ml induces only submaximal level of cell proliferation (data not shown), and there is evidence that lower concentrations of Con A preferentially activate CD4 T-lymphocytes (45). Thus, most likely only a part of the total thymocytes responds to Con A and subsequently to ANP. CD4 positive thymocytes may be the main target cells for ANP, and the peptide should elicit a quite significant effect on the basis of single responsive cells. Elucidation of the exact cell type bearing NP receptors will be helpful to find a distinct role for ANP in the thymus. Thymocytes proceed through distinct stages of proliferation and differentiation: for instance, thymocyte precursors rapidly proliferate and then cease proliferation to go through maturation and selection processes (44). ANP might inhibit thymocyte proliferation to allow differentiation to occur. Similar roles have been proposed for met-enkephalin-containing peptides in the thymus (46) and for a variety of other neuropeptides such as OT and AVP (47) as well as PRL (48).

In conclusion, we have demonstrated an effect of endogenous atrial natriuretic peptide on thymocyte proliferation. This effect seems to be mediated by the Npra receptor, which was shown to be up-regulated following stimulation with Con A. The functional significance of thymic CNP, however, remains to be elucidated. The data suggest that endogenous natriuretic peptides affect thymus physiology in a similar manner as cytokines or growth factors. This is a novel aspect in the biology of natriuretic peptides.

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