Proliferative Actions of Natriuretic Peptides on Neuroblastoma Cells

INVOLVEMENT OF GUANYLYL CYCLASE AND NON-GUANYLYL CYCLASE PATHWAYS

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To identify neural tumor cell lines that could be used as models to study growth-related natriuretic peptide actions, we determined the effects of these peptides on the proliferation of human and rodent neuroblastoma cell lines. Subnanomolar concentrations of atrial natriuretic peptide (ANP) and type C natriuretic peptide (CNP) stimulated proliferation in all four cell lines. These actions were associated with cGMP elevation and were blocked by a protein kinase G inhibitor. These data imply the involvement of guanylyl cyclase (GC)-coupled natriuretic receptors. However, higher concentrations of ANP and CNP, and low concentrations of des-[Gln\(^{18}\),Ser\(^{19}\),Gly\(^{20}\),Leu\(^{21}\),Gly\(^{22}\)]-ANP\(_{4-23}\)-NH\(_{2}\) (desANP\(_{4-23}\)) (analog for NPR-C receptor) exerted antiproliferative actions in three of the cell lines. These effects were insensitive to a protein kinase G inhibitor and to HS-142-1, suggesting that growth-inhibitory actions involved a non-GC receptor. They did not appear to involve cAMP, protein kinase A, protein kinase C, or calcium mobilization but were abolished when constitutive mitogen-activated protein kinase activity was inhibited. Radioligand binding experiments revealed the presence of a uniform class of binding sites in NG108 cells and multiple binding sites in Neuro2a cells. Northern and reverse transcriptase-polymerase chain reaction analyses revealed differential gene expression for NPR-A/B/C in NG108 and Neuro2a cells. The results indicate that natriuretic peptides stimulate neuroblastoma cell proliferation through type NPR-A/B (GC) receptors. Higher concentrations of ANP and CNP exerted a mitogen-activated protein kinase-dependent antiproliferative action mediated by a non-GC receptor that interacts with desANP\(_{4-23}\) with relatively high affinity.

Natriuretic peptides constitute a family of structurally related hormones that includes atrial natriuretic peptide (ANP), brain natriuretic peptide, and the type C natriuretic peptide (CNP) (1, 2). These peptides interact with overlapping specificity on three known receptors (3, 4). Receptor subtypes A and B (NPR-A and NPR-B, respectively) contain a single transmembrane domain and possess intrinsic GC activity (5, 6). The type C receptor (NPR-C) is similar to types A and B but lacks the intracellular GC domain (7). Despite widespread expression of NPR-C, initial studies were unable to identify its signaling pathways. Because NPR-C was found to internalize after binding natriuretic peptides, it was proposed that the receptor is involved in removing ANP peptides from the circulation (8). It has thus been commonly referred to as the “clearance” receptor. However, more recent studies that have utilized relatively specific ligands for NPR-C suggest that it may be positively coupled to phospholipase C (9) and adenylyl cyclase (10) or negatively coupled to adenylyl cyclase (reviewed in Ref. 4) and MAP kinase pathways (11).

Natriuretic peptides have been shown to inhibit the proliferation of several cell types, including vascular smooth muscle cells (12), kidney mesangial cells (13–15), chondrocytes (16), osteoblast-like cells (17), and hepatoblastoma cells (18). In addition, a recent report indicated that the NPR-C-mediated inhibition of astrocyte proliferation occurs in association with decreased MAP kinase activation (11). In contrast to these inhibitory actions, ANP stimulated the proliferation of embryonic cardiomyocytes (19), and both CNP and brain natriuretic peptide were found to stimulate longitudinal bone growth in vitro (20). Moreover, NPR-C knockout mice and transgenic mice overexpressing brain natriuretic peptide exhibit pronounced skeletal overgrowth (21, 22). Data suggest that elevation of cGMP may be involved in some of type A and B receptor-mediated proliferative responses (13, 15, 17, 19), possibly through regulation of the MAP kinase-selective phosphatase MPK-1 (23) and/or phosphorylation of platelet-derived growth factor receptor (24).

Natriuretic binding sites are reported to be present in the proliferative zones of the embryonic brain (25) and in neuroblastoma tumor cell lines (26). Neuroblastoma cells have also been reported to express natriuretic peptides (27) and to secrete certain endopeptidases (28) that are capable of cleaving natriuretic peptides. Despite these findings, relevant biological actions of natriuretic peptides in embryonic neuroblasts and neuroblastoma cells have not been elucidated. Here, we investigated the hypothesis that natriuretic peptides regulate the proliferation of neuroblastoma cells through specific ANP receptor subtypes. These studies utilized ANP, which binds with high affinity to NPR-A and NPR-C; CNP, which binds with...
highest affinity to NPR-B; and an NPR-C-specific agonist des-(Gln23, Ser26, Gly27, Leu28) ANP4-23. We also studied the sensitivity of the proliferative actions to various kinase inhibitors and characterized at molecular and pharmacological levels the type of ANP receptors that were expressed. Finally, we measured the peptide-induced changes in the levels of cyclic second messengers, cAMP and cGMP, by radiomunoassay and intracellular calcium by fura-2 cytofluorometry. The data obtained indicate that natriuretic peptides stimulate proliferation through NPR-A and/or NPR-B (GC) receptors, whereas antiproliferative actions appear to be mediated by a non-GC natriuretic receptor that interacts with desANP4-23 with relatively high affinity.

MATERIALS AND METHODS

Cell Cultures—Neuro2a neuroblastoma cells were obtained from American Type Tissue Collection (Manassas, VA). SK-N-SH neuroblastoma sublines SHIN and SY-5Y were obtained from Dr. June Biedler (Sloan Kettering Cancer Institute, Rye, NY). NG108 neuroblastoma/glioma hybrid cells were obtained from Dr. Chris Evans (UCLA). All cell lines were maintained in 75-cm² flasks (Falcon) in 20 ml of Dulbecco’s modified Eagle’s medium (Cellbio/Fisher). Medium was supplemented with 10% bovine serum (FBS) (Life Technologies), 1% penicillin/streptomycin, 2 mM glutamine, 20 mM HEPES, 100 units/ml of penicillin (Life Technologies), and 100 μg/ml of streptomycin (Sigma). Time course and saturation studies were performed in six-well plates (Falcon) seeded 24 h earlier with 400,000 cells/well in 1 ml of medium and cultured for 24 h. Medium was then replaced with fresh serum-free medium. After incubation for 15 min at 37°C, cells were preincubated for an additional 1 h with vehicle or signal transduction inhibitors (GF109203X, H89, and Rp-8-pCTP-cGMPS from Calbiochem and PD908059 from New England Biolabs) or the NPR-A/NPR-B antagonist HS-142-1 (kindly provided by Kyowa Hakko Kogyo Co., Shizuoka, Japan). Inhibitor efficiencies and specificities were tested in preliminary studies across a range of concentrations (from 1 to 100 μM) using stimulators of the specific kinases (PACAP for protein kinase A, platelet-derived growth factor for mitogen-activated protein kinase, and ANP for G kinase). The lowest doses causing full but specific inhibitions were used in subsequent assays. After preincubation with the specified inhibitors, peptides (desANP4-23, ANP, and CNP from Sigma) were added for 1 h prior to distribution of [3H]thymidine (1 μCi/well). Four hours after radiotracer addition, cells were rinsed with phosphate-buffered saline. Total RNA from control tissues (containing 8% serum), and cells were centrifuged and then rinsed with phosphate-buffered saline. Total RNA from control tissues was extracted and purified according to the method of Chomczynski and Sacchi (31). Subsequent poly(A)-selection of mRNA was accomplished using the Poly(A)Pure™ kit (Ambion) according to the manufacturer’s instructions.

RT-PCR Analysis of Natriuretic Peptide Receptor Gene Expression in Neuro2a Cells—RT-PCR was performed on RNA from Neuro2a cells and NG108 neuroblastoma cells, as well as control mouse brain, kidney, and liver tissues were loaded (6 μg/lane) on a 0.8% agarose gel/final concentration of 3.5%, and 279–271 for natriuretic receptor subtypes A, B, and C respectively.

Purification of mRNA—Neuro2a and NG108 cells were cultured in seven flasks (75 cm²) and harvested at 90% confluence with 0.05% trypsin, 0.02% EDTA. Trypsin was neutralized with the tissue culture medium (containing 8% serum), and cells were centrifuged and then rinsed with phosphate-buffered saline. Total RNA from control tissues (brain, kidney, and lung) was obtained from two male ND4 mice. Total RNA from cells and tissues was extracted and purified according to the protocol of Chomczynski and Sacchi (31). Subsequent poly(A)-selection of mRNA was accomplished using the Poly(A)Pure™ kit (Ambion) according to the manufacturer’s instructions.

Western Blot Analyses—Poly(A)-selected mRNA from Neuro2a and NG108 neuroblastoma cells, as well as control mouse brain, kidney, and liver tissues were loaded (6 μg/lane) on a 0.8% agarose 2% formaldehyde–MOPS gel (30, 32). After transfer to nylon membranes (MSI), blots were prehybridized for 2 h and then hybridized for 16 h in Ultrahyb™ hybridization solution (Ambion) at 44°C. Northern hybridizations were performed sequentially (NPR-C, NPR-B, and NPR-A, respectively) using 100 ng/ml of each probe. Probes consisted of EcoRI-excised cDNA inserts (see below) labeled by random incorporation of [32P]dCTP (Random Primers kit; Life Technologies, Inc.). Membranes were washed twice in 2× SSC, 0.1% SDS solution at 44°C for 5 min, and then washed twice in 0.1× SSC, 0.1% SDS for 15 min at 44°C. Blots were exposed for 3 days and signals detected with a PhosphorImager (Molecular Dynamics, Inc.).

Natriuretic receptor probes were obtained by RT-PCR using mouse kidney total RNA as template. Primers (Life Technologies, Inc.) were designed using the on-line Primer3 software (33) based on mouse or rat natriuretic peptide receptor sequences published in the NCBI data base (GenBank accession numbers L31932, X14177, and D78517). The sense primers for natriuretic receptor subtype A, B, and C were 5′-AAGGTGAGGAGTCTGGTAGATGGACGTTTT-3′, 5′-GTTCTCCAGGTTGCTCATGAC-3′, and 5′-GCGAAGCTATGATCCAATGGC-3′, respectively. These were designed to generate PCR fragments of 389, 379, and 492 base pairs, corresponding to nucleotides 1790–1831, 1771–1764, and 1754–1734 of the sequences of natriuretic receptor subtypes A, B, and C, respectively.

Amplifications were carried out for 35 cycles of denaturation (94°C, 30 s), annealing (54°C, 45 s), and extension (72°C, 45 s). PCR was finished by an incubation for 5 min at 72°C. RT-PCR reactions yielded products of the expected size. To validate the nature of the PCR products, one-fifth of the PCR products were run on 2% agarose gels, followed by overnight transfer to nylon membrane (Magna, MSI). Membranes were baked, UV-cross-linked, and then prehybridized for 6 h at 37°C in the hybridization solution described (30). Hybridizations were performed at 37°C for 6 h, using the following [32P]-end-labeled internal oligonucleotides: 5′-GCGTGGTAGATGGACGTTTT-3′, 5′-GTCTCAGGTTGCTCCAGCACAG-3′, and 5′-GTTGAGGAGTCTGGTAGATGGACGTTTT-3′. These corresponded to the nucleotides 1996–2015, 579–598, and 715–734 of the sequences of natriuretic receptor subtypes A, B, and C respectively.

These primers corresponded to highly conserved regions between mouse and rat natriuretic receptor subtypes A, B, and C. Amplifications were carried out for 35 cycles of denaturation (94°C, 30 s), annealing (54°C, 45 s), and extension (72°C, 45 s). PCR was finished by an incubation for 5 min at 72°C. RT-PCR reactions yielded products of the expected size. To validate the nature of the PCR products, one-fifth of the PCR products were run on 2% agarose gels, followed by overnight transfer to nylon membrane (Magna, MSI). Membranes were baked, UV-cross-linked, and then prehybridized for 6 h at 37°C in the hybridization solution described (30). Hybridizations were performed at 37°C for 6 h, using the following [32P]-end-labeled internal oligonucleotides: 5′-GCGTGGTAGATGGACGTTTT-3′, 5′-GTCTCAGGTTGCTCCAGCACAG-3′, and 5′-GTTGAGGAGTCTGGTAGATGGACGTTTT-3′. These corresponded to the nucleotides 1996–2015, 579–598, and 715–734 of the sequences of natriuretic receptor subtypes A, B, and C respectively.
buffer (1× SSC, 0.1% SDS) at 40 °C. Signals were detected using a PhosphorImager (Molecular Dynamics) (exposure time from 2 h to overnight).

RESULTS

Action of ANP Analogs on Proliferation—Thymidine incorporation was determined during the four final hours of a 5-h period of peptide treatment. These studies revealed that natriuretic peptides modulated proliferation in all neuroblastoma cell lines tested. Natriuretic peptides stimulated NG108 growth dose-dependently (Fig. 1A). Proliferation was significantly stimulated by ANP at 0.1 nM and reached a nearly maximum 33% increase in proliferation at 10 nM. CNP also stimulated the growth of these cells, although less potently. In contrast, the NPR-C-selective analog desANP4–23 (29) had no effect on NG108 cells. In all other neuroblastoma cell lines, a biphasic action of ANP and CNP was observed (Fig. 1, B–D). ANP and CNP slightly but significantly stimulated proliferation in all of these cell lines at subnanomolar concentrations but inhibited cell proliferation at higher doses. Treatment with desANP4–23 did not stimulate proliferation at any concentration but inhibited growth beginning at 0.1 nM. Stimulatory and inhibitory effects of natriuretic peptides were unaffected by o-phenanthroline and bacitracin (data not shown), which have been shown to potently inhibit endopeptidase activities that cleave natriuretic peptides in other neuroblastoma cells (28). This indicates that the actions of natriuretic analogs were not likely to be due to degradation products.

To obtain information regarding the signaling pathways used by natriuretic peptides to control cell proliferation, peptide actions were studied in the presence and absence of various protein kinase inhibitors. NG108 and Neuro2a cells were selected for these studies. To determine the role of cGMP signaling, cells were pretreated with Rp-8-pCTP-cGMPS (20 μM), a protein kinase G (PKG) inhibitor (34). Stimulatory effects of natriuretic peptides in both cell lines were completely blocked by Rp-8-pCTP-cGMPS (Fig. 2, A and B). In Neuro2a cells, blockade of this stimulation revealed more potent and pronounced growth inhibitory effects of ANP and CNP (Fig. 2B versus Fig. 1D).

In contrast to the PKG inhibitor, the MEK1/2 kinase inhibitor PD98059 (35) did not block the induction of proliferation by ANP or CNP, although it significantly decreased the basal rate

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**FIG. 1.** Thymidine incorporation in four different neuroblastoma cell lines. Cells were incubated for 5 h with increasing concentrations of natriuretic peptide analogs, and assays were performed as described under “Materials and Methods.” A, effects of natriuretic peptides on thymidine incorporation in NG108 cells. B, effects of natriuretic peptides on thymidine incorporation in SH-IN. C, effects of natriuretic peptides on thymidine incorporation in SY-5Y. D, effects of natriuretic peptides on thymidine incorporation in Neuro2a. Peptide order is as follows: control, ANP, CNP, and DesANP4–23. A key for the bars is shown in the first graph. Data (mean ± S.E.) are representative of four independent experiments, each performed in triplicate. Statistical analysis of the data (analysis of variance) has been performed (*, p < 0.05; ***, p < 0.01; ***, p < 0.005).

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**FIG. 2.** Effects of PKG inhibitor Rp-8-pCTP-cGMPS on natriuretic peptide-induced modulation of thymidine incorporation in NG108 (A) and Neuro2a (B) cells. Cells were stimulated for 5 h with increasing concentrations of natriuretic peptides in the presence of 20 μM Rp-8-pCTP-cGMPS, and assays were performed as described under “Materials and Methods.” Basal incorporation is indicated as Control bars in the absence and presence of kinase inhibitor. The inset at the bottom shows a key for peptide bars. Effects of natriuretic peptides in the absence of Rp-8-pCTP-cGMPS are shown in Fig. 1. Data (mean ± S.E.) are representative of two independent experiments each performed in triplicate. Statistical analysis of the data (analysis of variance) has been performed (*, p < 0.05; **, p < 0.01; ***, p < 0.005).
of proliferation of NG108 cells at the concentration of 20 μM (Fig. 3A). The same was true in Neuro2a cells (Fig. 3B).

Growth-inhibitory actions of higher doses of ANP, CNP, and desANP4-23 in Neuro2a cells were no longer observed in the presence of PD98059. Thus, the antiproliferative actions of natriuretic peptides appeared to require basal activity of a MEK1/2 pathway. Inhibitors of protein kinase A (H89, 20 μM) (36) and PKC (GF109203X, 10 μM) (37) did not significantly alter the inhibitory actions of any of the natriuretic analogs tested (data not shown).

One possible explanation for the inhibitory actions of high concentrations of ANP and CNP in SK-N-SH subclones and Neuro2a cells is that NPR-A and/or NPR-B switch from GC activation to MAP kinase inhibition when presented with higher concentrations of ligands and thereby inhibit proliferation. To determine if both effects were mediated by NPR-A and/or NPR-B, DNA synthesis was determined in Neuro2a and NG108 cells incubated with natriuretic peptides in the presence or absence of the NPR-A/NPR-B-selective antagonist HS-142-1 (38) (Fig. 4). The stimulatory actions of the natriuretic peptides ANP and CNP on NG108 cells were antagonized by HS-142-1, confirming that the growth-stimulatory actions of natriuretic peptides were mediated by GC-coupled receptors (Fig. 4, A and B). In contrast, the antiproliferative actions of ANP, CNP, and desANP4-23 in Neuro2a cells were unaffected by this drug (Fig. 4C), indicating that the growth-inhibitory effects were probably not mediated by NPR-A or NPR-B. HS-142-1 (20 μg/ml), by itself, had a negligible effect on DNA synthesis in untreated Neuro2a and NG108 cells.

**FIG. 3.** Effects of the MAP kinase inhibitor PD98059 on natriuretic peptide-induced modulation of thymidine incorporation in NG108 (A) and Neuro2a (B) cells. Cells were stimulated for 5 h with increasing concentrations of natriuretic peptides in the presence of inhibitor (30 μM), and assays were performed as described under “Materials and Methods.” The inset at the bottom shows peptide representations. Basal incorporation is indicated as Control bars in the absence and presence of kinase inhibitor. Effects of natriuretic peptides in the absence of inhibitor are shown in Fig. 1. Data (mean ± S.E.) are representative of two independent experiments each performed in triplicate. Statistical analysis of the data (analysis of variance) has been performed (*, p < 0.05; **, p < 0.01; ***, p < 0.005).

**FIG. 4.** Thymidine incorporation assays in NG108 (A and B) and Neuro2a (C) cells stimulated for 5 h with increasing concentrations of natriuretic peptides in the absence or presence of 10 or 20 μg/ml HS-142-1 antagonist. Data (mean ± S.E.) are representative of two independent experiments, each performed in triplicate. Statistical analysis of the data (analysis of variance) showed significant differences in peptides versus control on thymidine incorporation (p < 0.05) but no differences on peptide effects due to HS-142-1 addition.
concentrations of the natriuretic peptides ANP, CNP, and desANP 4–23 were tested and compared with peptide histidine isoleucine (PHI), an analogue of the neuropeptide VIP. Peptide symbols are explained in the figure.

isobutylmethylxanthine with or without forskolin. None of the ANP analogs affected cAMP levels under these conditions (data not shown). Changes in intracellular calcium levels were measured in Neuro2a cells grown on glass coverslips, in response to 0.1, 10, or 20 nM concentrations of natriuretic peptides ANP, CNP, and desANP 4–23. None of the natriuretic peptides induced changes in intracellular calcium levels (data not shown). In contrast, other neuropeptides (1 nM PACAP-38, 10 nM PACAP-27, or 20 nM VIP), used as internal positive controls, stimulated increases in intracellular calcium, presumably due to action of these peptides on the PACAP-preferring PAC1 receptor in these cells (30).

**Pharmacological Characterization of Natriuretic Receptors**—The radiotracer 125I-ANP bound specifically to both NG108 and Neuro2a cells, in a time-dependent and saturable fashion. Nonspecific binding was less than 25% of the total binding at the equilibrium. Kinetic (Figs. 6, A and B) and saturation (Figs. 6, C and D) experiments were performed in both Neuro2a and NG108 cells to assess binding parameters. In absence of native peptide, total binding in both cell lines increased nonlinearly with time and did not reach a maximum by 200 min. However, after subtraction of nonspecific binding, binding equilibrium was reached after a 50–100-min incubation at 4 °C in NG108 and Neuro2a, respectively. Subsequent experiments were done after a 120-min incubation at 4 °C. Under these conditions, specific 125I-ANP binding increased with concentration of the radiotracer and appeared saturable in both cell lines (Fig. 6, C and D). Linearization of the time course plots (data not shown) revealed one class of binding sites in NG108 cells, characterized by an apparent association constant (K_d) of 0.034 min⁻¹, whereas two classes of binding sites were revealed in Neuro2a cells. These two classes are characterized by different K_d values (0.018 and 0.06 min⁻¹). Scatchard plots (data not shown) allowed a direct access to the binding parameters. However, to determine more precisely the values of B_max and K_d, a nonlinear regression was run using Graphprier software. In NG108 cells, a single class of binding sites was observed with a K_d of 42 ± 1.3 pm and a B_max of about 650 sites/cell. Neuro2a had high (18.6 pm) and low (K_d = 0.64 nM) affinity binding sites. The density of these binding sites is given by B_max values of about 210 and 310 sites/cell for high and low affinity sites, respectively.

Pharmacological profiles of ANP binding sites were determined on NG108 and Neuro2a cells, using displacements of 125I-ANP by increasing concentrations of ANP, CNP, and ANP 4–23. For NG108 cells, displacement curves (Fig. 7A) and derived IC50 values (Table I) indicated that natriuretic peptides displaced 125I-ANP with the following potency: ANP > CNP > desANP 4–23. Hill values were near unity for all analogs, suggesting that ligand/receptor interactions occurred on an apparent single 125I-ANP binding site. The observed analog rank potency suggests that the main receptor on NG108 cells is NPR-A (3).

In Neuro2a cells, displacement curves revealed a more complex situation. Both CNP and desANP 4–23 were as potent as unlabeled ANP in displacing 125I-ANP (Fig. 7B). Using a one-site competition equation to graph the displacement curves, Hill values obtained were below 0.7 (Table I). This suggested that natriuretic peptides interact on Neuro2a cells through more than one binding site. This is more apparent in Fig. 8A, which shows the full set of data points for displacement by CNP and desANP 4–23. However, these data suggest that two distinguishable binding sites are present in Neuro2a cells.

To further analyze the nature of the binding sites on Neuro2a cells, we performed displacement of 125I-ANP with the NPR-A/NPR-B antagonist HS-142-1. This compound was able to maximally inhibit about 65% of 125I-ANP–specific binding, with an IC50 of about 7 µg/ml (Fig. 8B). This suggested that about 65% of the binding was due to interaction of 125I-ANP with NPR-A and/or NPR-B, while the remaining 35% was due to interaction with another site. To determine if the residual HS-142-1-insensitive 125I-ANP binding sites could be displaced by desANP 4–23, cells were preincubated with 25 µg/ml HS-142-1, a concentration that maximally inhibited 125I-ANP displacement by native ANP. Under these conditions, desANP 4–23 was able to displace 125I-ANP binding with high affinity (IC50 of about 22 ± 3.5 pm) (Fig. 8C).

Natriuretic Peptide Receptor Gene Expression in Neuroblastoma Cells—Northern analysis on poly(A)-selected RNA was used to detect the three known natriuretic receptor mRNAs in NG108 and Neuro2a cells. Positive controls for hybridization were brain, kidney, and lung samples. NG108 cells displayed an appropriately sized band for NPR-A, two weak bands for NPR-B (surrounding the expected size observed in control tissues), and no signals for NPR-C (Fig. 9). Neuro2a cells also exhibited weak bands for NPR-A and NPR-B and no signal for NPR-C. To confirm the Northern data and to detect very low levels of gene expression for these receptors, we performed RT-PCR on poly(A)-selected RNA using the same receptor-specific primer sets that generated cDNA probes used in the Northern analysis. In NG108 cells, bands of the expected sizes were obtained in NPR-A, NPR-B, and NPR-C lanes (Fig. 10).
Southern analysis with internal probes confirmed specificities of hybridization signals for NPR-A and NPR-C (Fig. 10). Because NPR-B could not be detected in NG108 cells in this experiment, RT-PCR was repeated on these cells. Cloning and sequencing of PCR products finally revealed the presence of a low level of NPR-B receptor gene expression in NG108 (data not shown). Thus, GC-coupled receptors (primarily NPR-A, but also perhaps a low level of NPR-B) are likely to mediate the natriuretic peptide-induced stimulation of NG108 proliferation. Although NPR-C mRNA was expressed at low levels in NG108 cells, the pharmacological data showed no evidence that a functional NPR-C was present in these cells.

DISCUSSION

The data described here indicate that natriuretic peptides regulate the proliferation of neuroblastoma cell lines in a cell-specific manner. In one cell line (NG108), ANP and CNP induced purely a dose-dependent increase in thymidine incorporation by a mechanism that involved cGMP-dependent PKG. Modest but significant growth-stimulatory effects were also observed in SK-N-SH subclones and Neuro2a cells with low concentrations of natriuretic peptides. At higher peptide concentrations, however, the growth-stimulatory effects in these cells were apparently overridden by a growth-inhibitory mechanism. This inhibitory effect was sensitive to the MEK1/2 inhibitor PD98059, which also significantly decreased proliferation in the absence of natriuretic peptides.

The interpretation of experiments in NG108 cells seems relatively straightforward. The dose-dependent stimulation of proliferation was completely blocked by the PKG inhibitor Rp-8-cPTP-cGMP. This suggests the involvement of NPR-A or NPR-B receptors. These contain a GC domain in the intracellular portion of the receptor that is activated by agonist bind-
The fact that ANP was significantly more potent than CNP in inducing proliferation and displacing 125I-ANP binding in NG108 cells suggests that the primary receptor subtype expressed was NPR-A. This was corroborated by Northern analysis, which clearly showed the presence of NPR-A gene transcripts in NG108 cells. Natriuretic peptide stimulation of proliferation via cGMP-coupled receptors has also been observed in other cell types, for example chick embryonic cardiomyocytes (19). The GC pathway also appears to mediate the stimulatory effects of natriuretic peptides on bone growth (20).

As shown by RT-PCR followed by Southern analysis and/or DNA sequencing, low levels of NPR-B and NPR-C mRNAs were also present in NG108 cells. Such a low level of expression may explain why the encoded receptors could not be distinguished from NPR-A binding sites in pharmacological studies. As such, they seem unlikely to have much significance in these cells. Moreover, there was no pharmacological evidence that functional NPR-C receptors were expressed in NG-108 cells.

NPR-A and NPR-B mRNAs were found to be present in Neuro2a cells, but at low levels. This expression was confirmed by hybridization and sequencing of RT-PCR products. In addition,

TABLE I

| Ligands     | ANP IC50 ± S.E. (nM) | Hill slope ± S.E. | CNP IC50 ± S.E. (nM) | Hill slope ± S.E. |
|-------------|----------------------|-------------------|----------------------|-------------------|
| ANP         | 13 ± 0.074           | 0.95 ± 0.03       | 24 ± 0.14            | 0.91 ± 0.01       |
| CNP         | 127 ± 1.2            | 0.89 ± 0.04       | 24 ± 0.04            | 0.26 ± 0.11       |
| desANP4-23  | 32 ± 0.4             | 0.49 ± 0.04       | 32 ± 0.4             | 0.49 ± 0.04       |

As shown by RT-PCR followed by Southern analysis and/or DNA sequencing, low levels of NPR-B and NPR-C mRNAs were also present in NG108 cells. Such a low level of expression may explain why the encoded receptors could not be distinguished from NPR-A binding sites in pharmacological studies. As such, they seem unlikely to have much significance in these cells. Moreover, there was no pharmacological evidence that functional NPR-C receptors were expressed in NG-108 cells.

The actions of natriuretic peptides on the growth of SK-N-SH subclones and Neuro2a cells appear more complex. Low concentrations of ANP and CNP induced an increase in DNA synthesis that was sensitive to PKG blockade, again indicative of action on NPR-A and/or NPR-B (GC) natriuretic receptors. On the other hand, higher concentrations of ANP and CNP inhibited proliferation in a manner that was insensitive to PKG blockade. This inhibitory action was fully abolished when cells were also incubated with the MEK1/2 inhibitor PD98059. This may imply that natriuretic peptides act downstream of a growth-stimulatory MEK1/2 pathway that is constitutively active in these cells under the study conditions. In support of this possibility, PD98059 inhibited basal proliferation by 57% in control Neuro2a cell cultures (Fig. 3). Alternatively, it is possible that growth-inhibitory concentrations of natriuretic peptides act via MEK1/2 induction, although this seems unlikely.

NPR-A and NPR-B mRNAs were found to be present in Neuro2a cells, but at low levels. This expression was confirmed by hybridization and sequencing of RT-PCR products. In addi-
tion, radioligand displacement experiments confirmed the existence of high affinity $^{125}$I-ANP binding sites consistent with NPR-A and/or NPR-B receptors. Thus, like in NG-108 cells, a GC-coupled receptor is likely to mediate the stimulatory action of natriuretic peptides on the proliferation of these cells. On the other hand, the identity of receptor that mediates the PKG-independent growth-inhibitory effects of natriuretic peptides is still unclear. One hypothesis is that NPR-A and/or NPR-B receptors mediate both growth-stimulatory and growth-inhibitory actions. For example, in the presence of higher concentrations of natriuretic peptides, these receptors might switch from proliferative to antiproliferative signaling pathways. To investigate whether or not NPR-A and/or NPR-B receptors might also mediate the growth-inhibitory actions, we tested whether or not these actions could be blocked by the NPR-A/NPR-B-selective antagonist HS-142-1. Although this reagent, as expected, blocked the growth-stimulatory action of natriuretic peptides, it did not prevent the growth-inhibitory effects (Fig. 4), suggesting that neither NPR-A nor NPR-B was involved in the antiproliferative actions.

Radiotracer experiments in Neuro2a cells suggested that another natriuretic peptide receptor (in addition to NPR-A and NPR-B) might be present in these cells. Most notably, these studies demonstrated the existence of both HS-142-1-sensitive and HS-142-1-insensitive $^{125}$I-ANP binding sites. This suggested that the antiproliferative actions of natriuretic peptides might be mediated by an HS-142-1-insensitive receptor. The NPR-C-specific analog desANP$_{4-23}$ effectively competed these HS-142-1-insensitive $^{125}$I-ANP binding sites, suggesting the presence of NPR-C or an NPR-C-like receptor in Neuro2a cells. That such a receptor might couple to growth is suggested by the fact that NPR-C-specific ligands such as desANP$_{4-23}$ have been reported to regulate cell growth or other cellular processes by way of increased intracellular calcium, adenylyl cyclase inhibition, or decreased MAP kinase activity (reviewed in Ref. 4). Interestingly, we found that relatively low concentrations of desANP$_{4-23}$ selectively mimicked the MEK1/2-sensitive antiproliferative actions of high concentrations of ANP and CNP in SK-N-SH subclones and Neuro2a cells. However, RT-PCR analysis revealed gene expression for NPR-A and NPR-B only, leaving unclear the molecular nature of the receptor that mediates the growth-inhibitory actions of the natriuretic peptides. Among the possibilities, 1) NPR-C is expressed in Neuro2a cells, but at levels below detection by Northern analysis and RT-PCR, and 2) a non-GC receptor other than NPR-C that binds both natural natriuretic peptides and desANP$_{4-23}$ mediates the growth-inhibitory actions. Considering the sensitivity of RT-PCR technique and the multiple primer sets used, the first hypothesis seems highly improbable. Interestingly, NPR-C as well as an NPR-C-like receptor (termed "type D") was recently cloned from eels. Curiously, expression of the type D receptor in COS cells confirmed its high affinity for...
desANP(1–23) but revealed an unexpected sensitivity to HS-142-1 (40). Other groups have proposed the existence of atypical “NPR-C-like” receptors in rats (25, 41).

Our interest in natriuretic peptide actions on neuroblastoma cells stems from previous studies on the proliferative actions of VIP- and PACAP-related peptides on both normal neuroblasts (32, 42) and neuroblastoma cell lines (30, 43). Because the actions of these peptides in Neuro2a cells could not be fully explained by the presence of known VIP and PACAP receptors (30), we postulated that the effects could be mediated by putative receptors that bind both VIP- and ANP-related peptides (44, 45). In fact, we found that a small portion of 125I-ANP binding sites in Neuro2a cells could be displaced by high concentrations of VIP-related neuropeptides (data not shown). Thus, VIP- and ANP-related peptides may in some way interact at the cell surface to control cellular functions (46). Studies that further address the interaction of VIP- and PACAP-related peptides with natriuretic peptides in neuroblastoma cells are in progress.

An interesting question raised by these studies is whether or not ANP-related ligands and their receptors also play a role in regulating the proliferation of neuroblasts in normal embryos or have other important actions in the developing nervous system. At least three different groups have shown by receptor autoradiography that natriuretic peptide binding sites are present in the ventricular (proliferative) zone of the embryonic rat brain (25, 47, 48). Furthermore, gene expression for CNP is present in the mouse brain at embryonic day 10.5 (49). Taken with the data obtained here in neuroblastoma cell lines, we propose that a natriuretic peptide ligand/receptor system functions in embryonic nervous system development.

Finally, the data reported here suggest that natriuretic peptides might be involved in neuroblastoma tumor growth. If the opposing actions of natriuretic peptides on neuroblastoma cell proliferation are mediated by different receptors, then the balance of these receptors might influence the overall growth rate of these cells in response to endogenous natriuretic peptides. A therapeutic approach might be to attempt to change the balance of these receptors or to utilize ligands that specifically antagonize NPR-A and/or NPR-B receptors or that stimulate non-GC growth receptors involved in growth inhibition.

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