Adenine nucleotides decrease the apparent $K_m$ of endogenous natriuretic peptide receptors for GTP

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Antos LK, Potter LR. Adenine nucleotides decrease the apparent $K_m$ of endogenous natriuretic peptide receptors for GTP. Am J Physiol Endocrinol Metab 293: E1756–E1763, 2007. First published September 11, 2007; doi:10.1152/ajpendo.00321.2007.—Natriuretic peptide receptors A (NPR-A) and B (NPR-B) mediate most effects of natriuretic peptides by synthesizing cGMP. ATP increases the activity of these receptors by an unknown mechanism. We recently reported that a nonhydrolyzable form of ATP, adenylyl imidodiphosphate (AMPPNP), stabilizes but is not required for the activation of NPR-A and NPR-B in membranes from highly overexpressing cells. Here, we repeated these studies on receptors expressed in endogenous settings. Kinetic analysis indicated that both AMPPNP and ATP dramatically decrease the apparent $K_m$ of both receptors for GTP but had little effect on the $V_{max}$. The $EC_{50}$ for AMPPNP decreased as substrate concentration increased whereas the magnitude of the effect was greater at lower GTP concentrations. ATP increased the activity of a mutant receptor containing glutamates substituted for all known phosphorylation sites similarly to the wild-type receptor, consistent with a phosphorylation independent mechanism. Finally, the putative ATP binding sites were investigated. Mutation of the ATP modulatory domain region had no effect, but mutation of K535A dramatically diminished ANP-dependent cyclase activity in a manner that was unresponsive to ATP. Mutation of the highly conserved 630-KSS to AAA (all alanines) resulted in an expressed receptor that had no detectable guanylyl cyclase activity.

We conclude that ATP is not required for the initial activation of NPRs but does increase activity over time by reducing the apparent $K_m$ for GTP.

guanylyl cyclase; cyclic guanosine monophosphate; guanosine triphosphate; adenosine triphosphate; Michaelis-Menten constant; heart failure; hypertension; bone growth

THERE ARE THREE KNOWN MAMMALIAN natriuretic peptides: atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) (31). ANP and BNP bind the transmembrane guanylyl cyclase known as natriuretic peptide receptor A (NPR-A) or guanylyl cyclase A, whereas CNP binds a related but distinct enzyme known as natriuretic peptide receptor B (NPR-B) or guanylyl cyclase B (31). ANP and BNP decrease blood pressure and inhibit cardiac hypertrophy and fibrosis. Blood pressure is reduced through the stimulation of natriuresis, diuresis, vasorelaxation, and increased endothelial permeability. CNP stimulates long bone growth, in part by reducing the apparent $K_m$ for GTP. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
indicate that ATP increases its activity only at later but not earlier periods of time (44, 45).

Two separate groups have recently challenged our findings because they were carried out in highly overexpressing synthetic cells (7, 20). Therefore, we investigated whether the same results would be obtained in membranes from cells that express these receptors endogenously. Here, we find that endogenously expressed NPR-A and NPR-B are strongly activated by natriuretic peptides in the absence of adenine nucleotides but that ATP increased activities at longer time periods. To further understand the role of ATP in the regulation of these receptors, we performed kinetic experiments in the presence or absence of ATP and the nonhydrolyzable ATP analog adenylylimidodiphosphate (AMPPNP). We find that both ATP and AMPPNP decrease the apparent Michaelis-Menten constant (Km) of the enzyme, whereas ATP has no statistically significant effect on the maximal velocity (Vmax) of the enzyme. Finally, we investigated the role of two putative ATP binding sites in the kinase homology domain of NPR-A by using a site-directed mutagenesis approach. We find that mutation of the putative ATP regulatory domain (ARM) had no effect but that mutation of Lyx353 to alanine disrupts ligand activation and yields an enzyme that is unaffected by the presence of ATP.

**MATERIALS AND METHODS**

**Cell culture and membrane preparation.** Bovine aortic endothelial cells (BAEC) were acquired from the Lonza Group (www.lonzabiotechnology.com) and cultured in 1:1 F-12-DMEM containing 10% FBS and 1% penicillin-streptomycin. NIH3T3 fibroblast cells were cultured in DMEM containing 1% penicillin-streptomycin and 10% calf serum as previously described (38), and HEK 293 neo cells were grown in 10% FBS with 1% penicillin-streptomycin. HEK 293T cells stably expressing NPR-A were grown and maintained as previously described (1, 14). Crude membranes were prepared by washing cells at 4°C with phosphate-buffered saline and scraping off the plate in the presence of phosphatase inhibitor buffer (PIB; 25 mM HEPES, pH 7.4, 20% glycerol, 50 mM NaCl, 50 mM NaF, 2 mM EDTA, 0.25 μM microcystin, and 2.5× Roche protease inhibitor tablet). Suspended cells were sonicated for 1–2 s and centrifuged at 20,000 g for 10 min at 4°C. The supernatant was aspirated and the pellet resuspended in PIB. Guanylyl cyclase assays contained 0.05–0.3 mg of total membrane protein. For experiments shown in Fig. 1, membranes were repeatedly resuspended in 1 ml of PIB and pelleted by centrifugation.

**Guanylyl cyclase assays.** Guanylyl cyclase activity for Figs. 1 and 2 were performed using 20 μl of membranes containing 1.5–3.5 mg/ml for the BAEC and 5–15 mg/ml for the NIH3T3 cells of total protein. The membranes were resuspended in PIB and treated with water, Mg2+-ATP, Mg2+-AMPPNP, ligand (ANP or CNP), or Mn2+-Triton, as indicated in the figure. Final reaction concentrations for the adenine nucleotides and ligands were 1 mM and 1 μM, respectively, except where indicated otherwise. The reactions were stimulated by addition of 50 μl of cocktail containing 4 mM magnesium, 1 mM GTP along with 25 mM HEPES, pH 7.4, 0.5 μM microcystin, 1 mM EDTA, and 0.75 mM 3-isobutyl-1-methylxanthine. For Figs. 3 and 4, 40 μl of membranes (1–3.5 mg/ml) was stimulated with 60 μl of the same cocktail as described above with or without 1 mM Mg-AMPPNP. Additionally, the cocktail in Fig. 3 contained 5 mM creatine phosphate and 0.1 μg/μl creatine phosphokinase, and membranes were prewarmed for 15 s before addition of cocktail. In Fig. 5, 25 μl of membranes was treated with 25 μl of the indicated amounts of AMPPNP and stimulated with 50 μl of a cocktail containing either 0.1 or 1 mM Mg-GTP. The reaction described in Fig. 6 contained 25 μl of membranes that contained either 25 μl of water and 4 mM Mg-ATP or Mg-AMPPNP and stimulated with cocktail containing varying amounts of GTP. In these experiments, 5 mM creatine phosphate and 0.1 μg/μl creatine phosphokinase were included in the reaction cocktail. All assays were stopped by adding 0.4 ml of 50 mM sodium acetate containing 5 mM EDTA buffer. Samples were then centrifuged for 10 min at 3,000 rpm, and a 100-μl aliquot was analyzed for cGMP by radioimmunoassay (PerkinElmer). In Fig. 5, top, membranes from HEK 293T cells stably expressing NPR-A cells were used as the enzyme source. Figure 7 was performed as described for Figs. 1 and 2 except that 300 μCi of [α-32P]GTP was added to cold GTP, and conversion of [α-32P]GTP to [32P]cGMP was measured as previously described (3).

**Creation and transfection of NPR-A mutants.** Mutants used in Fig. 6 were engineered with a Stratagene Quikchange Site-Directed Mutagenesis Kit using a pCMV3 wild-type NPR-A backbone (36) and 5′ to 3′ primers gctgaccctgagtgcgcgagcctccaattatggc, gcaaccttgtggctgt-aag, and ccattgatttcccatgggaacctgcccggcagccaactgt-ggc to generate the desired mutants. Mutants used in Fig. 6 were engineered with a Stratagene Quikchange Site-Directed Mutagenesis Kit using a pCMV3 wild-type NPR-A backbone (36) and 5′ to 3′ primers gctgaccctgagtgcgcgagcctccaattatggc, gcaaccttgtggctgt-aag, and ccattgatttcccatgggaacctgcccggcagccaactgt-ggc to generate the desired mutants. Constructs were transfected into NIH3T3 cells using FuGene 6 (Roche Applied Science). Transfected cells were selected for resistance to the antibiotic G418. Western blots. Membrane proteins (15 μg) were fractionated by SDS-PAGE and electroblotted to a polyvinylidene fluoride membrane and probed with polyclonal antiserum from rabbit 6325 (2). NPR-A was visualized by enhanced chemiluminescence. β-Actin was used as a loading control and blotted with an anti-β-actin antibody purchased from Sigma.

**ATP detection.** One hundred microliters of membranes prepared as described above was tested for the presence of ATP using an ATPlite ATP detection kit (PerkinElmer). The reaction described in Fig. 6 contained 25 μl of membranes that contained either 25 μl of water and 4 mM Mg-ATP or Mg-AMPPNP and stimulated with cocktail containing varying amounts of GTP. In these experiments, 5 mM creatine phosphate and 0.1 μg/μl creatine phosphokinase were included in the reaction cocktail. All assays were stopped by adding 0.4 ml of 50 mM sodium acetate containing 5 mM EDTA buffer. Samples were then centrifuged for 10 min at 3,000 rpm, and a 100-μl aliquot was analyzed for cGMP by radioimmunoassay (PerkinElmer). In Fig. 5, top, membranes from HEK 293T cells stably expressing NPR-A cells were used as the enzyme source. Figure 7 was performed as described for Figs. 1 and 2 except that 300 μCi of [α-32P]GTP was added to cold GTP, and conversion of [α-32P]GTP to [32P]cGMP was measured as previously described (3).

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Statistical analysis. Data were analyzed via a Student’s unpaired t-test using Prism 4.0 GraphPad software. This software was also used to perform nonlinear regression analysis of kinetic data. All data are represented as mean ± SE.

RESULTS

ATP is not required for activation of endogenous NPR-A and NPR-B.

We have previously reported that adenine nucleotides are not required to activate NPR-A and NPR-B in membranes from 293T cells highly overexpressing NPR-A or NPR-B (3). However, whether or not these results reflect physiological activation has been called into question (7, 20). To address this issue, we tested guanylyl cyclase activity in membranes from BAEC or NIH3T3 fibroblast membranes that endogenously express NPR-A or NPR-B, respectively.

Addition of ANP to BAEC membranes increased guanylyl cyclase activities 7.6-fold over those observed under basal conditions when measured for a short 15 s time period (Fig. 1, top). Addition of ATP or AMPPNP to reactions containing ANP increased cyclase activity slightly, but these activities were not significantly different from those observed in reactions containing no adenine nucleotides. Similarly, CNP addition to reactions containing CNP increased cyclase activity slightly, but these activities were not significantly different from those observed in reactions containing no adenine nucleotides.

Fig. 2. Sequential washing of membranes does not sensitize NPRs to adenine nucleotide. Membranes from BAEC (top) or NIH3T3 cells (middle and bottom) were harvested and washed as indicated. Membranes were assayed in the presence or absence of 1 mM concentrations of adenine nucleotide (ATP or AMPPNP) and/or 1 μM concentrations of ANP (top) or CNP (middle). Mn²⁺ and Triton X-100 were used as synthetic activators of guanylyl cyclase activity. Bottom: ATP concentration (expressed as pmol ATP/mg protein) found in NIH3T3 cell extracts or membranes after successive washes. Data are shown as means ± SE and are representative of 3 separate experiments; n = 6. *,†,#P < 0.001, 0.01, and < 0.005, respectively, vs. no-wash sample.

Fig. 3. AMPPNP stabilizes the guanylyl cyclase activity of NPR-A and NPR-B. Membranes from BAEC (top) or NIH3T3 cells (bottom) were prepared and stimulated with 1 μM ANP (top) or CNP (bottom) in the presence or absence of 1 mM AMPPNP for indicated periods of time in reaction cocktail containing 0.1 μg/μl creatine phosphokinase and 5 mM creatine phosphate. Data are shown as means ± SE and are representative of 3 separate experiments; n = 6.

Fig. 4. A constitutively phosphorylated version of NPR-A is regulated by AMPPNP. Membranes were prepared from 293T cells stably overexpressing a version of NPR-A containing glutamates substituted for all 6 of its known phosphorylation sites (NPR-A-6E) and stimulated with ANP in the presence or absence of ATP for indicated periods of time. Graph is a compilation of 4 experiments; n = 7. Data are means ± SE.
tion to NIH3T3 cell membranes resulted in a sixfold increase over basal guanylyl cyclase activities when measured for 15 s (Fig. 1, bottom). The inclusion of ATP or AMPPNP with CNP did not result in a significant increase in cGMP production compared with reactions lacking adenine nucleotides. These data indicate that in these physiological systems ligand alone is fully capable of activating NPR-A and NPR-B and that additional supplementation with adenine nucleotide does not increase initial guanylyl cyclase activities at early time periods.

Membrane washing does not decrease ATP-independent activity of NPRs. To rule out the possibility that ATP may still be present in the membranes due to cellular contamination, membranes were repeatedly washed and then tested for responsiveness to ligand stimulation. This procedure is likely to remove ATP, because the EC50 for ATP-dependent activation of NPR-A and NPR-B is ~0.1 mM (8, 18, 24), which is indicative of very weak binding. Hence, if natriuretic peptides are activating these receptors because the membranes contain residual cellular ATP, then with each successive wash the membranes should contain less ATP and the receptor should be less responsive to natriuretic peptide stimulation.

Our data indicate that natriuretic peptides are not activating NPR-A or NPR-B as a result of residual ATP, because even after four washes ANP alone increased guanylyl cyclase activity 3- to 4-fold, and CNP increased activity 7- to 13-fold over basal levels (Fig. 2). The addition of ATP or AMPPNP to membranes from the BAEC slightly increased cyclase activity, but it was not statistically significant after any wash (Fig. 2, top). Similarly, the addition of ATP increased the cyclase activity measured in membranes from the NIH3T3 cells about twofold, but it was only statistically significant when measured after two washes only (Fig. 2, middle). AMPPNP also increased activity about twofold, but the differences were not statistically significantly after any wash. The slight reduction in CNP-dependent activation with washing is most likely due to protein loss, as activities measured in the presence of manganese and Triton X-100 were reduced similarly. These data demonstrate that the ligand activation observed in Fig. 1 is not due to residual ATP contamination from the membrane preparation. Interestingly, we observed slightly higher activity in the presence of adenine nucleotide in the wash data than observed in straight stimulation activity in Fig. 1, bottom. The reason for this is not known, but one explanation is that the additional washes require the membranes to be left on ice for longer periods of time, which decreases the responsiveness of the enzyme and therefore could increase the requirement for adenine nucleotide.

To prove that ATP was being removed through membrane washing, we measured ATP levels in NIH3T3 cell membranes by use of a coupled ATP-luciferase assay. Figure 2 demonstrates that separation of membrane protein from the cytosolic fraction (crude to no-wash samples) dramatically decreases ATP concentrations. Washing the membranes once decreases ATP concentration by almost one-half, whereas samples washed two to four times showed no significant reduction in ATP concentration beyond the amount of ATP detected in the samples that were washed only once. Therefore, membrane washing effectively decreases the amount of ATP in our samples, although the vast majority of ATP is removed in the first and second washes.

**Effect of adenine nucleotides on NPR-A and NPR-B as a function of time.** To determine whether adenine nucleotides stabilize cyclase activity of endogenously expressed NPR-A and NPR-B as they do receptors overexpressed in 293T cells, ANP- or CNP-dependent guanylyl cyclase activity was measured in the presence or absence of AMPPNP as a function of time. AMPPNP was used instead of ATP to isolate the allosteric effect of the adenine nucleotide from its ability to serve as a substrate for the kinase that phosphorylates these receptors (15). Since GTP is more likely to be degraded in the longer assays, we included a cyclic nucleotide regeneration system (creatine phosphokinase and creatine phosphatase).

Our data indicate that, at short time points (<60 s), there is little difference between AMPPNP-treated and untreated
BAEC or NIH3T3 cell membranes. However, at the longer time points, the presence of AMPPNP causes about a twofold increase in activity (Fig. 3, bottom). Hence, ATP is not required for long-term activation of the enzyme, but it does increase the amount of activity observed about twofold. These data closely resemble the previous time course data obtained in 293T-overexpressing cell membranes. However, at the longer time points (as high as 15-fold at 30 s), and lower-level stimulations are observed at the longer time points (~2- to 3-fold after 180 s).

**Effect of AMPPNP on kinetic parameters of NPRs.** To determine how AMPPNP elevates the enzymatic activity of NPR-A and NPR-B, we examined the ability of increasing concentrations of AMPPNP to increase ligand-dependent guanylyl cyclase activity in the presence of high (1 mM GTP) or low (0.1 mM GTP) substrate concentrations. These concentrations were chosen because our group typically uses the higher GTP levels, whereas groups that report different findings typically used lower GTP concentrations. As predicted by Michaelis-Menton kinetics, activities were higher in reactions containing 1 mM GTP vs. 0.1 mM GTP (Fig. 5). The EC50 of AMPPNP for the high and low GTP concentrations was 15 and 115 μM in BAEC membranes (Fig. 5, A and B) and 31 and 112 μM in membranes from NIH3T3 cells (Fig. 5, C and D). Interestingly, AMPPNP increased cyclase activity ~10- and 3-fold or 18- and 2.5-fold at the lower and higher GTP concentrations in BAEC and NIH3T3 membranes, respectively. These data are consistent with AMPPNP increasing the K_m of the enzyme, since it has a greater effect at lower substrate concentrations. Finally, we found that concentrations of AMPPNP greater than 1.5 mM inhibited cGMP formation, which most likely results from competition at the substrate binding site.

**Effect of ATP on a dephosphorylation-resistant NPR-A mutant.** To further separate the allosteric effects of ATP from its role as a substrate in the phosphorylation of NPR-A, membranes from a mutant form of NPR-A containing glutamates substituted for all six of its known phosphorylation sites were assayed for guanylyl cyclase activities in the presence and absence of ATP. Unlike a mutant receptor consisting of alanines for the known phosphorylation sites, this receptor is responsive to ANP stimulation and demonstrates a blunted desensitization response (34).

ATP had no effect at the early 5 s or 15 s time points but produced a significant increase at every time point thereafter (Fig. 4). The average fold increase in activity with AMPPNP is 5.6, but higher fold stimulations are observed at the midlevel time points (as high as 15-fold at 30 s), and lower-level stimulations are observed at the longer time points (~2- to 3-fold after 180 s).

**Table 1. Kinetic analysis of endogenous NPRs**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Control</th>
<th>AMPPNP</th>
<th>ATP</th>
<th>Control</th>
<th>AMPPNP</th>
<th>ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAEC</td>
<td>1,435±772.9</td>
<td>63.1±56.2*</td>
<td>126.6±87.5*</td>
<td>269.2±48.18</td>
<td>237.1±24.86</td>
<td>350.6±36.79</td>
</tr>
<tr>
<td>NIH3T3</td>
<td>2,314±483.5</td>
<td>213±58.9*</td>
<td>242.8±46.5*</td>
<td>120.2±9.7</td>
<td>73.7±3.6</td>
<td>154.6±5.5</td>
</tr>
<tr>
<td>NPR-A-6E</td>
<td>7,384±2395</td>
<td>852.3±232.3</td>
<td>730.0±221</td>
<td>522.3±99.4</td>
<td>702.3±49.2</td>
<td>1078±82.5</td>
</tr>
</tbody>
</table>

Values are representative and given as means ± SE; n = 4. BAEC, bovine aortic endothelial cells; AMPPNP, adenylyl imidodiphosphate; NPR, natriuretic peptide receptor. *P < 0.02 vs. control K_m.
AMPPNP decreased the containing form of NPR-A from 7,384 to 730 receptor guanylyl cyclases (29), was shown to dramatically line membranes, ATP decreased the addition of adenine nucleotide. Similar to the endogenous cell ylation sites. For NPR-A does not involve changes in the known phosphor-

Fig. 7. Effect of putative ATP binding domain mutations on activity of NPR-A. HEK 293 neo cells were transfected with indicated NPR-A constructs or GFP control vector. Crude membranes from transfected cells were prepared and assayed for guanylyl cyclase activity for 15 min. WT, wild type; AAA, all alanines. Data are shown as means ± SE and are representative of 3 separate experiments; n = 6. Insets: Western blot analysis of NPR-A and β-actin (loading control) levels are shown.

Discussed with AMPPNP for the BAEC membranes and from 2,314 to 243 μM GTP with ATP or to 213 with AMPPNP for the NIH3T3 cell membranes (Fig. 6). In contrast to previous reports, the Vmax was not statistically significantly affected by addition of adenine nucleotide. Similar to the endogenous cell line membranes, ATP decreased the Km of the glutamate-containing form of NPR-A from 7,384 to 730 μM GTP where AMPPNP decreased the Km to 852.3 μM GTP (Fig. 6, bottom). Therefore, the ability of AMPPNP or ATP to decrease the Km for NPR-A does not involve changes in the known phosphorylation sites.

Mutational analysis of putative ATP binding sites. Whether ATP binds directly to NPR-A and NPR-B is controversial. Initial reports suggested that the specific intracellular glycine region called the ARM within the kinase homology domain is required for adenine nucleotide regulation (12, 13, 19). However, a subsequent report indicated that this region is not involved in the regulation of NPR-A (22). More recently, the mutation of Lys535 to alanine in NPR-A resulted in a dramatic decrease in ANP-dependent, but not detergent-dependent, guanylyl cyclase activity. Interestingly, ATP had no effect on the cyclase activity of this mutant (Fig. 7, top).

Recently, 8-azido-ATP was cross-linked to the kinase homology domain of NPR-A purified from bacteria (7). MALDI-TOF mass spectrometry analysis indicated that Cys634 in the sequence 630-KSSNCVVDGR-639 was photoaffinity modified. On the basis of these data, a mutant receptor was created by mutating both Cys634 and Val635 to tryptophan. Analysis of the ANP-dependent guanylyl cyclase activity of this double mutant indicated that activity was markedly reduced, although ATP was still able to increase activity about twofold. Interestingly, all human particulate guanylyl cyclase receptors contain the 630-KS motif, whereas NPR-A and NPR-B have an additional conserved serine. To test the role of this KSS region in the regulation of NPR-A, we constructed a triple mutant consisting of all alanines (AAA) substituted for the lysine and serines. Although this receptor was expressed at similar levels to the wild-type receptor based on Western blot analysis, the guanylyl cyclase activities obtained in membranes from cells transfected with this receptor were not greater than activities measure in cells transfected with a GFP plasmid that has no guanylyl cyclase activity (Fig. 7, bottom). Hence, since the triple mutant has no detergent-dependent cyclase activity, we believe that the mutation of KSS to AAA results in an improperly folded or destabilized receptor.

Discussion

This report is the first to show that endogenous natriuretic peptide receptors are maximally stimulated in the absence of adenine nucleotides at early time periods but are modulated by ATP at longer time periods. We also separate the allosteric effect of ATP from its role in phosphorylating NPR-A by demonstrating marked increases in the ANP-dependent activity of a “constitutively phosphorylated” form of NPR-A. Kinetic analysis indicates for the first time that ATP and AMPPNP both dramatically decrease the Km of these receptors while having no significant effect on Vmax. The greater effect of adenine nucleotides observed at lower GTP concentrations may explain why previous investigators reported an absolute requirement of ATP for receptor activation, since they used lower GTP concentrations in their guanylyl cyclase assays (15). Mutagenesis studies indicate that the GxGxxxG region within NPR-A is completely dispensable for ATP regulation of these receptors whereas the mutation of K535 nearly abolishes the ability of ANP but not detergent to activate NPR-A. Finally, our data demonstrating that ATP increases the activity of NPR-A and NPR-B only at longer periods of time coincides with our previous results in overexpressing cells (3).
During the determination of the EC₅₀ for AMPPNP, we found that higher concentrations of GTP result in a lower EC₅₀ for AMPPNP and that the difference between maximum and minimum activities was higher at lower substrate concentrations; i.e., AMPPNP had a greater effect at lower GTP concentrations. Subsequent experiments indicated that both ATP and AMPNP dramatically decrease the Kₘ of NPR-A and NPR-B but have no significant affect the Vₘₐₓ. Previous kinetic experiments performed on NPR-A (24, 49) and GC-C (17) reported no effect of ATP on the Kₘ of either enzyme. One possibility for the discrepancy is that the membranes were not prepared in the presence of phosphatase inhibitors. Furthermore, previous investigators used different ATP analogs (caged ATP and ATPγS) instead of ATP or AMPNP, which were used in our experiments. Since caged ATP has been shown to yield higher guananyl cyclase activity than ATP in rat lung membranes in both the presence and absence of ANP, it may not be an accurate indicator of how ATP regulates these receptors (8). However, a recent report also showed a decrease in the Kₘ upon addition of ATP in rat glomular membranes (50).

Our investigation into the regions responsible for adenosine nucleotide regulation clearly indicated that the glycine-rich putative ARM is not involved in the regulation of NPR-A. Our results further revealed that mutation of K535 to alanine resulted in a mutant receptor with reduced hormone-dependent, but not detergent-dependent, cyclase activity that is not affected by the presence of ATP. This is in contrast to wild-type NPR-A or the AxA mutation where the addition of ATP results in an approximately threefold increase over cyclase activities observed with ANP alone. These data are consistent with K535 being required for ATP-dependent modulation of NPR-A. Alternatively, it is possible that this mutation disrupts the normal conformation of the receptor and blocks the ability of ATP or an ATP-binding protein to interact with a separate normal conformation of the receptor and blocks the ability of ATP signaling.

In conclusion, we report that endogenously expressed NPR-A and NPR-B are maximally activated in the absence of adenine nucleotides at short time periods. The ATP-independent activation of these receptors is in agreement with most reports. Please note, however, that we are neither currently suggesting nor have we previously suggested that ATP does not increase the guananyl cyclase activities of these receptors, as has been implied (7, 20). With the exception of the very early time points, we, as all other investigators, observe more activity in the presence of ATP (3). Importantly, we now demonstrate that the increased activities result from decreases in the Kₘ of these enzymes, not from increases in maximal velocities.

GRANTS

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REFERENCES

EFFECT OF ATP ON NATRIURETIC PEPTIDE RECEPTORS


