Natriuretic peptide receptor A (NPR-A) is an essential cardiovascular regulator that is stimulated by atrial natriuretic peptide and B-type natriuretic peptide, whereas natriuretic peptide receptor B (NPR-B) stimulates long bone growth in a C-type natriuretic peptide-dependent manner. Many reports indicate that ATP is essential for NPR-A and NPR-B activation. Current models suggest that natriuretic peptide binding to receptor extracellular domains causes ATP binding to intracellular kinase homology domains, which derepresses adjacent catalytic domains. Here, we report 100-fold activations of natriuretic peptide receptors in the absence of ATP. The addition of a nonhydrolyzable ATP analog had no effect at early time periods (measured in seconds) but increased cGMP production about 2-fold after longer incubations (measured in minutes), consistent with a stabilization, not activation, mechanism. These data indicate that ATP does not activate natriuretic peptide receptors as has been repeatedly reported. Instead, ATP increases activity primarily by maintaining proper receptor phosphorylation status but also serves a previously unappreciated enzyme stabilizing function.
activation. For the first time, these data demonstrate that ATP is not an allosteric activator of NPR-A and NPR-B. Instead, they indicate that the primary effect of ATP is to keep NPR-A and NPR-B phosphorylated and that a secondary effect is to stabilize the cyclase activity of these receptors. Together, these data support a new single-step model for natriuretic peptide receptor activation.

**Experimental Procedures**

**Membrane Preparation**—HEK293T or NIH3T3 cells stably expressing NPR-A (293T-NPR-A or 3T3-NPR-A) or NPR-B (293T-NPR-B) were grown and maintained as described previously (33, 34). Crude membranes were prepared by washing cells at 4 °C with phosphate-buffered saline and scraping them off the plate in the presence of either phosphatase inhibitor buffer (25 mM Hepes, 20% glycerol, 50 mM NaCl, 50 mM NaF, 2 mM EDTA, 0.25 μM microcystin, and 8.6 mg/10 ml Sigma protease inhibitor mixture (P8465)) or homogenizing buffer (50 mM Hepes, 10% glycerol, 50 mM NaCl, 10 mM NaN₃, 0.1 mM GTP, and 10–30 μCi of [α-³²P]GTP) as described originally (32). Then water (basal), 1 μM ANP, or 1 mM AMPNP and 1 mM AMPPNP were added, and the reactions were incubated for an additional 10 min. The experiment shown in the bottom panel of Fig. 4 was conducted exactly as described for the top panel of Fig. 4 except that the membranes were prepared in phosphatase inhibitor buffer and the cyclase reaction contained mixture 1.

**Immunoprecipitations**—Membranes were prepared and assayed as described above in the presence of either mixture 1 or mixture 2, except cyclase reactions were stopped with the addition of 1 ml of ice-cold immunoprecipitation buffer containing microcystin and protease inhibitors. Samples were then transferred to a microcentrifuge tube and precleared by rotating for 20 min at 4 °C with 50 μl of protein A beads followed by low speed centrifugation. 800 μl of sample were transferred to a new tube and incubated overnight with 2 μl of rabbit polyclonal antiserum 6325 against NPR-A. Immunocomplexes were washed three times with immunoprecipitation buffer and fractionated by SDS-PAGE as described previously (36, 37). The phosphorylation state and protein levels of NPR-A were determined by ProQ Diamond and SYPRO Ruby staining of the gel, respectively, as reported for NPR-B (37).

**Results**

**ATP Is Not Required for Maximal Natriuretic Peptide Receptor Activation**—In the process of investigating the calcium-dependent inhibition of NPR-B, we performed guanylyl cyclase assays on membranes prepared from HEK293T cells stably expressing NPR-B (293T-NPR-B). In contrast to previous re-
ports where the absence of ATP was associated with a complete failure of CNP to activate NPR-B (21), CNP alone increased cyclase activity over 200-fold in our assay (Fig. 1, top left). The inclusion of AMPPNP did not further increase CNP-dependent activity, and the inclusion of ATP resulted in slight increases that were not statistically significant. Similarly, the addition of ANP to membranes from HEK-293T-NPR-A cells increased guanylyl cyclase activity about 50-fold (Fig. 1, top right). As with NPR-B, the inclusion of AMPPNP did not increase cyclase activity, and the ATP-dependent increases were not statistically significant. These data demonstrate that NPR-A and NPR-B are maximally activated in the absence of ATP, which is not consistent with a model in which ATP binding is required for receptor activation.

To determine whether adenine nucleotides affect the sensitivity of NPR-A and NPR-B to natriuretic peptide stimulation, we performed concentration-response experiments in the presence or absence of AMPPNP or ATP (Fig. 1, bottom panels). We observed no statistically significant differences in the CNP or ANP concentrations required to elicit half of the maximum response in the presence or absence of the adenine nucleotides (Fig. 1, insets, bottom panels). Thus, our data indicate that adenine nucleotides neither increase natriuretic peptide-dependent receptor sensitivity (Fig. 1, bottom panels) nor increase maximal activation levels (Fig. 1, top panels).

Because it was formally possible that the activation of our crude membranes was due to cellular ATP contamination, we performed guanylyl cyclase assays on membranes that were washed successively three or four times in phosphatase inhibitor buffer. Membranes were then assayed for 1 min at 37 °C in the presence of 1 mM ATP, 1 μM ANP or CNP, ATP and natriuretic peptide or manganese, and 1% Triton X-100. The data are from 3 to 4 individual experiments for which each column represents the average of 6–8 determinations.

FIG. 2. Membrane washing does not decrease the ATP-independent activity of natriuretic peptide receptors. Crude membranes from 293T-NPR-B (top), 293T-NPR-A (middle), or 3T3-NPR-A (bottom) cells were prepared and washed the indicated number of times in phosphatase inhibitor buffer. Membranes were then assayed for 1 min at 37 °C in the presence of 1 mM ATP, 1 μM ANP or CNP, ATP and natriuretic peptide or manganese, and 1% Triton X-100. The data are from 3 to 4 individual experiments for which each column represents the average of 6–8 determinations.

FIG. 3. AMPPNP stabilizes the guanylyl cyclase activity of NPR-A and NPR-B. Crude membranes from 293T-NPR-A (top panel) or 293T-NPR-B (bottom panel) cells were assayed at 37 °C for the indicated time periods in the presence or absence of the activators shown. Data from early time points are expanded and shown in the inset. The data are from three separate experiments where n = 6–10.
ATP Stabilizes the Guanylyl Cyclase Activity of NPR-A and NPR-B—One possible explanation for the discrepancy between the effects of ATP on the cyclase activities measured in Figs. 1 and 2 is the reaction time. The membranes described in Fig. 1 were assayed for 15 s, whereas in Fig. 2 they were assayed for 1 min. Therefore, we investigated the ability of AMPPNP to increase the guanylyl cyclase activity of NPR-A and NPR-B as a function of time. We used AMPPNP instead of ATP to rule out the effect of phosphorylation on potential activity changes. As shown in the top panel of Fig. 3, AMPPNP had no statistically significant effect on the activity of NPR-A until the membranes had been incubated for 300 s. However, for every subsequent time point, the amount of activity measured in the presence of AMPPNP was significantly higher than that measured in its absence. Similar results were obtained for NPR-B except that a significant effect of AMPPNP was observed after 180 s (Fig. 3, bottom panel). These are the first data to demonstrate that the increased natriuretic peptide-dependent cyclase activities result from the ability of AMPPNP to maintain initial reaction rates. In other words, these data indicate that AMPPNP is stabilizing, not activating, NPR-A and NPR-B.

Buffers That Yield Highest Cyclase Activity Yield More Highly Phosphorylated Receptors—Finally, we asked why we observed robust ATP-independent activations of natriuretic peptide receptors when other investigations observed either no activation or severely diminished responses. Clearly one reason is the short duration of our assays (15 s). All other reports describe much longer assay periods (10 min). However, another contributing factor became apparent during our attempts to demonstrate ATP- $S$-dependent sensitization of NPR-A to ANP stimulation as originally reported (32). In this assay, membranes from 3T3-NPR-A cells were prepared in a phosphatase inhibitor-deficient buffer and incubated in the presence of 10 $\mu$M concentrations of AMPPNP, ATP, or ATP$-S$ at 37 °C to sensitize NPR-A to subsequent 10-min activations with ANP in the presence or absence of 1 mM AMPPMP.

As in the original report (32), we found that membranes preincubated with ATP$-S$ were more active than those preincubated with ATP, AMPPNP, or buffer, presumably because thiophosphorylated NPR-A is resistant to dephosphorylation (Fig. 4, top). We also found that cyclase activities were higher in reaction mixtures containing ANP and AMPPNP compared with ANP alone, although unlike in the original report we observed a statistically significant ($p = 0.05$) 2.5-fold activation in the absence of AMPPNP. Interestingly, the specific activities obtained under these conditions were dramatically lower than those observed in our previous assays using membranes from the same cells (Fig. 2, middle). When we repeated this assay using our normal cell lysis (phosphatase inhibitor buffer) and cyclase reaction buffers that contain phosphatase inhibitors, EDTA, and higher GTP concentrations, specific activities were restored to the previous levels (Fig. 4, middle; please note y axis scale change). Importantly, the ability of ANP to prominently activate NPR-A in the absence of adenine nucleotides was also restored. Although cyclase levels in this assay were increased about 2-fold by AMPPNP, this is what would be expected because of stabilization in a 10-min assay (Fig. 3). These data indicate that the more than 100-fold disparity between our activities and the activities obtained by other laboratories is due to differences in the composition of the buffers used to prepare the membranes and measure cyclase activity.

Given that our lysis and cyclase reaction buffers included phosphatase inhibitors (mixture 1), whereas the buffers that resulted in diminished activity did not (mixture 2), we asked whether the phosphorylation state of NPR-A isolated in the presence of phosphatase inhibitors was higher than that of NPR-A isolated from buffers lacking these inhibitors. To accomplish this goal, we treated 3T3-NPR-A membranes exactly as described in the top and middle panels of Fig. 4. However, instead of stopping the cyclase reactions with zinc acetate as is normally done for a cyclase assay, we solubilized the receptors in immunoprecipitation buffer containing phosphatase inhibitors and purified it by immunoprecipitation and SDS-PAGE. We then measured NPR-A phosphate content and protein levels by incubating the resulting gel with ProQ Diamond and SYPRO Ruby to determine NPR-A phosphate and protein levels, respectively. Results from two separate experiments are shown that are representative of four individual experiments.

![Image](http://www.jbc.org/Downloaded from)

**Fig. 4.** Phosphatase inhibitors increase the guanylyl cyclase activity and phosphorylation state of NPR-A. Crude membranes from 3T3-NPR-A cells were incubated for 10 min at 37 °C in the presence of 10 $\mu$M AMPPNP, ATP, ATP$-S$, or water. The effect of the first incubation on the hormone responsiveness of NPR-A was accessed by measuring the guanylyl cyclase activity of the membranes in water (basal), 1 $\mu$M ANP, or 1 $\mu$M ANP and 1 $\mu$M AMPPNP for 10 min at 37 °C as indicated. In the top panel, membranes were prepared, and the cyclase reaction was conducted in buffers lacking phosphatase inhibitors (mixture 2, CT2), whereas in the middle panel phosphatase inhibitors were included in both buffers (mixture 1, CT1). The data are from 3–4 separate experiments where $n = 6–8$. In the bottom panel, membranes were treated exactly as described above, but NPR-A was purified by immunoprecipitation and SDS-PAGE. The resulting gel was stained with ProQ Diamond and SYPRO Ruby to determine NPR-A phosphate and protein levels, respectively. Results from two separate experiments are shown that are representative of four individual experiments.

NPR-A isolated from buffers lacking these inhibitors. To accomplish this goal, we treated 3T3-NPR-A membranes exactly as described in the top and middle panels of Fig. 4. However, instead of stopping the cyclase reactions with zinc acetate as is normally done for a cyclase assay, we solubilized the receptors in immunoprecipitation buffer containing phosphatase inhibitors and purified it by immunoprecipitation and SDS-PAGE. We then measured NPR-A phosphate content and protein levels by incubating the resulting gel with ProQ Diamond and SYPRO Ruby dyes, respectively (Fig. 4, bottom). We found that the amount of NPR-A isolated using the two procedures was unaffected by the buffer constituents (SYPRO Ruby), whereas the phosphorylation state of NPR-A was markedly higher when isolated with buffers containing phosphatase inhibitors (ProQ Diamond). Data from three separate experiments ($n = 6$) indicated that NPR-A purified in the presence of phosphatase inhibitors (mixture 1 = 1 arbitrary units ± 0.05) contained...
more than twice as much phosphate as NPR-A purified in the absence of phosphatase inhibitors (mixture 2).

The contribution of these regions to the stabilization process has not been formally investigated. However, the other ATP-regulated receptor guanylyl cyclases, GC-C and GC-E, completely lack this motif, suggesting that another region common to all four receptors either binds ATP directly or interacts with an ATP-binding regulatory protein.

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