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The Protein Kinase Domain of the ANP Receptor Is Required for Signaling

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A plasma membrane form of guanylate cyclase is a cell surface receptor for atrial natriuretic peptide (ANP). In response to ANP binding, the receptor-enzyme produces increased amounts of the second messenger, guanosine 3′,5′-monophosphate. Maximal activation of the cyclase requires the presence of adenosine 5′-triphosphate (ATP) or nonhydrolyzable ATP analogs. The intracellular region of the receptor contains at least two domains with homology to other proteins, one possessing sequence similarity to protein kinase catalytic domains, the other to regions of unknown function in a cytoplasmic form of guanylate cyclase and in adenylate cyclase. It is now shown that the protein kinase–like domain functions as a regulatory element and that the second domain possesses catalytic activity. When the kinase-like domain was removed by deletion mutagenesis, the resulting ANP receptor retained guanylate cyclase activity, but this activity was independent of ANP and its stimulation by ATP was markedly reduced. A model for signal transduction is suggested in which binding of ANP to the extracellular domain of its receptor initiates a conformational change in the protein kinase–like domain, resulting in derepression of guanylate cyclase activity.

ATRIAL NATRIURETIC PEPTIDE (ANP), synthesized in cardiac atria and released into the bloodstream in response to increased central blood volume, lowers blood pressure by stimulating vasodilation, diuresis, and natriuresis (*1*). Two cell surface receptors for ANP have been described: a low molecular weight "clearance" receptor having a small cytoplasmic domain (*2*) and a high molecular weight, guanosine 3′,5′-monophosphate (cGMP)–coupled receptor, containing a much larger cytoplasmic domain (*3, 4*). The latter receptor, which is the subject of this report, appears to mediate the physiological effects of ANP through its intrinsic ANP-activated guanylate cyclase activity; cGMP is believed to act as a second messenger for ANP in many, but not all, circumstances (*5*). The intracellular portion of the ANP receptor has been divided into two domains on the basis of sequence analysis: one, adjacent to the membrane, has similarity to protein kinases; the other, at the COOH-terminus, is similar to regions of both a soluble guanylate cyclase and a brain adenylate cyclase (*3, 4, 6, 7*).

Mutants of the cDNA for the rat brain ANP receptor were prepared in which most of either the kinase-like domain or the COOH-terminal domain was deleted (*8*) (Fig. 1A). Mutant and wild-type receptor clones were inserted into an expression vector (pSVL) under the control of the simian virus 40 late promoter and transfected into COS-7 cells (*9*), which have low concentrations of endogenous ANP receptor and guanylate cyclase. Analysis of the transfected cells by cross-linking to ^{125}I-labeled ANP demonstrated the expression of mutant receptors of the predicted sizes (Fig. 1B). Comparison of ^{125}I-labeled ANP binding by cells transfected with mutant and wild-type receptor clones and the guanylate cyclase activity of particulate fractions of the cells, measured in the presence of nonionic detergent and Mn^{2+}, suggested that the wild-type receptor (pSVL-ANPR) and the kinase deletion mutant (pSVL-kin$^-$) had similar specific guanylate cyclase activities (Fig. 1C). That is, we have consistently observed five- to sevenfold higher expression of both ^{125}I-labeled ANP binding and guanylate cyclase activity in cells transfected with pSVL-kin$^-$ than in cells transfected with pSVL-ANPR. In the absence of an antibody with which to quantitate the expressed proteins, however, this comparison of "specific activities" must be interpreted with caution. In contrast, the COOH-terminal deletion mutant (pSVL-cyc$^-$) lacked enzyme activity (Fig. 1C). These experiments demonstrate that guanylate cyclase catalytic activity resides in the COOH-terminal domain and that deletion of the protein kinase–like domain does not interfere with cyclase activity.

These observations were surprising given that the protein kinase–like domain, but not the COOH-terminal domain, is well conserved in the membrane guanylate cyclase from the sea urchin *Arbacia punctulata* (Fig. 1A). It is possible that the structure of the catalytic domain of the enzyme from this primitive sea urchin is quite different from that of other known guanylate cyclases. The localization of guanylate cyclase activity to the COOH-terminal sequences suggests that similar sequences found in a bovine adenylate cyclase (*7*) and in a subunit of a soluble guanylate cyclase (*6*) represent the catalytic domains of those enzymes.

We next examined the effects of the receptor deletions on the regulation of cGMP concentration by ANP in intact cells. Cells transfected with wild-type and mutant ANP receptor clones were incubated in the absence or presence of ANP, and cellular cGMP content was determined (Table 1). In cells transfected with pSVL or pSVL-cyc$^-$, less than 0.2 pmol of cGMP per well was detectable in the absence or presence of

Table 1. Effect of ANP on cGMP content of transfected cells. COS-7 cells in 35-mm wells, transfected with the indicated plasmids, were incubated in the absence (−) or presence (+) of ANP for 5 min at 37°C (*15*). Medium was aspirated and cGMP was extracted with ice-cold 0.5*N* perchloric acid and assayed (*3*). Results are expressed as the mean of triplicate samples ± SE.

Transfection	ANP	cGMP (pmol per well)
pSVL	−	<0.2
	+	<0.2
pSVL-ANPR	−	<0.2
	+	19.0 ± 1.8
pSVL-kin$^-$	−	10.5 ± 0.4
	+	10.7 ± 0.9
pSVL-cyc$^-$	−	<0.2
	+	<0.2

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ANP. In cells transfected with pSVL-ANPR, less than 0.2 pmol of cGMP per well was present in the absence of ANP, but cGMP concentrations were markedly increased after incubation with the hormone. In cells transfected with pSVL-kin$^-$, high concentrations of cGMP were observed in the absence or presence of ANP; no effect of ANP was observed, even though this mutant receptor binds ANP. Thus the guanylate cyclase activity of the wild-type ANP receptor is tightly regulated in the intact cell, whereas the kin$^-$ mutant appears to be constitutively activated. This suggests that the kinase-like domain may normally repress the activity of the catalytic domain and that this repression may be removed after binding of ANP.

The observation that the kin$^-$ mutant seems active in intact cells in the absence of ANP (Table 1) would at first seem to be inconsistent with the observation that this mutant appears to have basal guanylate cyclase–specific activity similar to that of the wild-type ANP receptor in vitro (Fig. 1C). However, these in vitro assays contained nonionic detergent and Mn^{2+}, both of which are known to activate membrane guanylate cyclases (*10*). Therefore, we examined the guanylate cyclase activity of particulate fractions from cells transfected with pSVL-ANPR or pSVL-kin$^-$ in the presence of Mg^{2+}, rather than Mn^{2+}, as the divalent cation, and in the absence of detergent (Table 2). Adenosine 5′-triphosphate (ATP) and nonhydrolyzable ATP analogs potentiate the stimulation by ANP of guanylate cyclase activity in vitro (*11*). One potential site of action for ATP and ATP analogs is the protein kinase–like domain. Therefore, we examined the effect of ATP on the activity of the wild-type and kin$^-$ guanylate cyclases.

In the presence of Mg^{2+} (Table 2), the guanylate cyclase activity of the wild-type receptor was ANP dependent, whereas that of the kin$^-$ mutant was ANP independent. The severalfold higher activity observed in cells transfected with pSVL-kin$^-$ may be due to the apparently higher levels of expression of the mutant receptor that we routinely observed (Fig. 1C). The stimulation of the wild-type guanylate cyclase by ANP was enhanced threefold in the presence of 0.5 m*M* ATP. The activity of the kin$^-$ mutant was enhanced only slightly (~25%) by ATP in the absence or presence of ANP (Table 2).

Our results suggest a model for signal transduction by the ANP receptor, in which binding of ANP to the extracellular domain initiates conformational changes that affect interactions between the cytoplasmic protein kinase–like and catalytic domains. The kinase-like domain ordinarily appears to repress guanylate cyclase activity, and this repression is removed after ligand binding, perhaps by disruption of an interaction between the two intracellular domains. The observation that the kinase-like domain is required for maximum stimulation by ATP is consistent with the possibility that this disruption is potentiated by binding of ATP to this domain, although other mechanisms for the ATP effect are equally plausible.

Although the simplest interpretation of our data is that the kin$^-$ mutant is constitutively active, our inability to rigorously quantitate the expressed proteins leaves open the possibility that this mutant is constitutively inactive but highly overexpressed. In either case, the kinase-like domain is required for regulation of guanylate cyclase

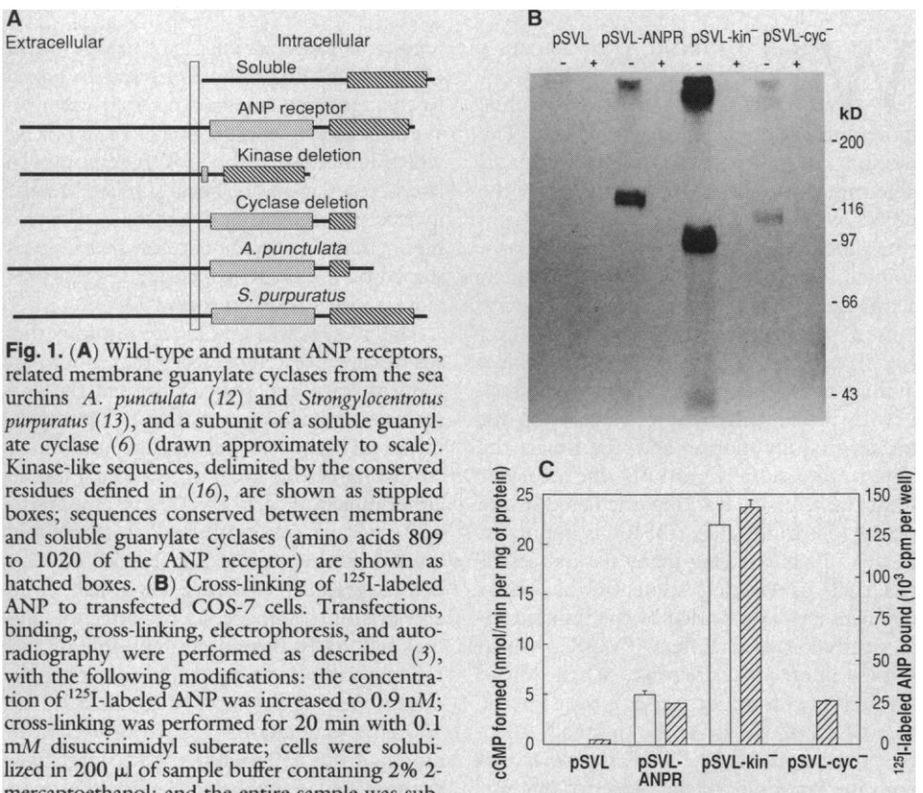

Fig. 1. (**A**) Wild-type and mutant ANP receptors, related membrane guanylate cyclases from the sea urchins *A. punctulata* (*12*) and *Strongylocentrotus purpuratus* (*13*), and a subunit of a soluble guanylate cyclase (*6*) (drawn approximately to scale). Kinase-like sequences, delimited by the conserved residues defined in (*16*), are shown as stippled boxes; sequences conserved between membrane and soluble guanylate cyclases (amino acids 809 to 1020 of the ANP receptor) are shown as hatched boxes. (**B**) Cross-linking of ^{125}I-labeled ANP to transfected COS-7 cells. Transfections, binding, cross-linking, electrophoresis, and autoradiography were performed as described (*3*), with the following modifications: the concentration of ^{125}I-labeled ANP was increased to 0.9 n*M*; cross-linking was performed for 20 min with 0.1 m*M* disuccinimidyl suberate; cells were solubilized in 200 μl of sample buffer containing 2% 2-mercaptoethanol; and the entire sample was subjected to electrophoresis. The absence (−) or presence (+) of unlabeled ANP in the binding incubations is as shown. (**C**) ^{125}I-labeled ANP binding (hatched bars) and guanylate cyclase activities (open bars) of transfected COS-7 cells were determined as described (*3*), with the following modifications: for binding, 0.4 n*M* ^{125}I-labeled ANP and 0.4 μ*M* unlabeled ANP were used; for measurement of guanylate cyclase activity the homogenization buffer contained 40 m*M* NaCl; buffers used in sample preparation contained 10 m*M* dithiothreitol; centrifugations were for 30 min at 42,000*g*; solubilization was for 1 hour; and reactions containing 0.2 m*M* methyl isobutylxanthine were performed for 10 min. Guanylate cyclase activities of solubilized particulate fractions from cells transfected with pSVL and pSVL-cyc$^-$ were 0.00023 ± 0.00009 and 0.0016 ± 0.0003 nmol of cGMP formed per minute per milligram of protein, respectively. Results are expressed as the mean of triplicate samples ± SE.

Table 2. Guanylate cyclase activity of wild-type ANP receptor and kin$^-$ mutant measured in the presence of Mg^{2+}. Particulate fractions were prepared from transfected cells as in Fig. 1C except that dithiothreitol was omitted from all buffers. The final pellet was suspended in 20 m*M* Hepes (*p*H 7.4), and samples containing 10 μg of protein were diluted into a 100-μl reaction mixture containing 20 m*M* Hepes (*p*H 7.4), 4 m*M* $MgCl_2$, 1 m*M* guanosine 5′-triphosphate, 0.2 m*M* methyl isobutylxanthine, 10 m*M* creatine phosphate, and creatine phosphokinase (100 U/ml). In addition, indicated samples contained 1 μ*M* rat ANP (Sigma) or 0.5 m*M* ATP. Samples were incubated for 15 min at 30°C, and the amount of cGMP formed was determined (*3*). Results are expressed as the mean of triplicate samples ± SE.

Transfection	ANP	ATP	Guanylate cyclase activity (pmol/min per mg of protein)
pSVL-ANPR	−	−	<3
	+	−	23.7 ± 1.4
	−	+	<3
	+	+	73.2 ± 3.5
pSVL-kin$^-$	−	−	385 ± 13
	+	−	419 ± 18
	−	+	533 ± 12
	+	+	523 ± 34

activity by ANP. A role for this domain in signal transduction is consistent with its high degree of conservation in all membrane guanylate cyclases sequenced thus far from sea urchins and mammals (*3, 4, 12, 13*).

We have noted the similarity in overall topology between the guanylate cyclase–ANP receptor and protein tyrosine kinase–growth factor receptors (*3*). Like the ANP receptor, these receptors contain an extracellular binding domain, a single transmembrane domain, a protein kinase domain adjacent to the membrane, and a COOH-terminal domain. A model has been proposed in which the protein kinase activity of the epidermal growth factor (EGF) receptor is sterically inhibited by interaction of the kinase domain with the COOH-terminal domain; this interaction is disrupted after EGF binding and the subsequent autophosphorylation of the COOH-terminal domain (*14*). In the case of the ANP receptor, the situation could be reversed: the kinase-like domain may be a negative regulator of the COOH-terminal, guanylate cyclase domain.

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8. To delete kinase-like sequences, we cloned a Hind III fragment, comprising nucleotides 1376 to 2722, of the ANP receptor cDNA into the Hind III site of the plasmid pSP72 (Promega), which had first been modified by insertion of an Nhe I linker into its Pvu II site. The Pvu II fragment, comprising nucleotides 1709 to 2549, of the ANP receptor cDNA (corresponding to amino acids 468 to 747) was then excised, the plasmid was religated, and the sequence of the truncated insert was determined [F. Sanger, S. Miklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977)] with a Sequenase kit (U.S. Biochemical Corporation). The truncated Hind III fragment was inserted in place of the wild-type Hind III fragment in the previously described ANP receptor construct in Bluescript (*3*). To delete COOH-terminal sequences, we excised the ANP receptor cDNA from Bluescript with Sal I and Sma I and subcloned the fragment into Sal I–Sma I–digested pSP72. After Kpn I and Sma I digestion, unidirectional exonuclease III digestion and S1 nuclease treatment [S. Henikoff, *Gene* **28**, 351 (1984)] was performed with the Promega Erase-a-Base kit; a termination codon was introduced by linker tailing [R. Lathe, M. P. Kieny, S. Skory, J. P. Lecocq, *DNA* **3**, 173 (1984); F. Cobianchi and S. H. Wilson, *Methods Enzymol.* **152**, 94 (1987)] with an Nhe I linker containing termination codons in all reading frames (New England Biolabs). Sequence analysis indicated that the clone terminates at nucleotide 2915, corresponding to Leu^{868}. The kinase deletion mutant was subcloned into pSVL as described (*3*), and the COOH-terminal deletion mutant was excised from pSP72 as a Sal I–Bgl II fragment and cloned into Xho I–Bam HI–digested pSVL.
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Ocular Responses to Linear Motion Are Inversely Proportional to Viewing Distance

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Eye movements exist to improve vision, in part by preventing excessive retinal image slip. A major threat to the stability of the retinal image comes from the observer's own movement, and there are visual and vestibular reflexes that operate to meet this challenge by generating compensatory eye movements. The ocular responses to translational disturbances of the observer and of the scene were recorded from monkeys. The associated vestibular and visual responses were both linearly dependent on the inverse of the viewing distance. Such dependence on proximity is appropriate for the vestibular reflex, which must transform signals from Cartesian to polar coordinates, but not for the visual reflex, which operates entirely in polar coordinates. However, such shared proximity effects in the visual reflex could compensate for known intrinsic limitations that would otherwise compromise performance at near viewing.

WHEN LOOKING OUT FROM A speeding train or bus, nearby objects are seen to rush by while more distant ones seem relatively stable. The motion of an image across the retina is determined by the angular velocity of the object with respect to the observer, and when the observer's motion is purely translational, as in the case of the passenger, simple geometry indicates that this velocity must depend on the proximity of the object (*1*). In order to scrutinize any given object in this passing scene, the observer must track it with his eyes, thereby compensating for his own bodily motion and, the nearer the object, the more vigorously the observer must track. Visual tracking, mediated by the ocular following reflex (OFR), is important in this (*2*), but during many natural activities such as walking some of the ocular compensaton is provided by the translational vestibulo-ocular reflex (TVOR), which senses linear accelerations of the head through the otolith organs embedded in the base of the skull (*3*). To be optimally effective, the output of the TVOR should accord with the proximity of the object of interest. Earlier reports did not specify proximal viewing and suggested that the TVOR was rather weak in humans (*4*). However, better responses are obtained if the subject attempts to fixate an imagined nearby target (*5*), and TVOR responses have been linked to the vergence angle of the two eyes (*6*). We now describe experiments on monkeys which indicate that the TVOR responses to lateral translation are linearly related to the inverse of the viewing distance (*1*). We also report that this dependence on proximity is shared by the OFR, which we suggest provides a visual back-up to the TVOR.

The TVOR was investigated in four rhesus monkeys seated on a sled that moved on a linear track and that accelerated the animals along the interaural axis (7). The movements of the sled were gentle and brief, consisting of one cycle of sinusoidal jerk in either direction (period, 200 ms; amplitude, 630 cm/s^2 per second), after which the sled cruised at the acquired speed for 200 ms before gradually slowing to a halt. Sled acceleration commenced only after the animal had satisfactorily fixated one of five randomly selected targets (light-emitting diodes at viewing distances of 16, 25, 50, 100, 150 cm) for randomly varied time periods ranging from 250 to 500 ms. The target was extinguished immediately before the onset of acceleration, leaving the room dark throughout the period of sled motion. A total of 60 responses was obtained for each stimulus at each viewing distance. To preclude learning, animals never experienced sled motion in the light.

Sled motion evoked consistent compensatory eye movements and, as Fig. 1A shows

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