Receptor proteins and biological effects of C-type natriuretic peptides in the renal glomerulus of the rat

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Brown, J., and Z. Zuo. Receptor proteins and biological effects of C-type natriuretic peptides in the renal glomerulus of the rat. *Am. J. Physiol.* 266 (*Regulatory Integrative Comp. Physiol.* 35): R1383–R1394, 1994.—Binding studies on rat glomeruli using ^{125}I-labeled Tyr^0-C-type natriuretic peptide-(1—22) [^{125}I-Tyr^0-CNP-(1—22)] and ^{125}I-labeled α-atrial natriuretic peptide (α-^{125}I-ANP), and the unlabeled ligands CNP-(1—22), α-ANP, and des-Gln^{18},Ser^{19},Gly^{20},Leu^{21},Gly^{22}-ANP-(4—23)-NH_2 (C-ANP) suggest that receptor-like sites that bind both α-ANP and C-ANP fall into two categories, one with high [dissociation constant (K_d) ~10^{-9}M] and one with low (K_d ~10^{-5}M) affinity for CNP-(1—22). Covalent attachment of ^{125}I-Tyr^0-CNP-(1—22) and α-^{125}I-ANP to these sites identifies two membrane proteins with corresponding properties. The first, which can be labeled by both radioligands, is a disulfide-bridged ~140-kDa protein that is reduced by dithiothreitol to ~67 kDa. This protein binds C-ANP and has K_d ~10^{-10} M for CNP-(1—22). The second protein, which is labeled only by α-^{125}I-ANP, also binds C-ANP, but has K_d ~10^{-5}M for CNP-(1—22). This ~77-kDa protein may also have a disulfide-bridged, high-molecular-mass form of ~140 kDa in the absence of dithiothreitol. Studies of glomerular function show that α-^{125}I-ANP is internalized whereas ^{125}I-Tyr^0-CNP-(1—22) is not. C-ANP abolishes the specific internalization of α-^{125}I-ANP. CNP-(1—22) inhibits internalization of 400 pM α-^{125}I-ANP weakly, only ~60% being inhibited by 10 μM CNP-(1—22). This implies that the ~77-kDa protein, with its low affinity for CNP-(1—22), mediates internalization. Furthermore, CNP-(1—22), as well as α-ANP and C-ANP, inhibits glomerular levels of adenosine 3′,5′-cyclic monophosphate (cAMP), and CNP-(1—22) does so with a high affinity, which corresponds to its affinity for the ~67-kDa protein. The results suggest that the ~67-kDa receptor is distinct from the natriuretic peptide clearance receptor and may control cAMP levels.

ligand binding; electrophoresis; receptor internalization; adenosine 3′,5′-cyclic monophosphate

C-TYPE NATRIURETIC PEPTIDES (CNPs) are the most recently identified class of natriuretic peptides. They have been isolated from porcine brain in two forms, the 22-amino acid CNP-(1—22) and the NH_2-terminally extended CNP-(1—53) (23, 34). These CNPs are probably posttranslational modifications of the product of a single gene (16), and their precursor mRNA has been found in rat brain (16). Immunoreactive CNPs are concentrated in specific cerebral areas (18), and localized receptor binding sites for CNP-(1—22) have now been demonstrated in rat brain (5). Furthermore, injections of CNP-(1—22) into the cerebral ventricles modulate drinking behavior as well as the secretion of luteinizing hormone (27, 28). Consequently, CNPs may be important neuropeptides.

Recently, evidence has also accumulated that CNPs may be local growth factors and even hormones. Cultured bovine carotid endothelial cells contain immunoreactive CNPs and release both CNP-(1—22) and CNP-(1—53) into the culture medium (36). Similarly, cultured human aortic endothelium contains CNP-(1—53), and CNP-(1—22) circulates in human plasma (32). mRNA for CNPs is present in bovine aorta (36). mRNA for CNPs is also expressed by rat heart (39), but CNPs have not been found in rat plasma (18). Nevertheless, significant levels of CNPs are found by radioimmunoassay in extracerebral rat tissues such as kidney (18), where they may be produced by endothelium to act on receptors that are present on glomeruli (4). CNP-(1—22) also has receptor binding sites on cultured vascular smooth muscle cells (35, 37) and potently inhibits the growth of these cells (13). Consequently, it has been proposed that CNP-(1—22) produced in vivo by endothelium may be a regulator of vascular growth at different sites in the circulation (4, 36).

CNPs resemble the two other classes of natriuretic peptide, the atrial and brain natriuretic peptides (ANPs and BNPs), in having a 17-residue, disulfide-bridged amino acid ring in their structure (2, 23, 33, 34) (Fig. 1). To an extent, therefore, the natriuretic peptides share a common pool of receptors (4, 5, 8, 17, 20, 21, 29, 35). Molecular cloning studies have identified three natriuretic peptide receptors. Two of these, ANPR-A and ANPR-B, are monomeric proteins with ~120-kDa primary structures and constitutive, agonist-dependent, guanylate cyclase activity (8, 9, 17, 20, 29). The third receptor, ANPR-C, is a disulfide-bridged homodimer of ~60-kDa units without intrinsic guanylate cyclase activity (12, 19, 20, 26). The hormone α-atrial natriuretic peptide (α-ANP) (Fig. 1) is a high-affinity agonist for ANPR-A, but CNP-(1—22) binds and activates this receptor poorly (8, 17, 29). Conversely, CNP-(1—22) is a potent agonist of cloned ANPR-B, whereas α-ANP has little affinity for this receptor (8, 17, 29). Finally, both α-ANP and CNP-(1—22) bind avidly to cloned human ANPR-C when this is expressed in transfected cells (17). Cloned ANPR-C also binds the synthetic ligand des-Gln^{18},Ser^{19},Gly^{20},Leu^{21},Gly^{22}-ANP-(4—23)-NH_2 (Fig. 1) (C-ANP), whereas ANPR-A has no significant affinity for this ligand (12, 20, 26). C-ANP inhibits the catabolism of α-ANP by competitively reducing its internalization to lysosomes (20), and the affinity of cloned ANPR-C for C-ANP is evidence that cloned ANPR-C is the endogenous clearance receptor (12, 20, 26). However, both α-ANP and C-ANP also inhibit adenylate cyclase activity in a variety of tissues (1, 19). This suggests either that ANPR-C has actions in addition to ligand internalization (1, 19) or that C-ANP actually binds to a heterogeneous group of receptors for α-ANP.

Little is known of the receptors for CNPs in vivo. Cloned ANPR-B can be expressed in transfected cells,

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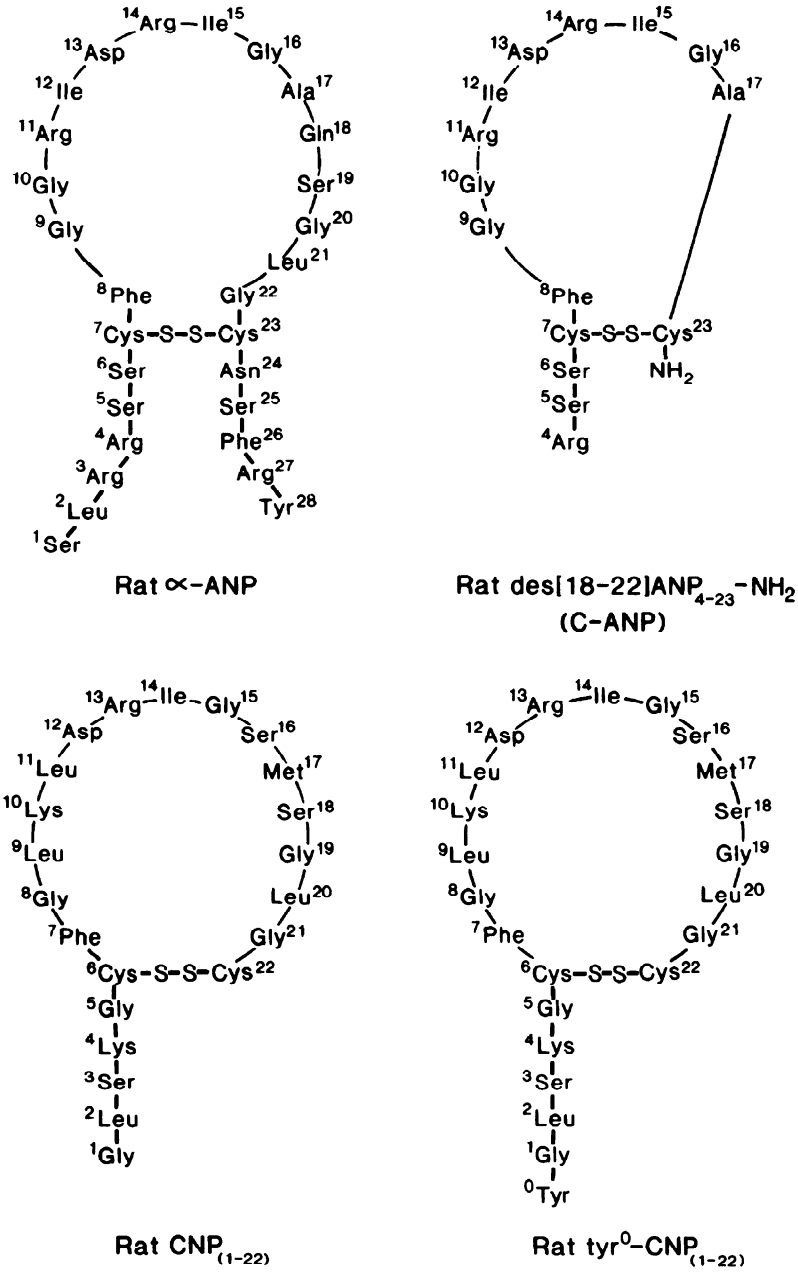

Fig. 1. Structures of the unlabeled natriuretic peptides used in this study. Standard abbreviations are given for amino acid residues. The carboxylic acid terminal amide of C-ANP is denoted as NH_2.

but evidence for it as a native protein mainly concerns vascular smooth muscle in culture (37). We have shown that all of the receptor-like binding sites for CNP-(1—22) that are detected autoradiographically in rat kidney and brain correspond to ANPR-C in their affinities for other ligands (4, 5). However, these ANPR-C-like sites are heterogeneous, some having high affinity and the rest having much lower affinity for CNP-(1—22) (4, 5). We now show that CNP-(1—22) binds to two glomerular membrane proteins that correspond to these ANPR-C-like binding sites. These proteins both resemble cloned ANPR-C in binding α-ANP and C-ANP, but differ from each other widely in their affinities for CNPs. We also show that CNP-(1—22) inhibits levels of glomerular adenosine 3′,5′-cyclic monophosphate (cAMP) with high affinity, whereas it prevents the internalization of α-ANP by glomerular cells with low affinity. The results suggest that the effects of CNP-(1—22) on cAMP and ligand internalization involve separate receptor proteins, both of which would previously have been classified as ANPR-C.

METHODS

Binding and internalization assays. Glomeruli from freshly dissected rat kidneys of 33 male Wistar rats (200–250 g) were isolated by differential sieving as we have described (4). Aliquots of 2,400 glomeruli were preincubated for 10 min at 4°C in 0.5 ml Hanks' balanced salt solution (HBSS) (pH 7.2) plus 0.2% bovine serum albumin (BSA) (fraction V) (Sigma) plus 1 mM phenanthroline (Sigma). They were then centrifuged for 4 min at 300 *g* and resuspended for 15 min at 37°C, or for 2 h at 4°C, in a fresh 0.5 ml of the same fluid plus 400 pM α-^{125}I-ANP (sp act 2,000 Ci/mmol) (Amersham) or 400 pM ^{125}I-labeled Tyr^0-CNP-(1—22) (sp act 1,300 Ci/mmol) (Peninsula Labs), with or without 0–1 μM unlabeled rat α-ANP,

0–10 μM rat CNP-(1—22), or 0–10 μM rat C-ANP (Peninsula) (Fig. 1), or 1 μM angiotensin II, 1 μM vasopressin, or 1 μM neuropeptide Y. Preliminary high-performance liquid chromatography (HPLC) and γ-counting of fractions, as described in detail before (4, 5), established that 1 mM phenanthroline prevented significant radioligand degradation under these conditions of incubation. Identical HPLC studies, but using the absorption of natriuretic peptides at 210 nm to monitor fractions (Waters 990 absorption spectrophotometer, Millipore), showed that there was also no significant degradation of either 10 μM α-ANP or 10 μM CNP-(1—22) under the conditions of incubation in the presence of 1 mM phenanthroline. Preliminary studies of the time courses of binding showed that maximum specific binding of both radioligands was reached by 15 min incubation at 37°C and by 2 h at 4°C. Incubations for the assay of the sum of surface-bound and internalized radioligand were stopped by centrifugation for 2 min at 1,000 *g* at 0°C. Incubations for assay of internalized radioligand alone were washed in 0.2 M acetic acid-0.5 M NaCl (pH 2.5) for 6 min at 4°C before the final centrifugation. ^{125}I associated with sedimented glomeruli was γ-counted.

Affinity cross-linking. Approximately 75,000 glomeruli were isolated as above by finely dicing and sieving the renal cortices from six male Wistar rats (200–250 g) in ice-cold HBSS (pH 7.2) plus 1 mM phenanthroline. Membranes were prepared from these glomeruli by homogenizing in 4 ml ice-cold HBSS/0.2% BSA/1 mM phenanthroline, pH 7.2, with three bursts of 30 s at 28,000 revolutions/min, each burst separated by 30 s (Polytron). The homogenate was centrifuged for 10 min at 0°C and 1,000 *g*. The supernatant was then centrifuged for 60 min at 4°C and 40,000 *g*. The pellet was washed three times in 1 ml phosphate-buffered saline (PBS) (pH 7.2) containing 1 mM phenanthroline and then stored in this fluid at −80°C in fractions containing 1,000 μg protein/ml. Aliquots of membranes (25–60 μg protein) were incubated for 60 min at 4°C with 50 pM α-^{125}I-ANP (rat) or 100 pM ^{125}I-Tyr^0-CNP-(1—22) in 0.5 ml PBS (pH 7.2), containing 0.2% BSA and 1 mM phenanthroline, in the presence or absence of the indicated unlabeled peptides. After incubation the membranes were deposited by centrifugation at 4°C for 60 min at 40,000 *g*. The pellet was washed three times in PBS (pH 7.2) plus 1 mM phenanthroline, and it was then resuspended in this solution plus 1 mM bis(sulfosuccinimidyl) suberate (Pierce Chemical) for 30 min at 4°C. The reaction was stopped by adding 0.5 ml of 100 mM Tris·HCl, pH 7.4, and the mixture was recentrifuged at 4°C for 60 min at 40,000 *g*. The pellet was washed once and resuspended in 0.1 ml of sample buffer [62.5 mM Tris·HCl, pH 6.8, with 10% glycerol (wt/vol) and 2.0% sodium dodecyl sulfate (SDS) in the presence or absence of 50 mM dithiothreitol (Sigma)]. The membranes were boiled for 5 min, and the resulting samples were resolved simultaneously with molecular weight standards by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), using a discontinuous buffer system, on 10% acrylamide separating gels. Preliminary experiments established that concentrations of dithiothreitol in the range 50–500 mM had no effects beyond those at 50 mM.

After electrophoresis, gels were stained in 0.05% Coomassie blue, destained, and dried before autoradiography on GRI-AX films (Genetic Research Instrumentation) with intensification screens at −80°C. ^{125}I standards (Amersham) were also exposed to the film. Autoradiograms were scanned densitometrically, and the optical densities of the autoradiograms of the ^{125}I standards were used to calibrate the autoradiograms of ^{125}I-labeled protein bands in femtomoles of radioligand bound.

Duplicate affinity cross-linking studies were also performed in the presence of a wider range of protease inhibitors. These studies were identical to those already described except for the addition of 10 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 100 μg/ml aprotinin, and 100 μg/ml bacitracin (Sigma) to all buffer solutions that already contained 1 mM phenanthroline, from the initial step of isolating glomeruli to the step of sample dissolution in sample buffer.

The ^{125}I contents of particular protein bands were also measured directly in a separate experiment in which seven pairs of 25-μg aliquots of glomerular membranes were cross-linked to 50 pM α-^{125}I-ANP (sp act 2,000 Ci/mmol), exactly as described above. One sample of each pair was then solubilized in the absence, and the other in the presence, of 50 mM dithiothreitol. Each pair of solubilized samples was resolved by SDS-PAGE on a 10% acrylamide separating gel. Autoradiograms of these gels were prepared, and regions of gel corresponding to each radiolabeled band on the autoradiograms were individually excised and γ-counted.

cAMP accumulation. Glomeruli were pooled from six male Wistar rats (200–250 g) for these experiments. Aliquots of glomeruli (7.5 μg protein/aliquot) were suspended in HBSS plus 0.2% BSA with 0.1 μM 5-hydroxytryptamine (5-HT) or 10 μM histamine (Sigma), both in the presence or absence of 0–1 μM rat α-ANP, 0–1 μM CNP-(1—22), or 1 μM C-ANP for 10 min at 20°C. Incubations were terminated by ice-cold trichloroacetic acid (Sigma) to a final concentration of 6% (wt/vol). Nine aliquots were incubated with each combination of 5-HT, histamine, and peptides. Aliquots were then centrifuged at 4,000 *g* for 10 min, and the supernatants were extracted four times, each time with three times their volume of water-saturated ether. cAMP was then measured in the supernatants by radioimmunoassay after acetylation (Amersham).

Protein assay. Protein content was determined by a bicinchoninic acid assay (Sigma).

Data analysis. The binding parameters for the binding of ligands to isolated glomeruli were estimated by the Ligand program (24). The fits to the data of models with up to three classes of binding sites were compared by the extra sum of squares principle (24). The significance of differences in apparent association constants (K_a) or in maximum binding capacities (B_{max}) was assessed from the change in the weighted residual sum of squares caused by constraining values of K_a or of B_{max} from different sets of data to be equal, rather than allowing them to vary independently during coanalysis of the sets of data by Ligand (24). Estimates of K_a of unlabeled ligands for protein bands on SDS-PAGE were also derived using Ligand to model the competitive inhibition of the binding of a fixed concentration of radioligand by increasing concentrations of unlabeled ligand. Binding was measured autoradiographically, as described above, only in autoradiograms in which the optical density of the protein bands was within the calibrated dose-response range of the film. The binding to each protein was modeled as a single affinity class of binding site. It was assumed, as has been shown for the natriuretic peptide binding sites on isolated glomeruli (4), that the affinity of α-ANP for each labeled glomerular protein was not significantly different to that of α-^{125}I-ANP and that, similarly, the affinity of CNP-(1—22) was not significantly different to that of ^{125}I-Tyr^0-CNP-(1—22). Estimates of K_a are more nearly log normally than normally distributed about their true mean (10). Consequently, each value of K_a (M^{-1}) is reported as the $-\log_{10}$ (pK_a) of the geometric mean and SE of nine determinations in the case of the binding data from isolated glomeruli and of three determinations in the case of the binding data from protein bands on SDS-PAGE (10). For reference, the values of K_a are also given where they first appear as their reciprocal, the apparent dissociation constant of the homologous or heterologous ligand (K_d). Other results are given as arithmetic means and SE of the number of

determinations indicated. Comparisons between these arithmetic means were made by unpaired t tests (31).

RESULTS

Equilibrium binding of ^{125}I-Tyr^0-CNP-(1—22) to glomeruli. The inhibition of the binding of 400 pM ^{125}I-Tyr^0-CNP-(1—22) by increasing concentrations of unlabeled CNP-(1—22) was consistent with a single class of binding sites for CNP-(1—22) of pK_a = 8.52 ± 0.12 (K_d = 3.0 nM) and B_{max} = 336 ± 18 fmol/mg protein (Fig. 2*A*). Similarly, the specific binding of up to 2 nM ^{125}I-Tyr^0-CNP-(1—22) was compatible with a single class of sites for Tyr^0-CNP-(1—22) of pK_a = 8.73 ± 0.30 (K_d = 1.9 nM) and B_{max} = 362 ± 203 fmol/mg protein (Fig. 2*B*). Thus both CNP-(1—22) and Tyr^0-CNP-(1—22) were probably binding to the same class of sites. Confirming this, 1 μM CNP-(1—22) alone or in combination with 1 μM Tyr^0-CNP-(1—22) respectively inhibited the binding of 400 pM ^{125}I-Tyr^0-CNP-(1—22) by 113 ± 7% and 103 ± 5% of that inhibited by 1 μM Tyr^0-CNP-(1—22) alone. These inhibitions were not significantly different. Angiotensin II, vasopressin or neuropeptide Y (all 1 μM) did not significantly affect specific radioligand binding. However, both α-ANP and C-ANP competed at all of the specific binding sites for ^{125}I-Tyr^0-CNP-(1—22) (e.g., Fig. 6*A*), relating these sites to ANPR-C.

Equilibrium binding of α-^{125}I-ANP to glomeruli. The specific binding of α-^{125}I-ANP was consistent with a single class of receptor sites for α-ANP with pK_a = 8.28 ± 0.09 (K_d = 5.3 nM) and B_{max} = 1,410 ± 240

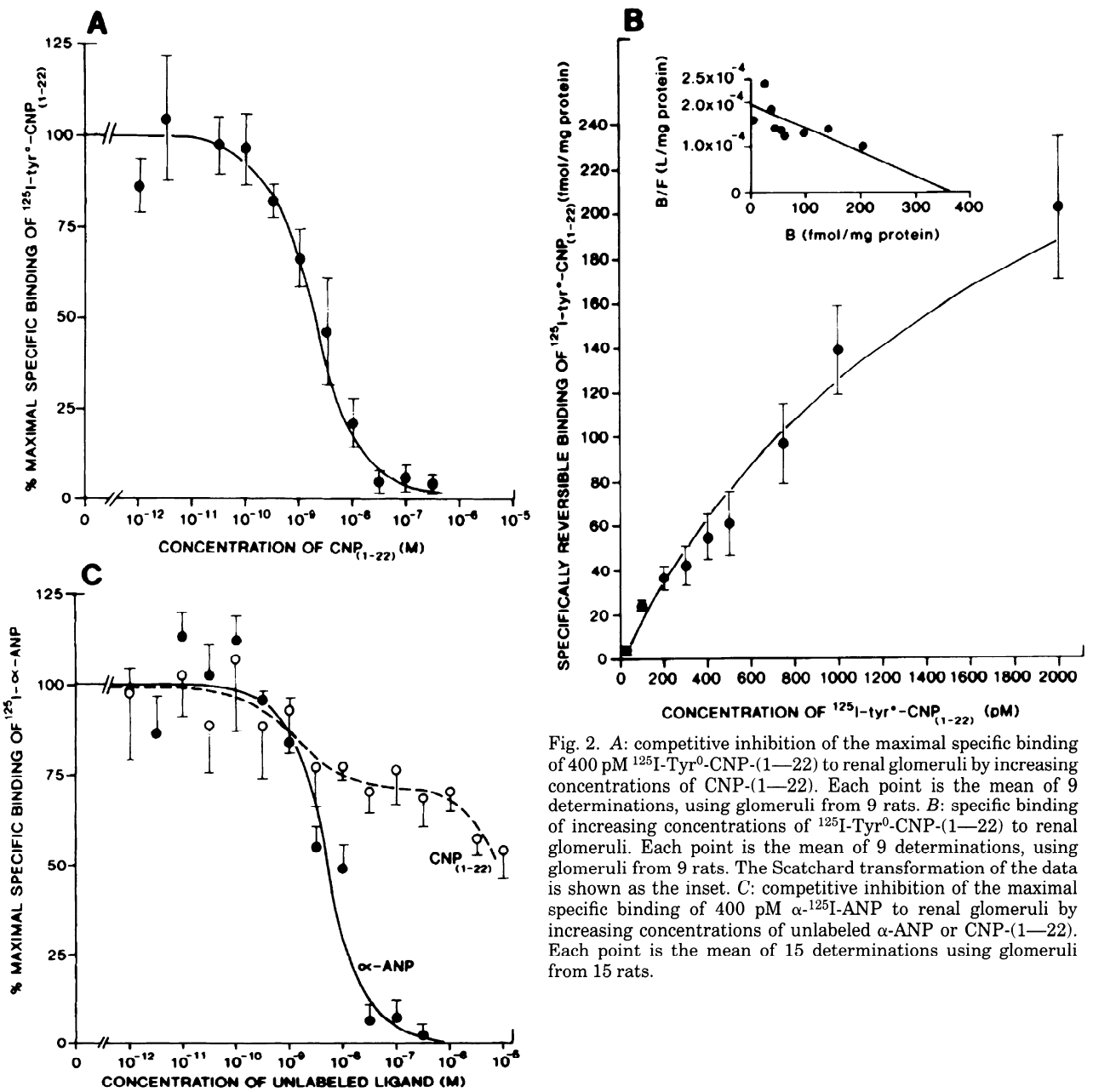

Fig. 2. *A*: competitive inhibition of the maximal specific binding of 400 pM ^{125}I-Tyr^0-CNP-(1—22) to renal glomeruli by increasing concentrations of CNP-(1—22). Each point is the mean of 9 determinations, using glomeruli from 9 rats. *B*: specific binding of increasing concentrations of ^{125}I-Tyr^0-CNP-(1—22) to renal glomeruli. Each point is the mean of 9 determinations, using glomeruli from 9 rats. The Scatchard transformation of the data is shown as the inset. *C*: competitive inhibition of the maximal specific binding of 400 pM α-^{125}I-ANP to renal glomeruli by increasing concentrations of unlabeled α-ANP or CNP-(1—22). Each point is the mean of 15 determinations using glomeruli from 15 rats.

fmol/mg protein. In keeping with previous measurements of the proportion of ANPR-C on glomeruli obtained by using excess (10 μM) C-ANP (3, 4), 10 μM C-ANP competed at 900 ± 100 fmol/mg protein of the specific glomerular binding sites for α-ANP in the present experiments. This estimate of the capacity of glomerular ANPR-C significantly exceeded both estimates (336 ± 18 and 362 ± 203 fmol/mg protein) of the ANPR-C-like sites that had high affinities for CNPs ($P < 0.001$ and $P < 0.05$, respectively). The discrepancy was resolved by examining the inhibition of the specific binding of α-^{125}I-ANP by CNP-(1—22). The inhibition was better modeled by two classes of binding site than by one ($P < 0.001$) (Fig. 2*C*). One class had pK_a = 8.64 ± 0.40 (K_d = 2.3 nM) and B_{max} = 570 ± 150 fmol/mg protein for CNP-(1—22). This class was therefore compatible in its high affinity for CNP-(1—22) and its B_{max}, as well as in its binding of α-ANP, with the single class of reversible binding detected by 400 pM ^{125}I-Tyr^0-CNP-(1—22). The other class of binding sites demonstrated with α-^{125}I-ANP had a substantially lower affinity for CNP-(1—22), with pK_a = 4.68 ± 0.20 (K_d = 21 μM) and B_{max} = 1,230 ± 300 fmol/mg protein. These sites with low affinity for CNP-(1—22) presumably bound ^{125}I-Tyr^0-CNP-(1—22) so poorly as not to be detected at the low concentrations of ^{125}I-Tyr^0-CNP-(1—22) that were used here (Fig. 2, *A* and *B*). Thus rat glomeruli exhibit two classes of ANPR-C-like binding sites, only one of which has high affinities for CNPs. Moreover, although even 10 μM CNP-(1—22) did not actually inhibit all of the specific binding of α-^{125}I-ANP, the pattern of inhibition produced by increasing concentrations of CNP-(1—22) was consistent with the idea that CNP-(1—22) would be able to compete with either high or low affinity at all of the specific binding sites for α-ANP (Fig. 2*C*). Therefore, the class of sites with low affinity for CNP-(1—22) is probably a mixture of ANPR-C-like sites and of ANPR-A. ANPR-A accounts for the high affinity, specifically reversible glomerular binding sites of α-ANP, which have no significant affinity for C-ANP (3, 4, 22), and it is a receptor that is already known to have low affinity for CNP-(1—22) (3, 4, 17).

Affinity cross-linking. The number and apparent molecular weights of the protein bands that were labeled by α-^{125}I-ANP were not altered by the addition of leupeptin, phenylmethylsulfonyl fluoride, aprotinin, and bacitracin to phenanthroline during the procedures leading to affinity cross-linking. However, the additional protease inhibitors produced a proportionate reduction of the intensity of all of the labeled protein bands, suggesting a general effect on ligand binding. Consequently, the ligand affinities of protein bands were measured in the presence of phenanthroline alone.

Affinity cross-linking with ^{125}I-Tyr^0-CNP-(1—22). A dose of 100 pM ^{125}I-Tyr^0-CNP-(1—22) labeled two major protein bands, of ~140 kDa and ~67 kDa, under nonreducing conditions (Fig. 3). The pK_a of the ~140-kDa band for CNP-(1—22) was 10.1 ± 0.3 (K_d = 80 pM) and that for the ~67-kDa band was 9.7 ± 0.4 (K_d = 200 pM). Addition of dithiothreitol abolished the ~140-kDa

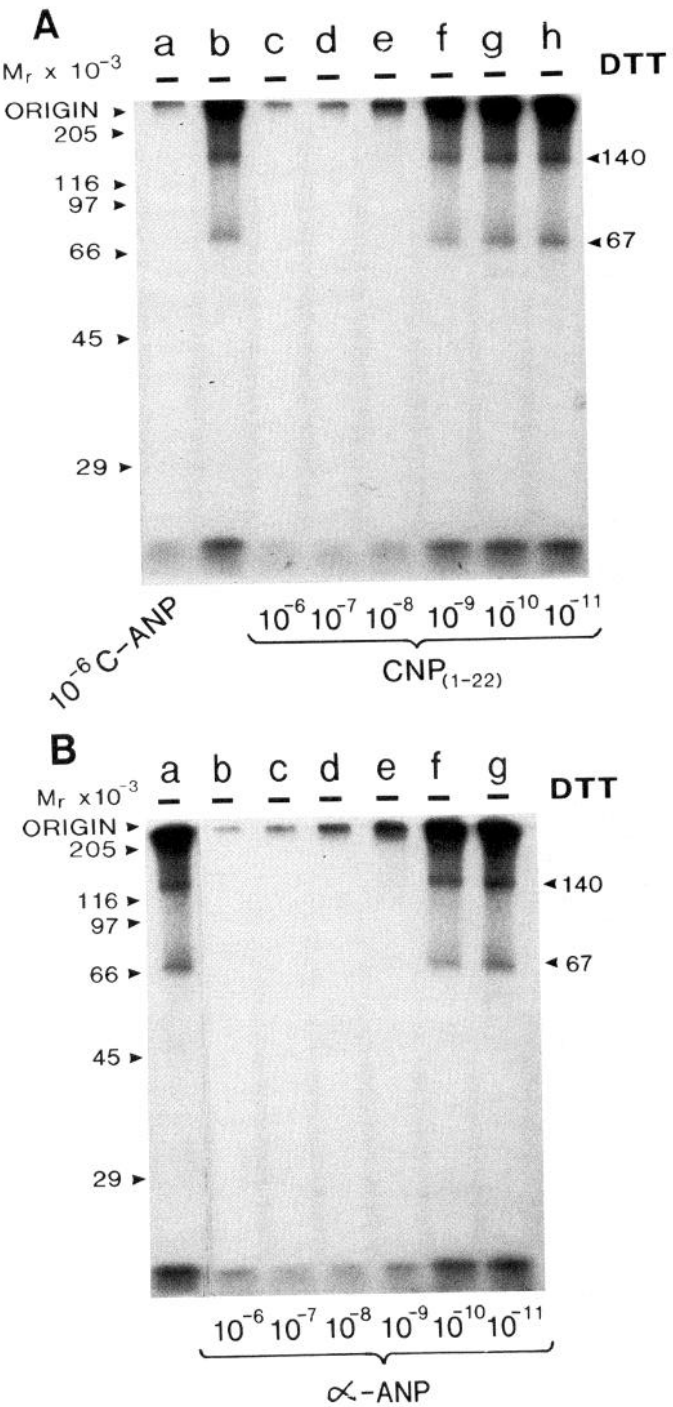

Fig. 3. Autoradiograms of SDS-PAGE of renal glomerular membranes covalently cross-linked to 100 pM ^{125}I-Tyr^0-CNP-(1—22). Cross-linked proteins were dissolved in the absence (−) of dithiothreitol (DTT) and were separated on 10% gels. Sixty micrograms of membrane protein were loaded per lane, and autoradiograms were exposed for 31 days at −80°C with intensification. Measurements of ligand affinities were made from the autoradiograms of 3 repetitions of each gel. *A*: radioligand was bound to membranes in the absence (*lane b*) or presence of either 10^{-6} M-10^{-11} M CNP-(1—22) (*lanes c-h*) or 10^{-6} M C-ANP (*lane a*). *B*: radioligand was bound to membranes in the absence (*lane a*) or presence of 10^{-6} M-10^{-11} M α-ANP (*lanes b-g*).

band and increased the ~67-kDa band. The ~67-kDa band retained its high affinity for CNP-(1—22) (pK_a = 9.6 ± 0.3) (K_d = 300 pM) (Fig. 4). This suggests that the higher relative molecular weight (M_r) band seen under nonreducing conditions may have been a disulfide-bridged dimer of ~67-kDa units. Furthermore, both the ~140-kDa and ~67-kDa bands had high affinities for α-ANP (pK_a = 10.4 ± 0.3 and 10.5 ± 0.3, respectively) (K_d = 40, and 30 pM, respectively) and C-ANP (pK_a = 9.6 ± 0.4, and 9.3 ± 0.4, respectively) (K_d = 300 and 500 pM, respectively). Therefore, the main glomerular membrane protein that cross-links to ^{125}I-Tyr^0-CNP-(1—22) is compatible with both the homodimeric structure and the known ligand selectivity of cloned ANPR-C (12, 26), and it matches the ligand selectivity of those ANPR-C-like sites that have high affinity for CNPs in freshly isolated glomeruli.

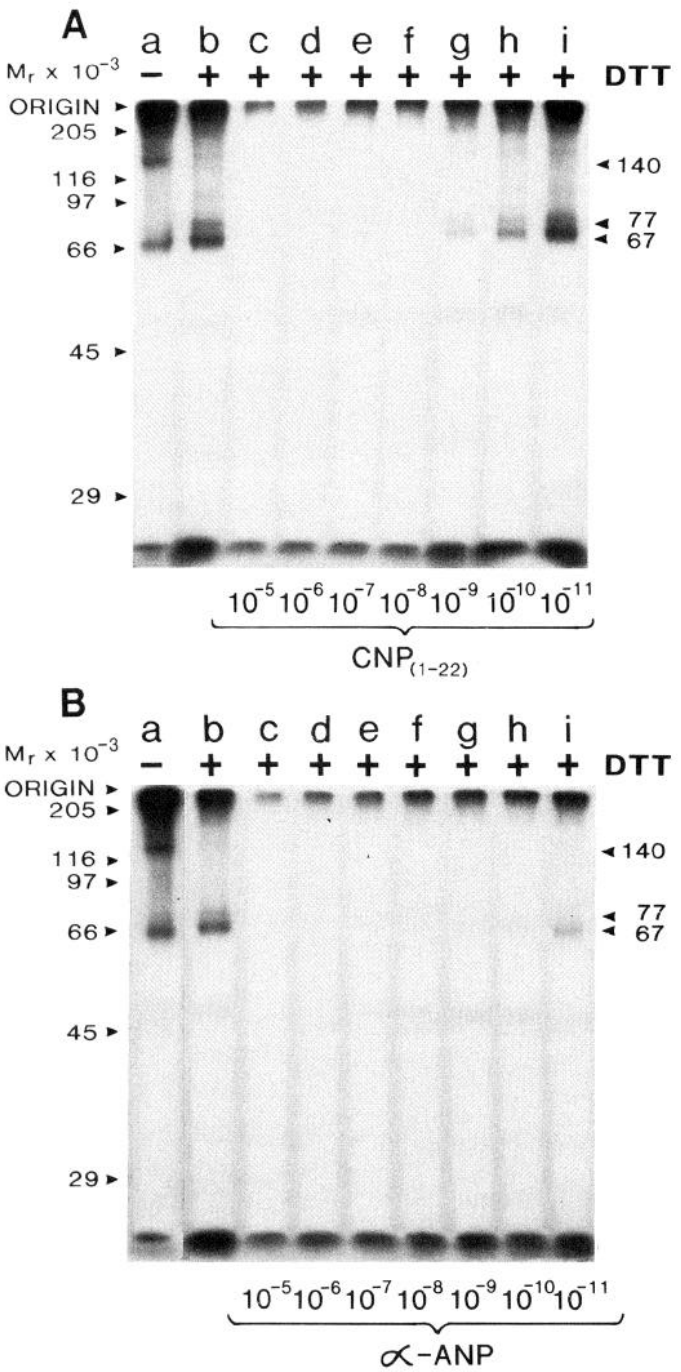

Fig. 4. Autoradiograms of SDS-PAGE of renal glomerular membranes covalently cross-linked to 100 pM ^{125}I-Tyr^0-CNP-(1—22). Cross-linked proteins were dissolved in the absence (−) or presence (+) of DTT and were separated on 10% gels. Sixty micrograms of membrane protein were loaded per lane, and autoradiograms were exposed for 31 days at −80°C with intensification. Measurements of ligand affinities were made from the autoradiograms of 3 repetitions of each gel. *A*: radioligand was bound to membranes in the absence (*lanes a* and *b*) or presence of 10^{-5} M-10^{-11} M CNP-(1—22) (*lanes c-i*). *B*: radioligand was bound to membranes in the absence (*lanes a* and *b*) or presence of 10^{-5} M-10^{-11} M α-ANP (*lanes c-i*).

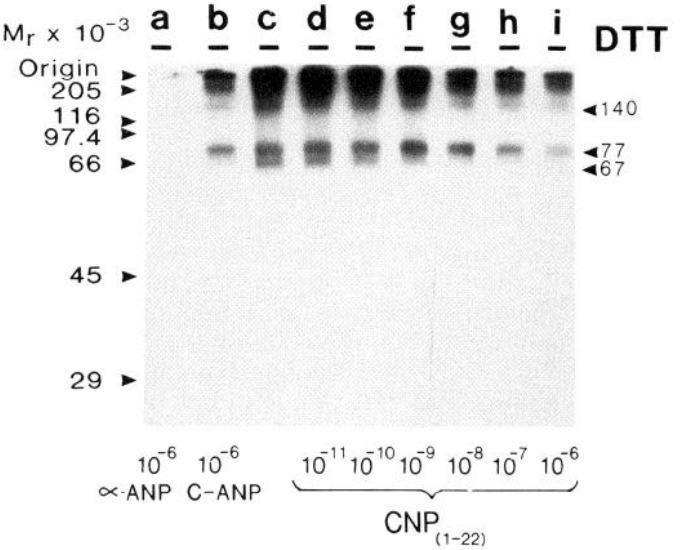

Fig. 5. Autoradiogram of SDS-PAGE of renal glomerular membranes covalently cross-linked to 50 pM α-^{125}I-ANP. Membranes were cross-linked either in the absence (*lane c*) or presence of 10^{-11} M-10^{-6} M CNP-(1—22) (*lanes d-i*), 10^{-6} M α-ANP (*lane a*), or 10^{-6} M C-ANP (*lane b*). Membranes were then dissolved in the absence (−) of DTT and proteins were separated on a 10% gel. Twenty-five micrograms of membrane protein were loaded per lane, and the autoradiogram was exposed for 7 days at −80°C with intensification. Measurements of ligand affinities were made from the autoradiograms of 3 repetitions of this experiment.

Affinity cross-linking with α-^{125}I-ANP. A concentration of 50 pM α-^{125}I-ANP labeled three major protein bands, at ~140, ~77, and ~67 kDa (Fig. 5). All of these bands bound α-ANP avidly under both reducing and nonreducing conditions. Under reducing conditions, for example, the pK_a for α-ANP were 9.5 ± 0.4, 9.3 ± 0.3, and 9.2 ± 0.4, respectively (K_d = 300, 500, and 600 pM, respectively), for the ~140-, ~77-, and ~67-kDa bands. The labeling of the ~140-kDa band was weakened but not abolished by excess dithiothreitol, whereas the other bands became more strongly labeled. A concentration of 1 μM C-ANP reduced but did not abolish the ~140-kDa band in the absence of dithiothreitol, and 1 μM C-ANP did not alter the labeling of the ~140-kDa band in the presence of dithiothreitol. Previous studies have obtained similar results with rat glomerular membranes and have concluded that, under nonreducing conditions, the ANP-binding proteins in the electrophoretic region corresponding to ~140 kDa are a mixture of the disulfide-bridged ANPR-C and of the monomeric ANPR-A,

which does not bind C-ANP (20, 22). However, despite the homodimeric structure of cloned ANPR-C (12, 26), we found that the reduction of the ~140-kDa band by dithiothreitol was associated with increased labeling of two distinct protein bands. Thus the counts in excised regions of gel corresponding to the ~140-kDa band fell from 456 ± 17 to 263 ± 10 counts/min ($P < 0.001$) in the presence of excess dithiothreitol, although both gels without and gels with dithiothreitol were loaded with equivalent amounts of protein. Similarly, the counts in the excised regions of gel corresponding to the ~67- and ~77-kDa bands under nonreducing conditions were, respectively, 213 ± 15 and 275 ± 29 counts/min, and these counts increased, respectively, to 248 ± 7 ($P < 0.05$) and 377 ± 12 counts/min ($P < 0.01$) in the presence of dithiothreitol. Both the ~67- and the ~77-kDa bands resembled monomeric ANPR-C in binding C-ANP, 1 μM C-ANP abolishing the labeling of the ~67-kDa band and attenuating that of the ~77-kDa band. However, the bands differed sharply in their affinities for CNP-(1—22). Under reducing conditions, for example, the pK_a for CNP-(1—22) was 8.7 ± 0.3 (K_d = 2 nM) for the ~67-kDa band and 5.2 ± 0.4 (K_d = 6 μM) for the ~77-kDa band. The ~67-kDa band labeled by α-^{125}I-ANP can therefore be identified with the ~67-kDa band labeled by ^{125}I-Tyr^0-CNP-(1—22) in having high affinities for CNP-(1—22), α-ANP, and C-ANP. This implies that one component of the ~140-kDa autoradiographic band labeled with α-^{125}I-ANP under nonreducing conditions is the ~140-kDa form of the ANPR-C-like glomerular membrane protein that can be cross-linked to ^{125}I-Tyr^0-CNP-(1—22). A high-M_r form of the ~77-kDa protein was not resolved under nonreducing conditions by labeling with α-^{125}I-ANP, but this would be explained if the form were to have an electrophoretic mobility that was not resolved from that of the high-M_r form of the ~67-kDa protein and from that of ANPR-A. Moreover, the small increment in the counts contained in the ~67-kDa band under reducing conditions does not account for the much greater decrement that dithiothreitol caused in the counts associated with the ~140-kDa band. Instead, the majority of the counts lost from the ~140-kDa band in the presence of dithiothreitol appeared as an increase in the ~77-kDa band. This suggests strongly that the ~77-kDa band can arise from one of the constituents of the ~140-kDa band, which exists under nonreducing conditions. The ~77-kDa protein labeled by α-^{125}I-ANP had poor affinity for at least one CNP, CNP-(1—22). However, a band that was labeled by 100 pM ^{125}I-Tyr^0-CNP-(1—22) could sometimes be detected at ~77 kDa with long autoradiographic exposure of gels of samples prepared with dithiothreitol. When present, this band was always relatively faint (e.g., Fig. 4), its binding of ^{125}I-Tyr^0-CNP-(1—22), determined autoradiographically, being only 10 ± 3% of that of the ~67-kDa protein band (n = 7 paired comparisons). Furthermore, the ~77-kDa protein band that was labeled by ^{125}I-Tyr^0-CNP-(1—22) had high affinities for both CNP-(1—22) and α-ANP (Fig. 4). That raises the possibility that this variable and quantitatively minor protein band is actually related to the ~67-kDa protein rather than to the major ~77-kDa protein that is labeled by α-^{125}I-ANP, has poor affinity for CNP-(1—22), and presumably also has low affinity for ^{125}I-Tyr^0-CNP-(1—22). Spurious cross-linking of membrane components to a small proportion of the ~67-kDa protein during the experimental procedure could, for example, give rise to an artifactual increase in the size of the 67-kDa protein without changing its characteristic ligand affinities.

Ligand internalization. The specific glomerular binding of ^{125}I-Tyr^0-CNP-(1—22) was abolished by removing surface-bound radioligand with acid washing (15) (Fig. 6). Thus the high-affinity specific binding sites of ^{125}I-Tyr^0-CNP-(1—22) do not internalize this ligand. In contrast, glomeruli did internalize α-^{125}I-ANP. The specific binding of α-^{125}I-ANP to glomeruli incubated to binding equilibrium with 400 pM α-^{125}I-ANP at 37°C was 143 ± 7 fmol/mg protein. This was reduced by acid washing to 34 ± 5 fmol/mg protein ($P < 0.001$), but it was not abolished ($P < 0.001$ vs. zero specific binding). The acid-resistant component of specific binding was abolished at 4°C, a temperature that is known to prevent

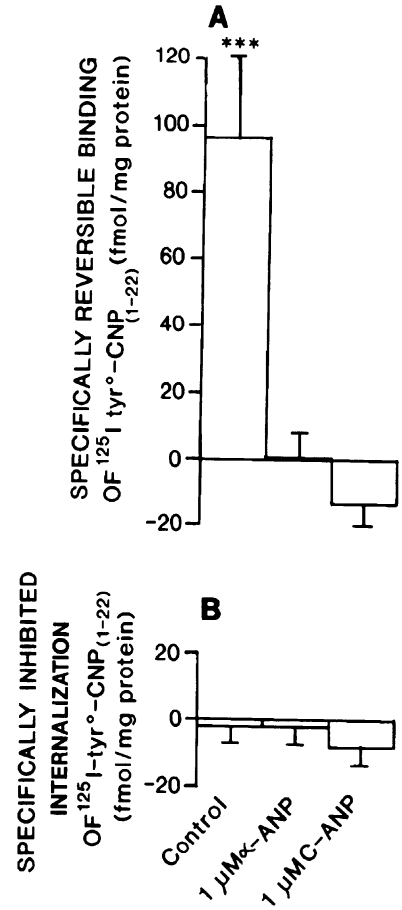

Fig. 6. Specific binding and internalization of 400 pM ^{125}I-Tyr^0-CNP-(1—22) at 37°C by renal glomeruli. *A*: specifically reversible binding of radioligand either alone (control) or in the presence of 1 μM α-ANP or 1 μM C-ANP. *B*: specifically inhibited internalization of radioligand measured as the acid-resistant binding of radioligand either in the absence of heterologous ligands (control) or in the presence of 1 μM α-ANP or 1 μM C-ANP. Results are the means of 9 determinations, using glomeruli from 9 rats. ***$P < 0.001$ for comparison of specifically reversible binding or specifically inhibited internalization vs. 0.

receptor internalization (15). Thus acid washing reduced the specifically reversible binding of radioligand from 264 ± 17 to 0 ± 4 fmol/mg protein when glomeruli were incubated to binding equilibrium with 400 pM α-^{125}I-ANP at 4°C. Finally, the glomerular internalization of α-^{125}I-ANP was prevented by high concentrations of C-ANP. The specific binding of radioligand to glomeruli incubated to binding equilibrium with 400 pM α-^{125}I-ANP in the presence of 1 μM C-ANP at 37°C was 68 ± 5 fmol/mg protein before acid washing and 21 ± 3 fmol/mg protein after acid washing, and the respective figures in the presence of 10 μM C-ANP were 31 ± 4 and 2 ± 3 fmol/mg protein.

^{125}I-Tyr^0-CNP-(1—22) was not internalized by the glomerular clearance receptor but may, nevertheless, have been bound. However, incubating glomeruli at 37°C to binding equilibrium with 400 pM α-^{125}I-ANP and increasing concentrations of CNP-(1—22) did not significantly reduce the acid-resistant fraction of the specific binding of α-^{125}I-ANP until concentrations of 10 μM CNP-(1—22) were reached (Fig. 7). This concentration of CNP-(1—22) is sufficient to allow substantial binding to the low-affinity ($pK_a \sim 5$) glomerular binding sites for the ligand, a fact reflected in the sharp fall in the specific binding of α-^{125}I-ANP at concentrations of CNP-(1—22) above 1 μM [Fig. 2*C*, $P < 0.05$, Fig. 7*A*, $P < 0.01$ for specific binding of α-^{125}I-ANP at 1 μM vs. 10 μM CNP-(1—22)]. Consequently, the internalizing natriuretic peptide receptor of rat renal glomeruli has an apparently low affinity for CNP-(1—22). A comparably poor affinity for ^{125}I-Tyr^0-CNP-(1—22) would explain why this radioligand is not detectably internalized from 400 pM solutions.

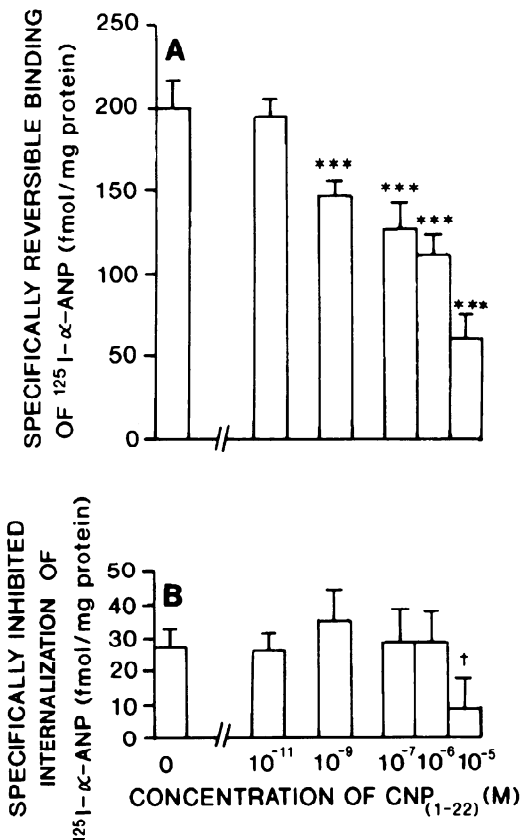

Fig. 7. Specific binding and internalization of 400 pM α-^{125}I-ANP at 37°C by renal glomeruli. *A*: specifically reversible binding of radioligand in the absence or presence of 10^{-11}-10^{-5} M CNP-(1—22). *B*: specifically inhibited internalization of radioligand measured as the acid-resistant binding of radioligand either in the absence or in the presence of 10^{-11} M-10^{-5} M CNP-(1—22). Results are means of 9 determinations, using glomeruli from 9 rats. ***$P < 0.001$ for comparison of specifically reversible radioligand binding in the presence vs. that in the absence of CNP-(1—22). †$P < 0.05$ for comparison of specifically inhibited radioligand internalization in the presence vs. that in the absence of CNP-(1—22).

cAMP accumulation. Both 10^{-5} M histamine and 10^{-7} M 5-HT increased glomerular cAMP content (Fig. 8) as has been described previously for rat glomeruli (30). These increases were significantly inhibited in the presence of either 1 μM CNP-(1—22) or 1 μM α-ANP (Fig. 8). A concentration of 1 μM C-ANP also inhibited the stimulation of cAMP levels produced by either 10^{-5} histamine ($0.10 > P > 0.05$) or 10^{-7} M 5-HT ($0.05 > P > 0.01$) (Fig. 8). The reduction of histamine-stimulated cAMP levels by CNP-(1—22) was substantial and dose dependent. It was achieved with high affinity so that 50% of the effect of histamine was inhibited by ~10^{-9} M CNP-(1—22) (Fig. 9). This contrasts to the markedly lower affinity with which CNP-(1—22) prevented the internalization of α-^{125}I-ANP (Fig. 7*B*).

DISCUSSION

The present binding data support previous evidence (4) that rat renal glomeruli express two classes of binding sites for CNP-(1—22). Both classes resemble cloned ANPR-C in their high affinity for α-ANP and in binding C-ANP. However, only one class has high affinity for CNP-(1—22) ($pK_a \sim 9$), the other having much lower affinity ($pK_a \sim 5$). We now show that these classes of glomerular binding site for CNP-(1—22) correspond to the properties of two glomerular membrane proteins of ~67 and ~77 kDa. Both proteins resemble the ~56-kDa monomer of cloned ANPR-C in binding α-ANP and C-ANP (12, 26). However, the ~67-kDa protein had high affinities ($pK_a \sim 10$) for CNPs, whereas the ~77-kDa protein had low affinity ($pK_a \sim 5$) for CNP-(1—22). The high affinity of the ~67-kDa protein for ^{125}I-Tyr^0-CNP-(1—22) allowed a ~140-kDa form of this protein to be differentiated from ANPR-A, which was not detectably labeled by 100 pM ^{125}I-Tyr^0-CNP-(1—22). The ~67-kDa protein may therefore form disulfide-bridged homodimers in the absence of dithiothreitol and would thus be compatible with cloned ANPR-C (12, 26). The results for the ~140-kDa glomerular protein with high affinity for CNP-(1—22) do not however exclude a disulfide-bridged heteromeric structure in which only a ~67-kDa subunit is cross-linked to ^{125}I-Tyr^0-CNP-(1—22). No highly selective radioligand was available for the ~77-kDa protein, and consequently it was not possible to demonstrate a high-M_r form of this protein in isolation. Nevertheless, the ~77-kDa band was augmented by dissolving glomerular membranes in the presence of dithiothreitol after covalently cross-linking them to α-^{125}I-ANP. The only pro-

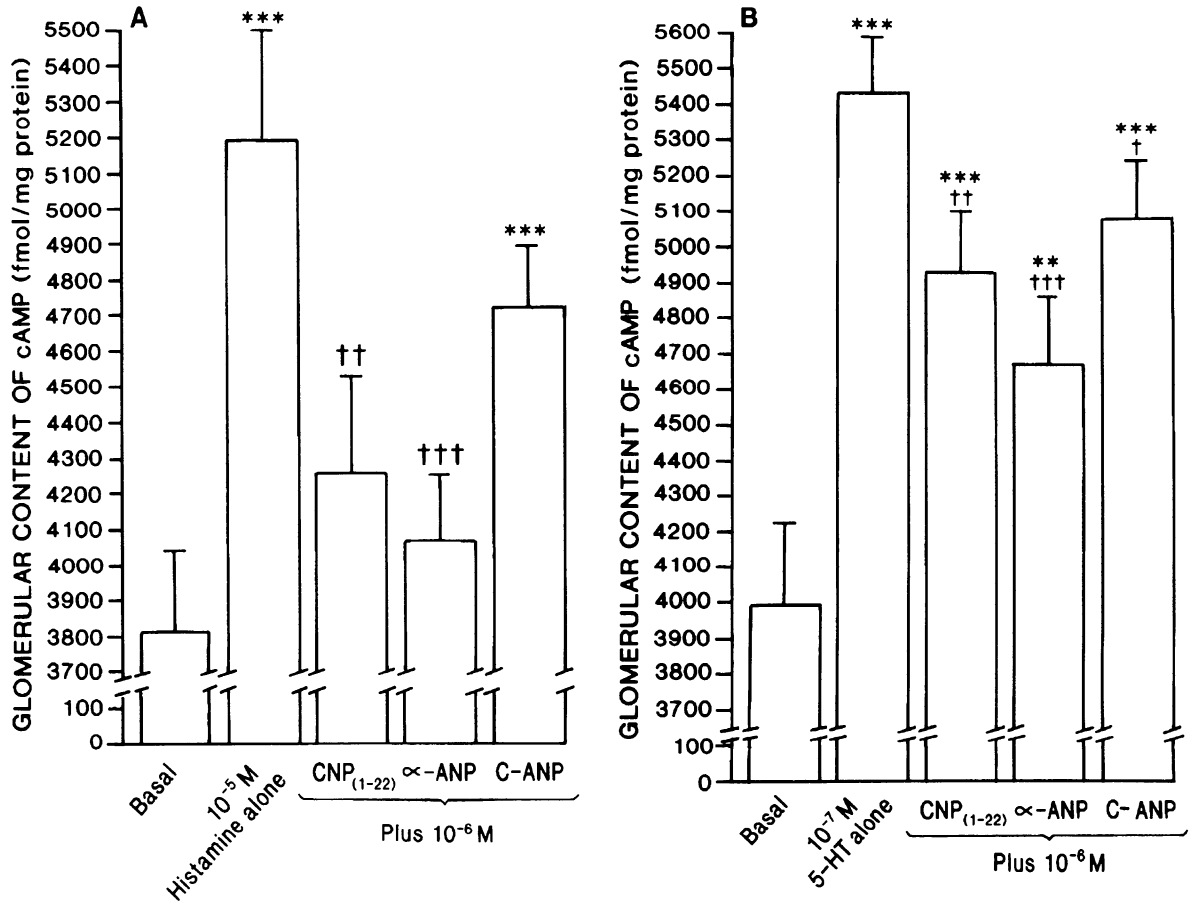

Fig. 8. Effect of histamine (*A*) or 5-hydroxytryptamine (5-HT) (*B*) either alone or plus 10^{-6} M CNP-(1—22), 10^{-6} M α-ANP, or 10^{-6} M C-ANP on the basal content of cAMP in renal glomeruli. Results are means of 9 determinations, using glomeruli from 9 rats. $^{**}P < 0.01$, $^{***}P < 0.001$ for comparison of glomerular cAMP in the presence of histamine or 5-HT vs. basal cAMP. $\dagger\dagger P < 0.01$, $\dagger\dagger\dagger P < 0.001$ for comparison of glomerular cAMP in the presence of the peptides combined with histamine or 5-HT vs. glomerular cAMP in the presence of histamine or 5-HT alone.

tein band to diminish under these circumstances was that labeled at ~140 kDa. Because the structure of the ~140-kDa ANPR-A has no disulfide-bridged subunits (9), these observations suggest that the ~77-kDa protein can arise from a different, reducible component of the ~140-kDa protein band. Consequently, the ~77-kDa protein also resembles cloned ANPR-C in having a high-M_r and possibly homodimeric form under nonreducing conditions. The results suggest that the ~67-kDa and ~77-kDa natriuretic peptide binding proteins of rat glomeruli differ from each other but share similarities to cloned ANPR-C.

The kidney is rich in proteases so that heterogeneity of ANPR-C-like proteins may have arisen artifactually during membrane preparation and cross-linking, even though these procedures were performed at 0–4°C, with one or more protease inhibitors. However, it would be unusual for a degradation product to have substantially higher specific ligand affinities than its parent protein, so it is difficult to reconcile the affinities of the ~67-kDa protein with those of a proteolytic product of the ~77-kDa protein. Furthermore, two classes of binding sites corresponding to the properties of the ~67- and ~77-kDa ANPR-C-like proteins were present on freshly isolated glomeruli. Most importantly, the widely different affinities of the ANPR-C-like proteins for CNP-(1—22) were reflected in the effects of CNP-(1—22) on the internalization of α-^{125}I-ANP and on the glomerular content of cAMP. Thus separate functional receptors with properties like those of the ~67- and ~77-kDa proteins must exist natively.

In this study, α-^{125}I-ANP was internalized by isolated glomeruli through a process blocked by low temperatures and by C-ANP. This was consistent with the operation of the natriuretic peptide clearance receptor. Similar observations in cultured rat mesangial cells have already shown that it is this receptor and not ANPR-A that is internalized (15). We found that the glomerular internalization of α-^{125}I-ANP at 37°C was partly blocked by 10 μM CNP-(1—22) but that lower concentrations of CNP-(1—22) were without significant effect. The experiments were performed in the presence of 1 mM phenanthroline, which prevented both ligand and radioligand degradation. Moreover, the glomeruli in these experiments were exposed simultaneously to both CNP-(1—22) and α-^{125}I-ANP, and they were incubated long enough to achieve binding equilibrium for α-^{125}I-ANP. It is unlikely that there were substantial differences in the rates at which the two peptides in solution reached glomerular receptors, particularly as the spe-

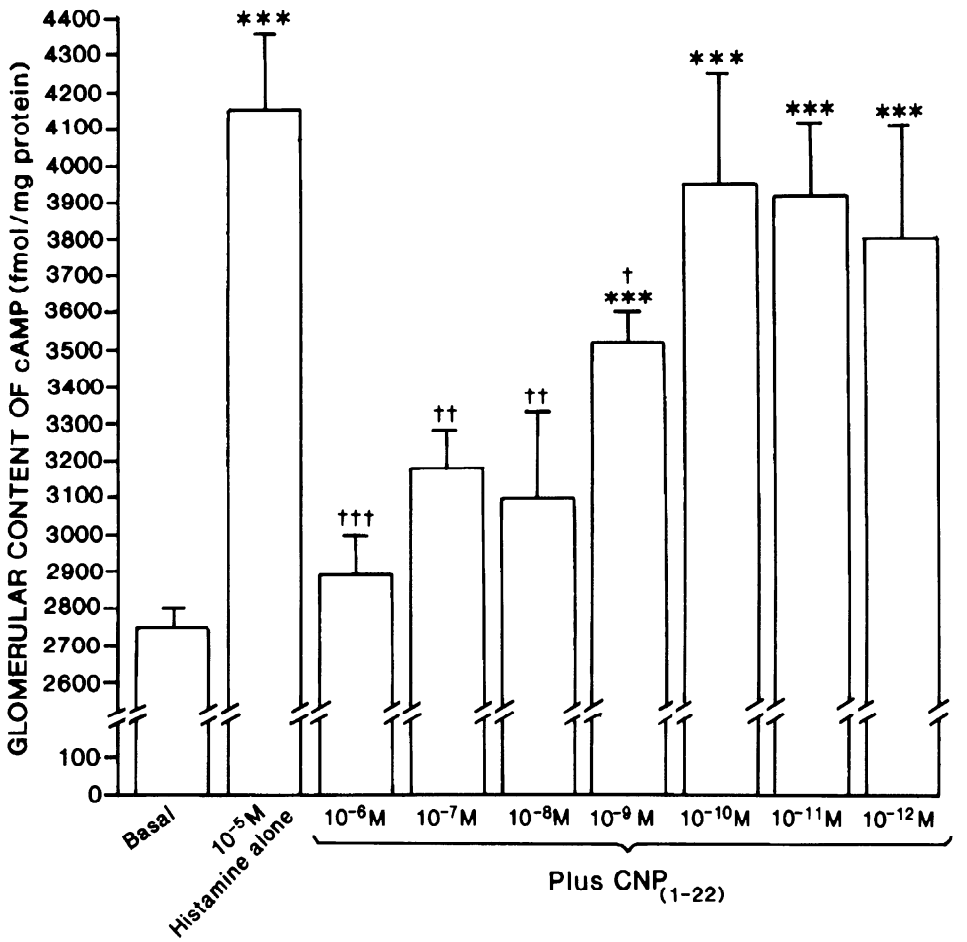

Fig. 9. Effects of different concentrations of CNP-(1—22) on the stimulation of glomerular cAMP content caused by histamine in renal glomeruli. The effect of histamine on basal glomerular cAMP is tested either alone or plus 10^{-6}-10^{-12} M CNP-(1—22). The results are each the means of 9 determinations, using glomeruli from 9 rats. ***P < 0.001 for comparison of glomerular cAMP in the presence of histamine vs. basal cAMP. †P < 0.05, ††P < 0.01, †††P < 0.001 for comparison of glomerular cAMP in the presence of different concentrations of CNP-(1—22) combined with histamine vs. the glomerular cAMP content with histamine alone.

cific glomerular binding of both α-^{125}I-ANP and ^{125}I-Tyr^0-CNP-(1—22) approached equilibrium at similar rates. It follows that the clearance receptor of α-ANP has low affinity for CNP-(1—22) and must correspond to some or all of the ANPR-C-like glomerular binding sites that have low affinity (pK_a ~5) for CNP-(1—22). Similarly, the ~77-kDa protein bound α-ANP, bound C-ANP, had pK_a ~5 for CNP-(1—22), and could account for the glomerular clearance receptor. The ~67-kDa ANPR-C-like protein had pK_a ~10 for CNP-(1—22) in both its dimeric and monomeric states and could not account for the properties of the clearance receptor of α-ANP. Confirming this, 400 pM ^{125}I-Tyr^0-CNP-(1—22) bound almost exclusively to the ~67-kDa protein and yet was not detectably internalized by isolated glomeruli.

We also found that CNP-(1—22), α-ANP, and C-ANP could inhibit the majority of the agonist-stimulated increases in glomerular cAMP content caused by 5-HT or histamine. α-ANP and C-ANP are already known to inhibit adenylate cyclase activity in several tissues, and this has been adduced as evidence that the clearance receptor of α-ANP also signals intracellularly via cAMP (1, 19). However, the binding sites corresponding to the glomerular clearance receptor for α-ANP had low affinity (pK_a ~5) for CNP-(1—22), whereas the effect of CNP-(1—22) on the glomerular content of cAMP occurred at low concentrations (~10^{-9}M). Both the ~67-kDa natriuretic peptide-binding protein (pK_a ~10) and the corresponding high-affinity glomerular binding sites for CNP-(1—22) (pK_a ~9) would be substantially occupied at 10^{-9} M CNP-(1—22), but an effect on cAMP levels through the clearance receptor would require that the intrinsic activity of CNP-(1—22) should compensate for a receptor occupancy of only ~0.01%. Thus it is probably the ~67-kDa ANPR-C-like protein, rather than the ~77-kDa ANPR-C-like protein with its low affinity for CNP-(1—22) that mediates the inhibition of glomerular cAMP content by CNP-(1—22), α-ANP, and C-ANP. Future work is needed to establish whether these distinct receptor proteins are preferentially expressed by different glomerular cells.

Cloned ANPR-C is not known to couple to the inhibition of cAMP levels, but neither has it been shown to mediate ligand internalization (12, 26). Consequently, the relationship of this cloned protein to the ANPR-C-like receptors of rat glomeruli is uncertain. We have found that deglycosylation of rat glomerular membranes with endoglycosidase F replaces the ~140-kDa, ~77-kDa, and ~67-kDa ANP-binding protein bands of untreated membranes with three new bands that are labeled by α-^{125}I-ANP (6). One of these new bands has an apparent M_r ~117,000, corresponding to the ~117-kDa primary structure of ANPR-A. The second has an apparent M_r ~58,000, resembling the 56-kDa primary structure of cloned bovine ANPR-C (12). It is therefore likely that at least one of the native ANPR-C-like

proteins of rat glomeruli actually is a glycosylated form of ANPR-C. The third ANP-binding protein produced by treatment with endoglycosidase F has an apparent M_r of only ~51,000 (6). This implies that the native ~67-kDa and ~77-kDa ANPR-C-like proteins of rat glomeruli differ in primary structure, but it has yet to be excluded that the ~51-kDa protein is an artifact of the proteolytic enzymes that can contaminate preparations of endoglycosidase F.

CNP-(1—22) does not significantly stimulate guanylate cyclase activity in rat glomeruli (4). Moreover, all of the specific binding sites for ^{125}I-Tyr^0-CNP-(1—22) that are detected in rat kidney by in vitro autoradiography have high affinity for α-ANP (4). Neither of these observations is compatible with the expression of ANPR-B in rat kidney. The present study of glomerular membranes also detects no ~120-kDa monomeric protein with high affinity for ^{125}I-Tyr^0-CNP-(1—22), confirming the absence of glomerular ANPR-B. Nevertheless, mRNA for ANPR-B has been found in homogenized rat kidney (29). One possibility, therefore, is that ANPR-B is expressed on renal tubules in the rat at levels too low to be detected autoradiographically. Such a situation would be analogous to the failure of in vitro autoradiography to detect the low levels of ANPR-A, which undoubtedly occur on the proximal tubule of the rat (3, 25, 38).

We have found that both CNP-(1—22) and α-ANP can activate a high-affinity glomerular receptor that inhibits cAMP. However, CNP-(1—22) is a much less potent diuretic and natriuretic agent than α-ANP (34), and it is likely that most of the acute renal effects of α-ANP are mediated through ANPR-A (2, 4, 20). Nevertheless, a natriuretic peptide receptor that is coupled to the cAMP system could still have important functions. α-ANP increases glomerular filtration partly by constricting the efferent glomerular arteriole (2), and this has been difficult to reconcile with an effect of cGMP, the vasodilatory intracellular messenger of ANPR-A (2). Action through a receptor that inhibits cAMP might explain this vasoconstrictory component of the renal effects of α-ANP. Moreover, such a receptor could have other vascular roles. Both C-ANP and α-ANP inhibit the production of endothelin by cultured endothelial cells, probably by lowering the level of endothelial cAMP (14). The interaction of CNP-(1—22) with this ANPR-C-like receptor has yet to be tested, but an effect of CNP-(1—22) on endothelin production might constitute a significant autocrine control of vascular events. Furthermore, C-ANP and α-ANP both inhibit cAMP levels in cultured rat aortic smooth muscle (1), and the ANPR-C-like protein in the solubilized membranes of vascular smooth muscle cells has high affinity for CNP-(1—22) (35). This raises the possibility that CNP-(1—22) may also inhibit cAMP levels in vascular smooth muscle, an effect that could contribute to the antiproliferative action of CNP-(1—22) (13, 19). It may be significant here that the vascular clearance receptor, with its low affinity for CNP-(1—22), would limit the actions of CNP-(1—22) poorly. Finally, ANPR-C-like binding sites with high and low affinity for CNP-(1—22) occur in the brain, where they are differentially distributed (5) and where they may have different functions. It is already known, for example, that α-ANP and C-ANP have neuromodulatory effects on neuroectodermal PC12 cells, and that these effects are probably mediated by a reduction in cellular cAMP (11). Whether some ANPR-C-like receptors of the brain might also modulate cAMP, and whether others act as clearance receptors are important remaining areas of study.

Perspectives

The structure of cloned ANPR-C includes only a short putative intracellular domain and therefore resembles other receptors that deliver extracellular ligands to lysosomes (12, 20). Consequently, it is widely disputed whether the intracellular domain of ANPR-C can also modulate second messengers (19, 20). Our evidence is that separate natriuretic peptide receptors clear α-ANP and inhibit cellular levels of cAMP. Nevertheless, these receptors resemble each other in their probable dimeric structures and in binding C-ANP. Thus they may be examples of a new family of dimeric natriuretic peptide receptors with widely differing intracellular effects. This hypothesis has recently been strengthened. The telencephalon of the fetal rat expresses a single CNP-binding protein that accounts for the ability of CNP-(1—22) to stimulate cGMP production in this tissue (7). The protein consists of ~70-kDa monomers that bind α-ANP and C-ANP (7), and we have recently found that the protein can also travel as a species of higher apparent molecular mass on SDS-PAGE in the absence of reducing agents (unpublished observations). Consequently, this fetal protein may be another member of the proposed family of dimeric natriuretic peptide receptors. It remains to be shown precisely how such receptors resemble each other, and how their structural differences translate into varied intracellular actions.

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REFERENCES

1. **Anand-Srivastava, M. B., M. R. Sairan, and M. Cantin.** Ring-deleted analogs of atrial natriuretic factor inhibit adenylate cyclase/cAMP system. *J. Biol. Chem.* 265: 8566–8572, 1990.
2. **Brenner, B. M., B. J. Ballermann, M. E. Gunning, and M. L. Zeidel.** Diverse biological actions of atrial natriuretic peptide. *Physiol. Rev.* 70: 665–699, 1990.
3. **Brown, J., S. P. Salas, A. Singleton, J. M. Polak, and C. T. Dollery.** Autoradiographic localization of atrial natriuretic peptide receptor subtypes in rat kidney. *Am. J. Physiol.* 259 (*Renal Fluid Electrolyte Physiol.* 28): F26–F39, 1990.
4. **Brown, J., and Z. Zuo.** Renal receptors for atrial and C-type natriuretic peptides in the rat. *Am. J. Physiol.* 263 (*Renal Fluid Electrolyte Physiol.* 32): F89–F96, 1992.
5. **Brown, J., and Z. Zuo.** C-type natriuretic peptide and atrial natriuretic peptide receptors of rat brain. *Am. J. Physiol.* 264 (*Regulatory Integrative Comp. Physiol.* 33): R513–R523, 1993.
6. **Brown, J., and Z. Zuo.** Heterogeneity in the C-ANP binding proteins of rat renal glomeruli (Abstract). *J. Physiol. Lond.* 459: 363P, 1993.
7. **Brown, J., and Z. Zuo.** A novel natriuretic peptide receptor in fetal rats (Abstract). *J. Physiol. Lond.* 459: 472P, 1993.

8. **Chang, M., D. G. Lowe, M. Lewis, R. Hellmiss, E. Chen, and D. V. Goeddel.** Differential activation by atrial and brain natriuretic peptides of two different receptor guanylate cyclases. *Nature Lond.* 341: 68–72, 1989.
9. **Chinkers, M., D. L. Garbers, M. S. Chang, D. G. Lowe, H. Chin, D. V. Goeddel, and S. Schulz.** A membrane form of guanylate cyclase is an atrial natriuretic peptide receptor. *Nature Lond.* 338: 78–83, 1989.
10. **DeLean, A., A. A. Hancock, and R. J. Lefkowitz.** Validation and statistical analysis of a computer modeling method for quantitative analysis of radioligand binding data for mixtures of pharmacological receptor subtypes. *Mol. Pharmacol.* 21: 5–16, 1982.
11. **Drewett, J. G., R. J. Ziegler, and G. J. Trachte.** Neuromodulatory effects of atrial natriuretic peptides correlate with an inhibition of adenylate cyclase but not an activation of guanylate cyclase. *J. Pharmacol. Exp. Ther.* 260: 689–696, 1992.
12. **Fuller, F., J. G. Porter, A. E. Arfsten, J. Miller, J. W. Schilling, R. M. Scarborough, D. B. Schenk, and J. A. Lewicki.** Atrial natriuretic peptide clearance receptor. Complete sequence and functional expression of cDNA clones. *J. Biol. Chem.* 263: 9395–9401, 1988.
13. **Furuya, M., M. Yoshida, Y. Hayashi, N. Ohnuma, N. Minamino, K. Kangawa, and H. Matsuo.** C-type natriuretic peptide is a growth inhibitor of rat vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* 177: 927–931, 1991.
14. **Hu, R. M., E. R. Levin, A. Pedram, and H. J. L. Frank.** Atrial natriuretic peptide inhibits the production and secretion of endothelin from cultured endothelial cells. *J. Biol. Chem.* 267: 17384–17389, 1992.
15. **Koh, G. Y., D. R. Nussenzveig, J. Okolicany, D. A. Price, and T. Maack.** Dynamics of atrial natriuretic factor-guanylate cyclase receptors and receptor-ligand complexes in cultured glomerular mesangial and renomedullary interstitial cells. *J. Biol. Chem.* 267: 11987–11994, 1992.
16. **Kojima, M., N. Minamino, K. Kangawa, and H. Matsuo.** Cloning and sequence analysis of a cDNA encoding a precursor for rat C-type natriuretic peptide (CNP). *FEBS Lett.* 276: 209–213, 1990.
17. **Koller, K. J., D. G. Lowe, G. L. Bennett, N. Minamino, K. Kangawa, H. Matsuo, and D. V. Goeddel.** Selective activation of the B natriuretic peptide receptor by C-type natriuretic peptide (CNP). *Science Wash. DC* 252: 120–123, 1991.
18. **Komatsu, Y., K. Nakao, S. Suga, Y. Ogawa, M. Mukoyama, H. Arai, G. Shirakami, K. Hosoda, O. Nakagawa, N. Hama, I. Kishimoto, and H. Imura.** C-type natriuretic peptide (CNP) in rats and humans. *Endocrinology* 129: 1104–1106, 1991.
19. **Levin, E. R.** Natriuretic peptide C-receptor: more than a clearance receptor. *Am. J. Physiol.* 264 (*Endocrinol. Metab.* 27): E483–E489, 1993.
20. **Maack, T.** Receptors of atrial natriuretic factor. *Annu. Rev. Physiol.* 54: 11–27, 1992.
21. **Maeda, T., M. Niwa, K. Shigematsuo, M. Kurihara, Y. Kataoka, K. Nakao, H. Imura, H. Matsuo, H. Tsuchiyama, and M. Ozaki.** Specific [^{125}I] brain natriuretic peptide-26 binding sites in rat and pig kidneys. *Eur. J. Pharmacol.* 176: 341–350, 1990.
22. **Martin, E. R., J. A. Lewicki, R. M. Scarborough, and B. J. Ballerman.** Expression and regulation of ANP receptor subtypes in rat renal glomeruli and papillae. *Am. J. Physiol.* 257 (*Renal Fluid Electrolyte Physiol.* 26): F649–F657, 1989.
23. **Minamino, N., K. Kangawa, and H. Matsuo.** *N*-terminally extended form of C-type natriuretic peptide (CNP-53) identified in porcine brain. *Biochem. Biophys. Res. Commun.* 170: 973–979, 1990.
24. **Munson, P. J., and D. Rodbard.** LIGAND; a versatile computerized approach for characterization of ligand binding system. *Anal. Biochem.* 107: 220–239, 1980.
25. **Nonoguchi, H., M. A. Knepper, and V. C. Manganiello.** Effects of atrial natriuretic factor on cyclic guanosine monophosphate and cyclic adenosine monophosphate accumulation in microdissected nephron segments from rats. *J. Clin. Invest.* 79: 500–507, 1987.
26. **Porter, J. G., Y. Huang, K. Schwartz, A. Arfsten, A. Lofredo, K. Spratt, D. B. Schenk, F. Fuller, R. M. Scarborough, and J. A. Lewicki.** Characterisation of the atrial natriuretic peptide clearance receptor using a vaccinia virus expression vector. *J. Biol. Chem.* 263: 18827–18833, 1988.
27. **Samson, W. K., K. D. Skala, and F. L. S. Huang.** CNP-22 stimulates, rather than inhibits, water drinking in the rat: evidence for a unique biological action of the C-type natriuretic peptides. *Brain Res.* 568: 285–288, 1991.
28. **Samson, W. K., F-L. S. Huang, and R. J. Fulton.** C-type natriuretic peptide mediates the hypothalamic actions of the natriuretic peptides to inhibit luteinizing hormone secretion. *Endocrinology* 132: 504–509, 1993
29. **Schulz, S., S. Singh, R. A. Bellet, G. Singh, D. J. Tubb, H. Chin, and D. L. Garbers.** The primary structure of a plasma membrane guanylate cyclase demonstrates diversity within this new receptor family. *Cell* 58: 1155–1162, 1989.
30. **Shah, S. V., T. E. Northrup, Y. S. F. Hui, and T. P. Dousa.** Action of serotonin (5-hydroxytryptamine) on cyclic nucleotides in glomeruli of rat renal cortex. *Kidney Int.* 15: 463–472, 1979.
31. **Snedecor, G. W., and W. G. Cochran.** *Statistical Methods.* Ames, IA: Iowa State Univ. Press, 1980.
32. **Stingo, A. J., A. L. Clawell, D. M. Heublein, C-M. Wei, M. R. Pittelkow, and J. C. Burnett.** Presence of C-type natriuretic peptide in cultured human endothelial cells and plasma. *Am. J. Physiol.* 263 (*Heart Circ. Physiol.* 32): H1318–H1321, 1992.
33. **Sudoh, T., K. Kangawa, N. Minamino, and H. Matsuo.** A new natriuretic peptide in porcine brain. *Nature Lond.* 332: 78–81, 1988.
34. **Sudoh, T., N. Minamino, K. Kangawa, and H. Matsuo.** C-type natriuretic peptide (CNP): a new member of natriuretic peptide family identified in porcine brain. *Biochem. Biophys. Res. Commun.* 168: 863–870, 1990.
35. **Suga, S., K. Nakao, H. Hosoda, M. Mukoyama, Y. Ogawa, G. Shirakami, H. Arai, Y. Saito, Y. Kambayashi, K. Inoye, and H. Imura.** Receptor selectivity of natriuretic peptide family, atrial natriuetic peptide, brain natriuretic peptide and C-type natriuretic peptide. *Endocrinology* 130: 229–239, 1992.
36. **Suga, S., K. Nakao, H. Itoh, Y. Komatsu, Y. Ogawa, N. Hama, and H. Imura.** Endothelial production of C-type natriuetic peptide and its marked augmentation by transforming growth factor-β. *J. Clin. Invest.* 90: 1145–1149, 1992.
37. **Suga, S., K. Nakao, I. Kishimoto, K. Hosoda, M. Mukoyama, H. Arai, G. Shirakami, Y. Ogawa, Y. Komatsu, O. Nakagawa, N. Hama, and H. Imura.** Phenotype-related alteration in expression of natriuretic peptide receptors in aortic smooth muscle cells. *Circ. Res.* 71: 34–39, 1992.
38. **Terada, Y., T. Moriyama, B. M. Martin, M. A. Knepper, and A. Garcia-Perez.** RT-PCR microlocalization of mRNA for guanylyl cyclase-coupled ANF receptor in rat kidney. *Am. J. Physiol.* 261 (*Renal Fluid Electrolyte Physiol.* 30): F1080–F1087, 1991.
39. **Vollmar, A. M., A. L. Gerbes, M. Nemer, and R. Schulz.** Detection of C-type natriuretic peptide (CNP) transcript in the rat heart and immune organs. *Endocrinology* 132: 1872–1874, 1993.