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Differential Regional Expression of Three Natriuretic Peptide Receptor Genes within Primate Tissues

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The natriuretic peptide receptors are three homologous cell surface proteins, each with a single transmembrane domain. The atrial natriuretic peptide receptor type A (ANPRA) and the homologous receptor type B (ANPRB) are both membrane guanylyl cyclases that synthesize cyclic GMP as an intracellular second messenger. The third receptor in this family, the atrial natriuretic peptide receptor type C (ANPRC), is not coupled to cyclic GMP production. We report on the distribution of the ANPRA, ANPRB, and ANPRC mRNAs in rhesus monkey tissues assayed by in situ hybridization. ANPRA mRNA is most abundantly expressed in the kidney glomerulus, adrenal zona glomerulosa, pituitary, cerebellum, and endocardial endothelial cells of the right and left atrium and right ventricle. In contrast, abundant ANPRB expression appears to be confined to the adrenal medulla, pituitary, and cerebellum. ANPRC mRNA appeared to be expressed very differently than ANPRA and ANPRB. In the heart, ANPRC mRNA is expressed most prominently in endocardial endothelial cells of all four chambers but is also found throughout the myocardium only in the right atrium. These data identify major sites of natriuretic peptide receptor mRNA expression and suggest that there may be prominent cell type-specific differential distribution of these receptors in central and peripheral targets for the natriuretic peptides.

Natriuretic peptides are a family of homologous polypeptide hormones that function in both central and peripheral control of fluid volume regulation. As such, these hormones are in dynamic mutual antagonism to the hypertensive renin/angiotensin II/aldosterone system. Of the three known hormones in this family, atrial natriuretic peptide (ANP) (reviewed in reference 13), brain natriuretic peptide (BNP) (37), and type C natriuretic peptide (CNP) (38), ANP has been the most intensely studied, with documented effects on the kidney, adrenals, vasculature, pituitary, and brain. Both ANP and the more recently described BNP are primarily cardiac hormones (26) that are released by the heart in its role as an endocrine organ, regulating fluid and electrolyte homeostasis. A variety of extra-atrial sites of ANP expression have been described (13), suggesting a localized paracrine role for ANP in some tissues. In contrast, the expression of the newly discovered hormone CNP appears to be limited to the nervous system (15).

Three members of the natriuretic peptide receptor family have been identified by molecular cloning. The atrial natriuretic peptide receptor type A (ANPRA) (20), also referred to as GC-A (5), is a membrane form of guanylyl cyclase that directly synthesizes the intracellular second messenger cyclic GMP (cGMP) in response to extracellular hormone binding. This receptor responds to stimulation by ANP and BNP (3, 5, 20, 32) but is not a hormonal target for CNP stimulation (15a). A second receptor/guanylyl cyclase homologous to ANPRA, referred to as ANPRB (3) or GC-B (32), displays a hormonal selectivity opposite that of ANPRA, with CNP as the apparent physiological hormone for cGMP stimulation (15a). The third receptor in this family, termed ANPRC (10, 19), is homologous to ANPRA and ANPRB throughout the extracellular domain of over 440 amino acids. However, ANPRC possesses only a 37-amino-acid cytoplasmic domain, in contrast to the large (greater than 500 amino acids) cytoplasmic domains of ANPRA and ANPRB. A role for ANPRC as a natriuretic peptide clearance receptor has been postulated (21), but there is also compelling evidence for signal transduction via this receptor with respect to attenuating intracellular cyclic AMP (cAMP) levels (1). ANPRC occupies a position intermediate between ANPRA and ANPRB in terms of hormone specificity, binding the three hormones with relative affinities of ANP $\cong$ CNP > BNP (20a).

Knowledge of what specific tissues express the three receptors is important for understanding the physiological actions of the natriuretic peptides and to demonstrate the in vivo significance of these receptors. Although in vitro autoradiography provides information on the location of ^{125}I-ANP binding sites (reviewed in reference 36), the molecular identity of the receptors cannot be established. However, these experiments do serve to highlight species differences in the distribution of ANP receptors. Northern (RNA) blotting experiments and cDNA cloning have demonstrated the expression of ANPRC in kidney (10, 19), ANPRA in kidney, adrenal, ileum, adipose tissue, placenta, pituitary, and brain (5, 20, 32), and ANPRB principally in pituitary, brain, and placenta (3). We report here the results of extensive in situ hybridization experiments in the rhesus monkey with cloned human ANPRA, ANPRB, and ANPRC cDNAs as probes. Our observations provide direct evidence for physiologically relevant abundant expression of natriuretic peptide receptor mRNAs in hormonal target tissues and illustrate an apparent marked differential localization for the mRNAs encoding these receptors.

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MATERIALS AND METHODS

Tissue preparation. Tissue samples were collected at necropsy from a 10-year-old normal adult male rhesus monkey. Samples were immediately put into 0.1 M $NaPO_4$ (pH 7.4)–4% paraformaldehyde fixative for 3 to 4 h at 4°C and then cryoprotected in 15% sucrose–phosphate-buffered saline overnight. For sectioning, the samples were embedded in optimal cutting temperature compound (O.C.T.; Miles Laboratories), snap frozen in liquid N_2, and stored at −70°C until use. Cryosections (10 μm) were thaw-mounted onto poly-L-lysine (Sigma)-coated glass slides, refrozen, and stored with desiccant at −70°C prior to hybridization.

Hybridization probes. Two ANPRA antisense probes were used. One was a 508-bp *Pst*I-*Bam*HI fragment covering codons 212 to 383 of the mature ANPRA extracellular domain (20) subcloned into pGEM4 (Promega Biotech). This vector was linearized with *Pst*I and transcribed with SP6 RNA polymerase (25). A longer ANPRA antisense probe was obtained from a pBluescript SK^- (Stratagene) subclone containing the translation initiation codon and extending 3′ to codon 795. This vector was linearized with *Eco*RI and transcribed with T7 RNA polymerase to give a 1,520-nucleotide antisense transcript. A control sense probe was generated from the ANPRA expression vector pRKANP-AR (20), which contains an SP6 promoter 5′ of the translation initiation codon. This vector was linearized at an engineered *Xho*I site at codons 795 and 796 of the mature ANPRA coding sequence to give an approximately 2,500-nucleotide transcript.

An ANPRB antisense probe was generated from a 314-bp *Eco*RI fragment from the 5′ end of cDNA clone 16.15 (3), covering codons 180 to 287 of the mature ANPRB coding sequence. A 1,520-nucleotide ANPRB antisense probe was synthesized by using T7 RNA polymerase transcription of a pBluescript SK^- ANPRB subclone, extending from the translation initiation codon to codon 790, and linearized with *Eco*RI. A control sense probe was made from the ANPRB expression vector pRKANP-BR (3) as described above for ANPRA, with plasmid linearization at an exactly analogous *Xho*I site in the ANPRB coding sequence. Similar results were obtained with both long and short probes for ANPRA and ANPRB.

The ANPRC antisense hybridization probe was from a 382-bp fragment, covering codons 258 to 386 of the human ANPRC precursor (19), subcloned in pGEM4. This plasmid was linearized with *Xba*I for SP6 RNA polymerase transcription.

In situ hybridization. In situ hybridizations were performed as described by Wilcox et al. (39, 40). Briefly, tissue cryosections were treated with paraformaldehyde and then with proteinase K and prehybridized at 42°C in 100 μl of hybridization buffer (50% formamide, 0.3 M NaCl, 20 mM Tris-HCl [pH 8.0], 5 mM EDTA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin, 10% dextran sulfate, 10 mM diothiothreitol). Overnight hybridization was started by adding 600,000 cpm of ^{35}S-UTP (Amersham; specific activity, 1,200 Ci/mmol)-labeled in vitro transcripts at 37°C. Slides were then washed in 2× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate [pH 7.0]) with 10 mM β-mercaptoethanol and 1 mM EDTA, treated with RNase, washed in the same buffer again, and washed at high stringency in 0.1× SSC with 10 mM β-mercaptoethanol and 1 mM EDTA at 52°C. Slides were rinsed in 0.5× SSC and dehydrated in graded alcohols containing 0.3 M ammonium acetate. Slides were dried, coated with NTB2 nuclear track emulsion (Kodak), and exposed in the dark at 4°C for 4, 8, or 12 weeks. After development, the sections were counterstained with hematoxylin and eosin for cell visualization. Sections were photographed under bright-field optics, with polarized-light epiluminescence (Lietz) illuminating the silver grains as white spots.

RESULTS AND DISCUSSION

Natriuretic peptide receptor mRNA in situ hybridization. To examine in detail the distribution of mRNAs encoding the three known natriuretic peptide receptors in a primate, we used in situ hybridization of rhesus monkey tissue sections. [^{35}S]cRNA probes were generated from the regions encoding the extracellular domains of all three receptors or also encompassing the kinase homology domain of ANPRA and ANPRB (Materials and Methods). In the case of each receptor, a high degree of cross-species sequence identity (>90%) facilitates hybridization of the human probes to the corresponding monkey mRNAs, and large differences in the sequences of the three receptors provide the necessary specificity. Tissue sections were hybridized concurrently with the various probes (see Materials and Methods) and subsequently exposed for 4, 8, or 12 weeks. Sense controls were compared with antisense probe hybridization signals for ANPRA and ANPRB to determine backgrounds. Controls routinely showed a low background of evenly distributed silver grains and were directly compared with antisense probe hybridizations to score positive results. For ANPRC antisense probe hybridization, positive signals were generally very specific within a given tissue and overall backgrounds within any particular set of hybridizations were also low. Figures 1, 2, and 3 show representative bright-field photomicrographs, with polarized-light epiluminescence illuminating the silver grains as white spots. A summary of our data is presented in Table 1. Positive hybridizations were detected most prominently in kidney, adrenal, heart, brain, and pituitary. The failure to detect positive hybridization with these probes in certain tissues may indicate one of two possibilities. Either these tissues are truly negative for receptor mRNAs, or the mRNAs could be present at a concentration below the minimum level of detection (currently unknown) for the in situ hybridization method used here (39, 40). Caution must therefore be exercised in the interpretation of negative hybridization results, and we should note that these data were obtained with tissues from only one animal.

Kidney. Counterstaining of cryosections taken through the rhesus monkey kidney cortex show cross sections of renal corpuscles surrounded by proximal and distal convoluted tubules of the nephrons (Fig. 1). ANPRA cRNA hybridization was intense in the glomerular core of the kidney corpuscle (Fig. 1A). ANPRA hybridization in the glomerulus was characterized by localized and discontinuous clustering of silver grains to what appears to be the visceral surface Bowman's capsule that surrounds the glomerular capillaries; this may be hybridization to the epithelial cells, mesangial cells, or both. The epithelial cells of this glomerular layer form interdigitated podocyte foot processes, between which the glomerular filtrate must pass. The epithelial cell layer is not, however, necessarily continuous over the entire surface of a glomerular tuft arteriole. Outside of the glomeruli, we observed infrequent clustering of silver grains over cell bodies, suggesting specific hybridization to cells of the nephrons, collecting ducts of the medullary rays, or arteri-

TABLE 1. Summary of natriuretic peptide receptor mRNA distribution in rhesus monkey tissues by in situ hybridization

Tissue	Hybridization[a]		
	ANPRA	ANPRB	ANPRC
Kidney	++	−	+
Adrenal cortex			
Zona glomerulosa	++	−	+[b]
Zona fasciculata	−	−	+[b]
Zona reticularis	−	−	+[b]
Adrenal medulla	+	+	+[b]
Heart			
Right atrium	+[c]	−	++[d]
Right ventricle	+[c]	−	+[c]
Left atrium	+[c]	−	+[c]
Left venticle	−	−	+[c]
Vasa vasorum	+	−	−
Pituitary			
Anterior	+/−	+	−
Intermediate	−	++	−
Posterior	−	+	−
Cerebral cortex	−	−	+
Cerebellum	+	+	+
Spleen	−	−	−
Thymus	−	−	−
Pancreas	−	−	−
Liver	−	−	−
Jejunum	−	−	−
Testis	−	−	−
Lung	−	−	−
Skin	−	+/−	−
Skeletal muscle	−	−	−
Thoracic aorta	−	−	−
Abdominal aorta	−	−	−
Aortic arch	−	−	−
Saphenous vein	−	−	−
Renal artery	−	−	−

[a] ++, highest-intensity hybridization; +, positive hybridization; +/−, positive signal in one experiment not reproduced; −, no hybridiztion signal.
[b] Discrete clusters of cells.
[c] Endocardial endothelial cell localization.
[d] Both myocardium and endocardial endothelial cell localization.

oles. However, for most of these structures, no specific hybridization was observed.

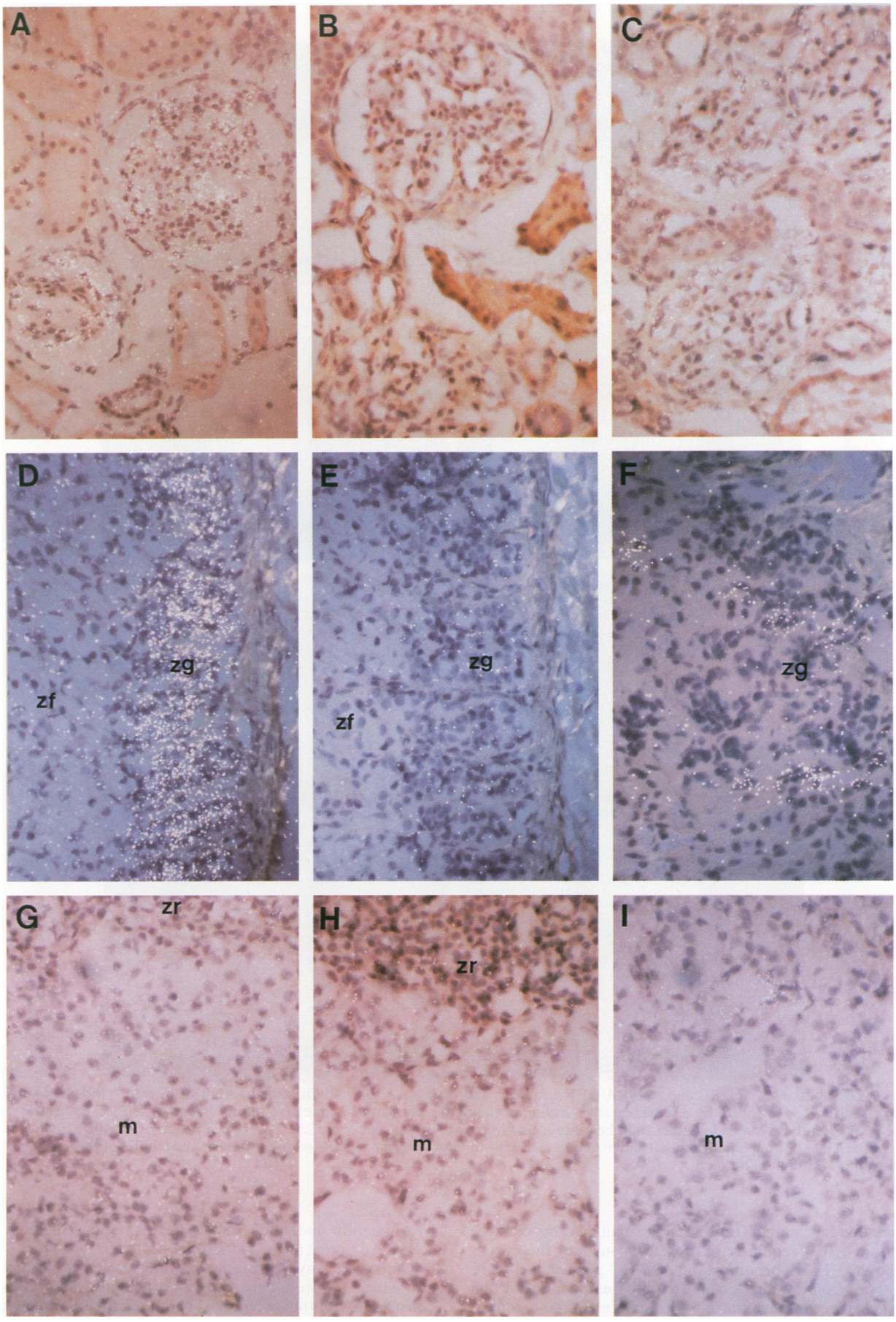
The kidney is a target organ for ANP action in every animal model studied, including rhesus monkeys (17). Experiments in rats have demonstrated that the second messenger cGMP is produced directly by glomerular cells concomitant with an increase in glomerular filtration rate in response to ANP, with no cGMP detectable in efferent arteriole blood (12). These data demonstrate the presence of functional natriuretic peptide receptor/guanylyl cyclase in either the mesangial or epithelial cells of the glomerulus. Northern analysis of mRNAs from human (20) and rat (32) demonstrates the presence of ANPRA mRNA in kidney. These data are consistent with the observation of intense ANPRA cRNA hybridization in the rhesus glomerulus and suggest that this receptor is the mediator of ANP responses in the kidney, at least in part through the production of cGMP as a second messenger. In addition to increasing glomerular filtration rate, ANP has actions distal to the glomerulus in regulating tubular (33) and collecting-duct (18) sodium resorption. Both ANP and cGMP inhibit a collecting-duct cation channel in electrophysiological cell-attached patch studies (18), indicating that ANPRA may be the transducer of this effect as well. Although scattered hybridization was observed outside of the glomerulus, we suggest that the majority of ANPRA mRNA is in fact present at too low a concentration to be detected by in situ hybridization in the kidney collecting ducts and tubles. Using in vitro autoradiographic receptor binding with ^{125}I-ANP on human, rat, and guinea pig kidney tissue sections, Mantyh et al. (22) observed intense labeling of the glomerular apparatus, in accordance with our data on ANPRA mRNA localization. They also observed labeling of renal arteries and the outer medulla, possibly corresponding to the collecting tubules, which would indicate the presence of ANPRA and/or ANPRC in this part of the kidney.

ANPRB cRNA did not show positive hybridization in the kidney cortex (Fig. 1B), indicating that this mRNA is below the level of detection by in situ hybridization or suggesting that CNP is not a physiological regulator of kidney function in the rhesus monkey. This latter possibility is supported by the observation that a human kidney cDNA library did not contain ANPRB cDNA clones (3). However, in the rat, ANPRB mRNA is detectable in the kidney by Northern blotting (32), and CNP has some renal effects in this species (38).

The ANPRC cRNA probe gave a less-intense positive hybridization signal throughout the kidney cortex than the ANPRA probe, with notable clustering of silver grains over cells in the glomerulus (Fig. 1C). We believe this to be a true positive hybridization signal from the behavior of this probe in the other tissue sections in the same experiment. This interpretation is further supported by the finding of ANPRC mRNA in human (19) and bovine (10) kidneys. The functional significance of ANPRC mRNA expression in the glomerulus and postglomerular structures is currently not known. Whereas an ANPRC-specific ANP analog had no effect on the function of in vitro-perfused rat kidneys (21), the same ANP analog has been reported to attenuate cAMP levels in a variety of rat tissues (1).

Adrenal cortex. Cryosections through the cortex of the Rhesus adrenal gland show the capsular fibroelastic connective tissue covering the secretory epithelial cells of the cortex (Fig. 1D and E). The thin zona glomerulosa layer can be seen immediately to the left of the capsule, with densely packed cells clustered in groups, followed by the more extensive zona fasciculata, with less tightly packed cells. Hybridization of the ANPRA cRNA was intense in the zona glomerulosa but scored negative in the zona fasciculata and zona reticularis of the cortex (Fig. 1D and G) in comparison with sense controls. In contrast, ANPRB cRNA did not hybridize to cells of the adrenal cortex (Fig. 1E and H), and ANPRC cRNA hybridization was localized to discrete and infrequent groupings penetrating from the zona glomerulosa

FIG. 1. Expression pattern of natriuretic peptide receptor mRNAs in rhesus monkey kidney and adrenal shown by in situ hybridization. Combination bright-field and polarized-light epiluminescence photomicrographs of representative sections were taken, showing the silver grains as white spots. In situ hybridization probes are (A, D, G) ANPRA, (B, E, H) ANPRB, and (C, F, I) ANPRC. (A, B, and C) Kidney cortex. (D, E, and F), Adrenal cortex, showing the zona glomerulose (zg) and zona fasciculata (zf). (G, H, and I) Adrenal medulla (m), with part of the cortical zone reticularis (zr) shown in panels G and H. Magnification, ×310 (all panels).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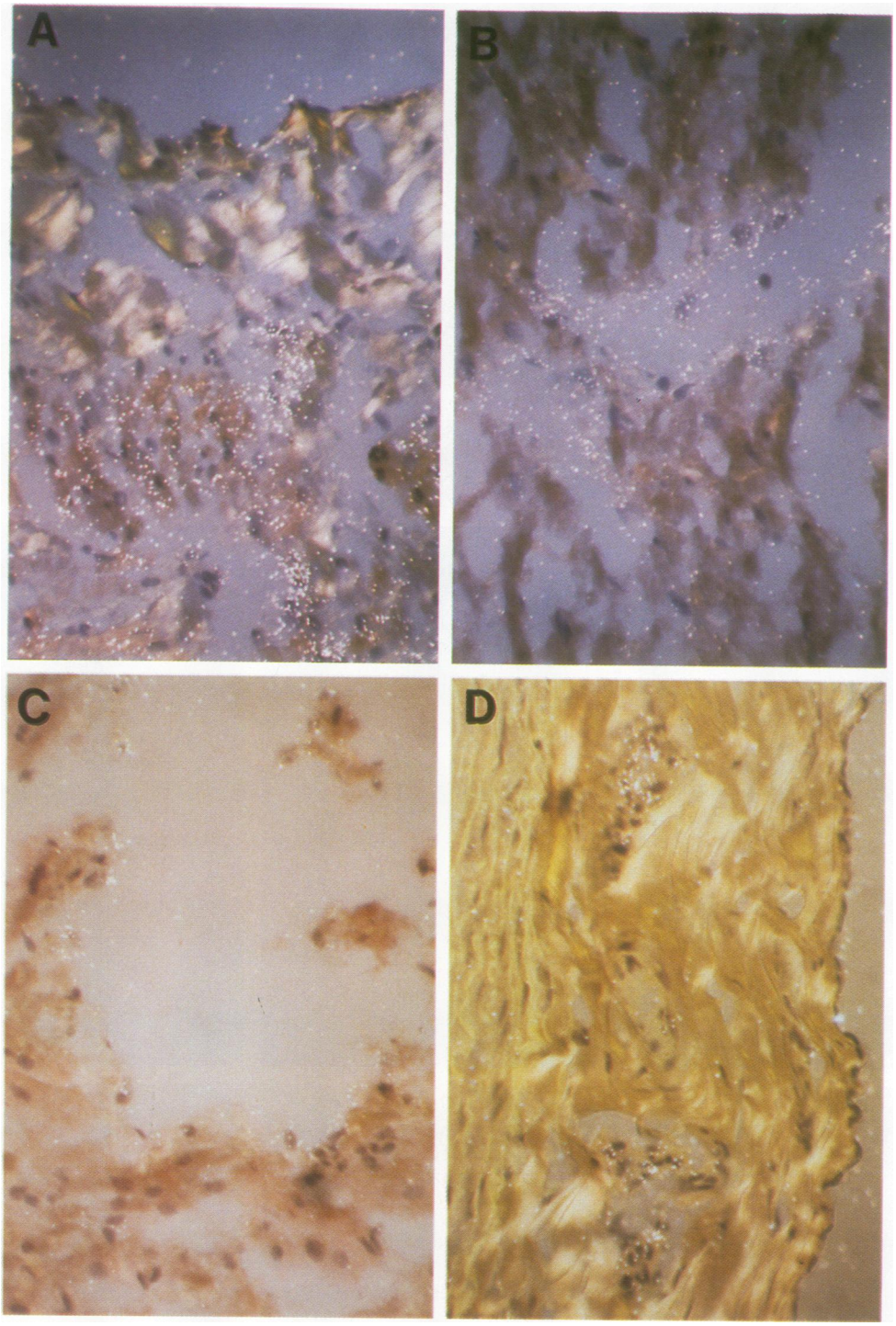

FIG. 2. Distribution of ANPRC and ANPRA mRNA in heart and vessels. In situ hybridization of ANPRC cRNA to the myocardial cells of the right atrium (A) is shown by a representative section including the pericardial connective tissue. Positive ANPRC hybridization was also seen consistently to the endocardial endothelial cells and is shown for the right ventricle (B). Hybridization of ANPRA is shown in the endothelial cell layer of the left atrium (C) and in endothelial cells of vasa vasorum vessels within the elastic adventitia of the aortic arch (D). Magnification, ×310 (all panels).

into the zona fasciculata (Fig. 1F) and small discrete clusters of positive cells irregularly scattered throughout the cortex (not shown). These groupings of ANPRC-positive cells are suggestive of endothelial cells of capillaries penetrating the adrenal cortex, not necessarily secretory cells. The extent of hybridization indicates that relatively less ANPRC than ANPRA is present in the adrenal cortex.

The specific hybridization of ANPRA cRNA to the mineralocorticoid-producing cells of the zona glomerulosa is consistent with this receptor's mediating the inhibitory effect

of ANP on aldosterone production by the zona glomerulosa (8, 16). Northern blot analysis has shown ANPRA mRNA to be as abundant in the human adrenal as in the kidney (20). In vitro autoradiography of ^{125}I-ANP binding to adrenal tissue sections from a variety of species, including cow, rat, and rhesus monkey, identified specific binding to the zona glomerulosa (23). Pharmacological studies in the rat have also implicated what we would now call ANPRA as the mediator of the ANP effect on aldosterone production (4, 31). Although we cannot rule out low levels of ANPRC or ANPRB mRNA in the zona glomerulosa, both ANP/receptor cross-linking and ANP-affinity purification of natriuretic peptide receptors from bovine adrenal zona glomerulosa revealed only a receptor guanylyl cyclase that most likely corresponds to ANPRA (24). The in situ hybridization data, together with these other observations, strongly suggest that ANPRA is the predominant natriuretic peptide receptor in the zona glomerulosa and mediates the inhibitory effect of ANP on aldosterone production.

Adrenal medulla. The neuroectodermal secretory cells of the adrenal medulla showed diffuse positive hybridization, with discrete and consistent clustering of silver grains over cells, for both the ANPRA and ANPRB cRNA probes (Fig. 1G and H). Silver grain density was higher in the medulla than in the zona reticularis for both of these probes, and comparison with sense control results (not shown) supports our interpretation. ANPRC cRNA hybridization in the medulla (Fig. 1I) was in a pattern similar to that seen in the adrenal cortex. No physiological role has been ascribed to the natriuretic peptides in the regulation of medullary chromaffin cell catecholamine production. Maurer and Reubi (23) observed specific binding of ^{125}I-ANP only to the adrenal medulla of guinea pigs by in vitro autoradiography, and not to the adrenal medulla of the rhesus monkey, mouse, hamster, or cow. ^{125}I-ANP would not bind to the ANPRB (15a) under the conditions of binding used by Mauer and Reubi (23), but our detection of ANPRA mRNA suggests that ^{125}I-ANP binding sites may be present. This discrepancy remains to be resolved. We note that Shionoiri et al. (35) observed the inhibition by ANP of catecholamine secretion from a human adrenomedullary pheochromocytoma; however, ANPRC was the predominant receptor in this tumor. This observation suggests that if ANP receptors are in fact present on the adrenal medullary chromaffin cells, then there could be negative regulation of catecholamine production by ANP and BNP. Such a possibility could also hold for the CNP/ANPRB ligand-receptor system.

Heart. Marked differential expression of the natriuretic peptide receptors was observed in cardiac tissues. In the heart, ANPRA cRNA hybridization was observed in endocardial endothelial cells lining the left atrium (Fig. 2C), right atrium, and right ventricle and was absent in the left ventricle (Table 1). Very dramatic hybridization of ANPRC cRNA was observed throughout the myocardium (Fig. 2A) and endocardial endothelial cells of the right atrium (Table 1). The right atrial section in Fig. 2A shows the thick layer of protective pericardial connective tissue above the positively hybridizing cells of the myocardium. Many distinct cells are positive for ANPRC mRNA; however, there were clearly cells that were not positive, suggesting specificity of these results. The expression of ANPRC mRNA throughout the myocardium of this tissue is interesting in light of the right atrium's being the major source of circulating ANP (reviewed in reference 13). The high level of ANPRC mRNA throughout the myocardium suggests that secreted ANP will immediately encounter this receptor before entering the circulation. ANPRC may then function to reduce the potential magnitude of fluctuations in circulating ANP, in accordance with the hypothesized hormonal buffering role for this receptor (21). Another possibility is functional feedback regulation on ANP secretion by ANPRC intracellular signalling (1, 11). At a difference with expression in the myocardial tissue of the right atrium, ANPRC mRNA is only found in the endocardial endothelial cells of the right ventricle (Fig. 2B), left ventricle, and left atrium (Table 1).

ANPRB cRNA hybridization was absent in the heart, thoracic aorta, abdominal aorta, aortic arch, vasa vasorum, saphenous vein, and renal artery (Table 1). Regional differences in ANPRA mRNA expression in vessels are illustrated in Fig. 2D; intense labeling of vasa vasorum endothelial cells by ANPRA was observed for this small vessel within the aortic adventitia, while the endothelial cells of the aortic arch were negative (not shown) (Table 1). ANPRC cRNA hybridization was negative in the peripheral vessels examined (Table 1).

ANP has potent cardiovascular effects, with cGMP playing a direct role as a second messenger in vasodilation (reviewed in reference 27). In dogs, rats and *Macaca fasicularis* monkeys, ANP reduced cardiac output and mean arterial pressure (34), with analogous observations also having been made in humans (6). In *Macaca* monkeys, isolated vessels display significant regional heterogeneity in response to relaxations by ANP, whereas sodium nitroprusside, an activator of soluble guanylyl cyclase, was equipotent in veins and arteries (14). The action of ANP as a vasodilator is likely transduced via cGMP production by ANPRA, with differences in the level of expression of ANPRA in blood vessels (Table 1) probably accounting for regional heterogeneity in ANP vasorelaxation. In the heart, the action of ANP in reducing cardiac output could be mediated via ANPRA or ANPRC signal transduction. Indeed, Anand-Srivastava et al. (2) have proposed that reduced cardiac output is mediated via antagonism of cAMP levels in Purkinje cells of the conduction system, possibly through ANPRC (1).

Pituitary. Sections through the rhesus pituitary show the darkly straining secretory epithelial cells of the anterior lobe (Fig. 3A). Cells of the intermediate lobe are more densely packed then those of the posterior lobe (Fig. 3B). Hybridization of the long ANPRB cRNA was observed throughout the pituitary (see Materials and Methods), with the most intense signal in the intermediate pituitary (Fig. 3A and B). Sense probe hybridizations performed concurrently showed a low background (not shown), reinforcing our interpretation of the positive signal. The hybridization observed with the short ANPRB probe (see Materials and Methods) was less intense than shown in Fig. 3A and B and was done without a matched sense control (not shown). We also observed a high abundance of ANPRB cDNA clones in a human pituitary cDNA library (3). Hybridization of ANPRA to anterior pituitary sections was positive in an initial experiment with a short probe but was not reproduced in a subsequent experiment with the longer sense and antisense probes in sections covering both lobes of the pituitary. These results with ANPRB and ANPRA mRNA hybridization suggest that slight uncontrolled differences in hybridization conditions could influence the limit of detection by this technique.

Data from a variety of sources indicate that ANPRA is present in the pituitary. ANPRA cDNA clones have been isolated from a human pituitary cDNA library (2a). In rats, ANP regulates the secretory activity of anterior pituitary corticotrophs in antagonism to arginine vasopressin and

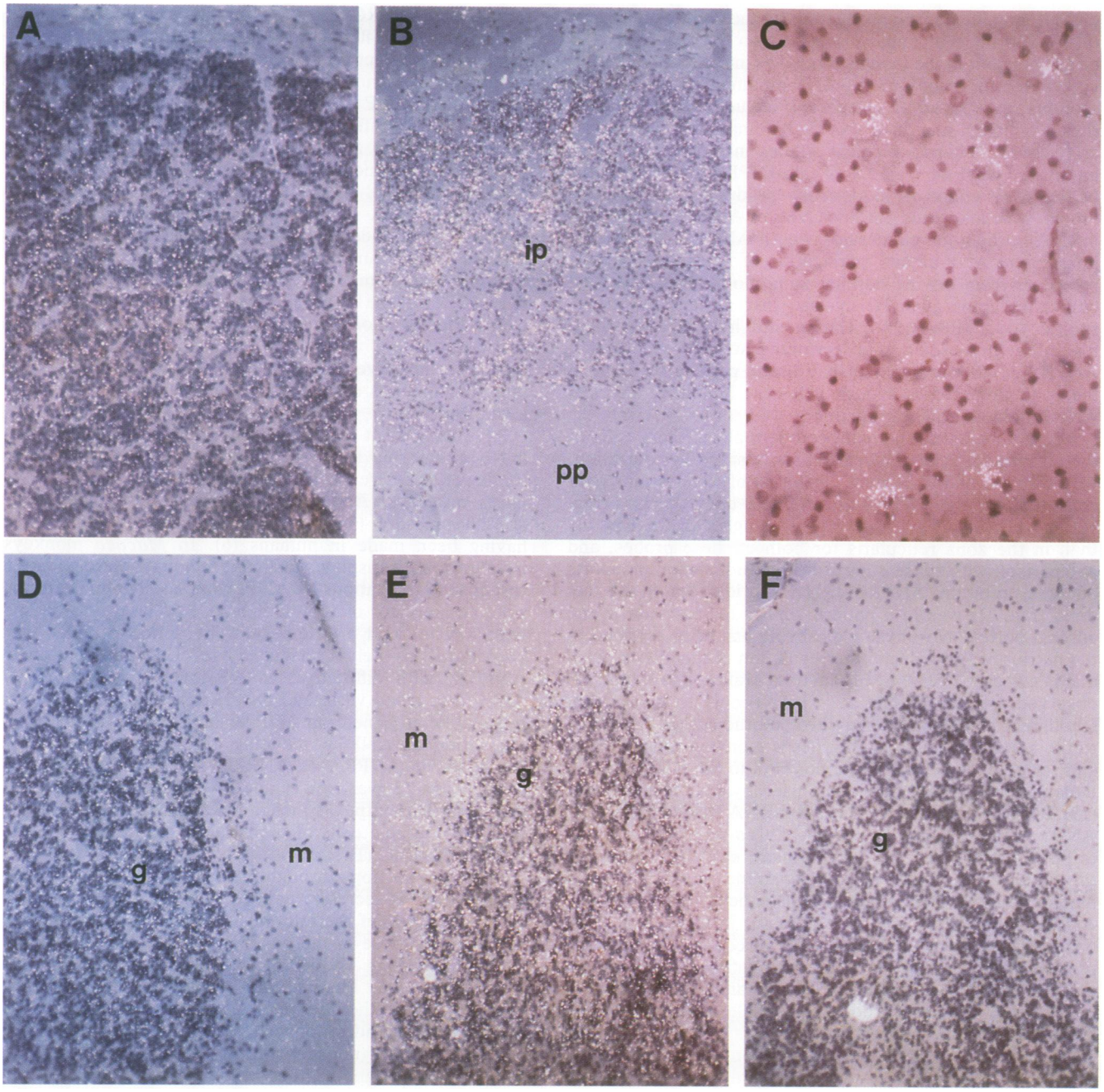

FIG. 3. Natriuretic peptide receptor mRNA distribution in rhesus monkey pituitary and brain by in situ hybridization. ANPRB hybridization is shown in the anterior pituitary (A) as well as the intermediate (ip) and posterior (pp) lobes of the pituitary (B). ANPRC hybridization in the cerebral cortex is shown in C. ANPRA (D) and ANPRB (E) mRNAs were found in the granular (g) and molecular (m) layers of the cerebellum. Negative hybridization in the cerebellum for ANPRC is shown in F. Magnification, ×125 (A, B, D, E, F), ×310 (C).

corticotrophin releasing factor (7). The inhibition of adrenocorticotrophin hormone secretion is also mimicked by cGMP analogs, further supporting a role for ANPRA in pituitary signal transduction. In humans, the infusion of ANP had no effect on circulating levels of growth hormone, lutinizing hormone, follicle-stimulating hormone, or thyroid-stimulating hormone but significantly suppressed prolactin-releasing hormone levels (28). ANP has also been identified immunologically in the human anterior pituitary (9). The abundance of ANPRB mRNA throughout the pituitary also suggests a role for CNP in regulating pituitary hormone secretion. In the anterior pituitary, a variety of trophic hormones are secreted, including adrenocorticotrophin hormone, a stimulator of aldosterone production. In the intermediate pituitary, β-endorphin and melanocyte-stimulating hormone are secreted, and in the posterior pituitary, the vasoactive hormones oxytocin and arginine vasopressin are secreted.

Brain. ANPRC cRNA hybridization was observed in the second and third layers of the cerebral cortex (Fig. 3C) in the absence of ANPRA or ANPRB hybridization (Table 1). This

signal was very intense and highly localized over scattered single-cell bodies. This hybridization pattern is unlike the ANPRC hybridization pattern in the adrenal cortex and medulla (Fig. 1F and I), which corresponds to clusters of cells. This pattern of ANPRC expression in the cortex was unexpected and may suggest a role for ANPRC in glial cell or neuronal regulation.

Positive hybridization for ANPRA and ANPRB was observed in the cerebellum (Fig. 3D and E), in both the dense granular layer and the more dispersed molecular layer. Hybridization was fairly evenly distributed throughout these two layers. In some sections, we observed ANPRC cRNA hybridizations to Bergman glial cells in the molecular layer immediately adjacent to the Purkinje cell layer, between the granular and molecular layers. These labeled cells were not always evident in photomicrographs, which otherwise appear negative (Fig. 3F).

The observation of ANPRA cRNA hybridization in the cerebellum is consistent with reports of intense ^{125}I-ANP binding in the cerebellum of vervet monkeys (29, 30). This binding distribution is different from that seen in other animals and highlights species differences in the brain distribution of these receptors. Our in situ hybridization data indicate that the ANP binding sites detected by Quirion et al. (29, 30) correspond to ANPRA, with coincident expression of ANPRB in the same region. The significance of natriuretic peptide receptor expression in the region of the brain involved in balance or motor control is not known.

Summary. Our observations on the localization of abundant ANPRA mRNA expression in a variety of hormonal target tissues provide a firm basis for the role of ANPRA in cellular regulation by ANP. The tissue distribution of ANPRA mRNA determined by in situ hybridization is in direct concordance with the observations on the peripheral and central control of body fluid homeostasis by ANP. Abundant sites of ANPRA mRNA expression correspond to major hormonal targets for ANP. In contrast, detectable levels of ANPRB mRNA expression are apparently limited to the pituitary, adrenal medullary neuroectoderm, and brain, suggesting a predominantly central role for this receptor in mediating the actions of CNP. We note that an ANPRB cDNA clone was isolated from a human ileum cDNA library (8), and we observed scattered positive epithelial cells in a rhesus monkey skin section in one experiment (Table 1), indicating that there are other sites of expression for this receptor outside of the nervous system.

Sites of abundant ANPRB mRNA expression detected by in situ hybridization may correspond to major hormonal target tissues for CNP. This inference is certainly true for ANP and the major sites of ANPRA expression detected by in situ hybridization in the kidney and adrenal cortex, for example. Our observations then define an apparent division between the abundant central expression of ANPRB in cells derived principally from the neural crest and the central and peripheral expression of ANPRA. In fact, this difference is apparently also reflected in the expression of the cognate hormones for ANPRB and ANPRA, CNP and ANP, respectively (15). There may be several reasons for this difference in the abundant expression of these two structurally related hormone/receptor guanylyl cyclase systems. Differential regulation of the expression of CNP versus ANP, in addition to the ligand specificity of the receptors, could provide distinct regulation of a subset of central functions also controlled by ANP. In addition to their shared property of catalytic synthesis of cGMP as a diffusible second messenger, there is also the possibility of divergent second-messenger systems originating from ANPRA and ANPRB, as reflected in the differences in their kinase homology domains (8, 32). Alternative receptor-sorting pathways may also exist as a means of differentially regulating responses to acute or chronic changes in hormone steady-state levels.

Abundant sites of ANPRC expression, although well defined by these studies, still present somewhat of an enigma, with no direct correlation between receptor mRNA expression and physiological actions of the natriuretic peptides. The most prominent site of ANPRC expression was found in the myocardium of the right atrium, a provocative finding given the production of ANP by this tissue. The functional significance of this high ANPRC level is not clear. However, the work of Anand-Srivastava et al. (1, 2), reporting on the antagonism by ANP of cAMP levels in various tissues, including the impulse-conducting system of the heart, together with the suggestion by Hirata et al. (11) that signal transduction by ANPRC regulates phosphatidylinositol polyphosphate metabolism, indicates that functional responses to altered ANP levels may be occurring through ANPRC. Experiments on the expression of cloned human ANPRC (19) have so far failed to reconstitute a signal transduction response (5a), suggesting that there may be a distinct cell type specificity for the apparent signal transduction mechanism.

Our observations on the localization of the mRNAs encoding the three natriuretic peptide receptors provide evidence for the physiological relevance of these molecules. Hybridization in tissues such as the adrenal medulla, myocardium, cerebral cortex, and cerebellum provides a basis for studying further roles of the natriuretic peptides in peripheral and central hormonal regulation of body fluid homeostasis.

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