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The Expression and Secretion of Atrial Natriuretic Factor and Brain Natriuretic Peptide by Rat Proximal Tubular Cells

Sanjay Mistry, Benedict Lussert,* Keith Stewart,* Gabrielle M. Hawksworth,† Alan Struthers‡ and James S. McLay*§*

Departments of *Medicine and Therapeutics and †Biomedical Sciences, Aberdeen University Medical School, Polwarth Building, Foresterhill, Aberdeen; and ‡Department of Clinical Pharmacology, Ninewells Teaching Hospital and Medical School, Dundee, U.K.

ABSTRACT. We examined the expression of both the natriuretic peptides and natriuretic peptide receptors (NPR) in primary cultures of rat proximal tubular (RPT) cells using Northern blot assay for peptides and receptors and radioimmunoassay and immunohistochemical analysis for atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), and C-type natriuretic peptide. Freshly isolated cells expressed mRNA coding for ANF, BNP, and the NPR-C. The presence of ANF and BNP in freshly isolated cells was confirmed by immunocytochemical staining. As cells approached confluence, there was a marked increase in mRNA expression for ANF and BNP. Immunocytochemical analysis and radioimmunoassay confirmed that both these peptides were co-localised in RPT cells and present in the cell supernatant. These changes in peptide expression were associated with a concurrent decrease in the expression of the NPR-C and the appearance of the NPR-A and -B. These results confirm that freshly isolated RPT cells possess the components of an autocrine natriuretic peptide system and that growth in primary culture is associated with changes in both peptide system and that growth in primary culture is associated with changes in both peptide and receptor subtype expression, raising the possibility that the endogenous production of ANF and BNP may be involved in the control of control cell growth. BIOCHEM PHARMACOL **59**;7:783–790, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. rat proximal tubule; atrial natriuretic factor; brain natriuretic peptide; cell growth; natriuretic peptide receptor

The natriuretic peptides (ANF, BNP, CNP)‖ and their binding sites (NPR-A, NPR-B, NPR-C), which have been localised to cardiovascular, renal, and brain tissue [1–3], are believed to be involved in the control of blood pressure homeostasis, cell growth and, possibly, immune response [4–6]. The physiological actions of the natriuretic peptides are mediated primarily by the guanyl cyclase-linked NPR-A and -B, although we and others have previously reported that the apparently 'quiescent' NPR-C may also play an important role in the modulation of cell growth and, possibly, immune function [7–9]. Previous studies have confirmed that the NPRs are present in the kidney and that their natural ligands ANF, BNP, and CNP may be synthesised within the kidney. ANF is reported to have actions in the late distal nephron, glomerulus, medulla, and the proximal tubule [10, 11]. Immunocytochemical studies have demonstrated immunostaining for ANF and BNP in segments of the human distal tubule and renal artery [12]; however, the evidence for natriuretic peptide distribution in the proximal tubule has remained controversial [13, 12].

It has been suggested that a possible autoregulatory role may exist for endogenously released natriuretic peptides and NPR development [14]. These findings suggested that locally secreted natriuretic peptides could act as a trigger for the observed changes in receptor expression during culture. To confirm a potential autocrine regulatory role for endogenously released natriuretic peptides in the process of NPR subtype development, we investigated natriuretic peptide and NPR expression in freshly isolated and primary cultures of RPT cells using Northern, radioimmuno-, and immunocytochemical assays at varying times in culture.

MATERIALS AND METHODS

RPT Cell Isolation

RPT cells were isolated from kidneys obtained from male Sprague–Dawley rats (200 g), as previously described [15]. Cortex was removed and minced in modified Hanks' buffer

§ Corresponding author: Dr. James S. McLay, Department of Medicine and Therapeutics, Aberdeen University Medical School, Polwarth Building, Foresterhill, Aberdeen, AB25 22D, U.K. Tel. +44 1224 681818 Ext 53015; FAX +44 1224 699884; E-mail: j.mclay@abdn.ac.uk

‖ *Abbreviations:* ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IgG, immunoglobulin G; NPR, natriuretic peptide receptor; RPT, rat proximal tubule; RT-PCR, reverse transcriptase–polymerase chain reaction; and SSC, standard saline citrate.

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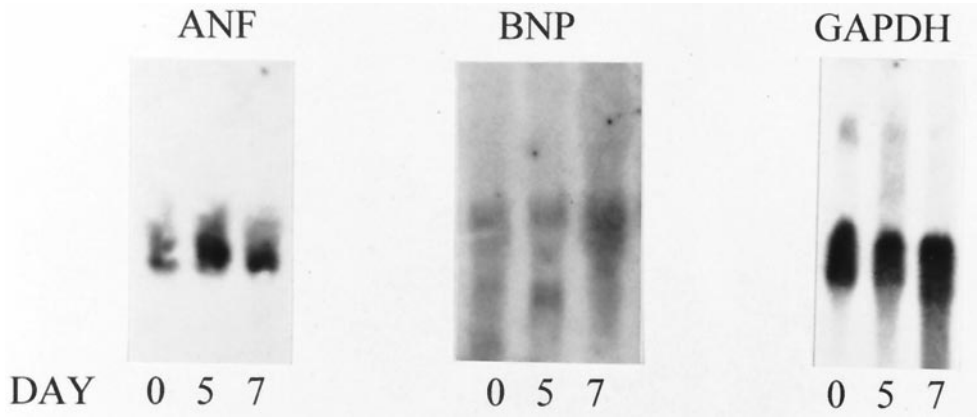

FIG. 1. ANF and BNP transcript expression in freshly isolated and primary cultures of RPT cells. Total RNA was extracted from freshly isolated (day 0) and cultured (days 5 and 7) cells after plating. GAPDH RNA was used to assess the RNA load of the lanes. Product sizes: ANF (1.35 kb), BNP (2.37 kb), and GAPDH (2.37 kb).

(containing EDTA) to remove excess blood. Subsequently, the minced cortical fragments were digested at 37° for 20 min in DMEM/Ham's F12 (GIBCO) containing 0.02% collagenase A (Boehringer Mannheim Biochemicals). Following digestion, the cell suspension was filtered through a 75-μM mesh (Lokertex) and the suspension washed and pelleted in DMEM/Ham's F12. The resultant pellet was resuspended in DMEM/Ham's F12 Percoll buffer solution and centrifuged at 20,000 *g* for 30 min at 4°. Following Percoll density centrifugation, three distinct bands were obtained at approximate densities of 1.04, 1.06, and 1.065 g/mL. The proximal tubular fraction (1.06 g/mL) was carefully removed and resuspended in DMEM/Ham's F12. Following isolation, the viability and total number of cells per unit volume, including individual cells making up tubular fragments, were then calculated [16]. The cells were then seeded at a density of 5×10^6 viable cells per 80-cm^2 flask (Costar) and incubated at 37° in an atmosphere of 95% air and 5% CO_2 in serum-replete medium (DMEM/Ham's F12 containing 10% fetal bovine serum.

Immunocytochemical Detection of ANF and BNP

KIDNEY SECTIONS. Kidneys from male Sprague–Dawley rats (200 g) were removed and dissected. Blocks of tissue were rapidly frozen in isopentane (Sigma) pre-cooled in liquid nitrogen, and tissue sections (5 μM) were cut on a cryostat (Leitz) and mounted onto glass slides. Slides for immunocytochemical analysis were air-dried (4 hr) and fixed in acetone for 10 min prior to storage.

CYTOSPINS. Freshly isolated RPT cells (1×10^5) were suspended in Tris-buffered saline (pH 7.6), pipetted into individual chambers of a Shandon Cytospin 2, and centrifuged at 100 *g* for 5 min. The resultant pellet of cells was air-dried (4 hr) and fixed in acetone for 10 min prior to storage.

CELL CULTURE USING LUX CHAMBERS. Cells were seeded (density 1×10^5 cells) in 8-well lux chambers (Sheldon) in 0.5 mL of DMEM/Ham's F12 containing 10% fetal bovine serum and cultured for up to seven days. Lux chambers were incubated at 37° in an atmosphere of 95% air and 5% CO_2. After primary culture, medium was removed and the lux chambers were fixed in acetone for 10 min prior to storage.

IMMUNOCYTOCHEMICAL ASSAY. Slides were brought to room temperature, marked using a Dako pen, and rinsed in Tris-buffered saline (TBS) (pH 7.4). Slides were immersed (10 min) in TBS containing 1% hydrogen peroxide to block endogenous peroxidase activity and then rinsed with TBS. Slides were incubated (20 min) with goat IgG (1:100

TABLE 1. The ratios of natriuretic peptide to GAPDH mRNA expression in freshly isolated (day 0) and primary cultures (days 5 and 7) of RPT cells

Day	ANF (dpm)	BNP (dpm)	GAPDH (dpm)
Freshly isolated (D0)	102,832 (0.19)	134,489 (0.24)	560,112
D5	151,544 (0.26)	215,310 (0.37)	578,809
D7	289,381 (0.53)	278,420 (0.42)	580,112

Values in parentheses are ratios to GAPDH. The results of 2 separate experiments are given.

TABLE 2. Immunoreactive ANF and BNP detected in cell supernatant from freshly isolated RPT cells (day 0) and during RPT cell primary culture (days 4, 5, and 7)

Time during culture	ANF production (pg/mg protein)	BNP production (pg/mg protein)	CNP production (pg/mg protein)
Freshly isolated (day 0)	1.16	9.28	1.02
Day 4	6.05	12.03	3.52
Day 5	43.26	12.96	4.68
Day 7	661.38	14.95	5.98

The actual results from two separate assays are shown.

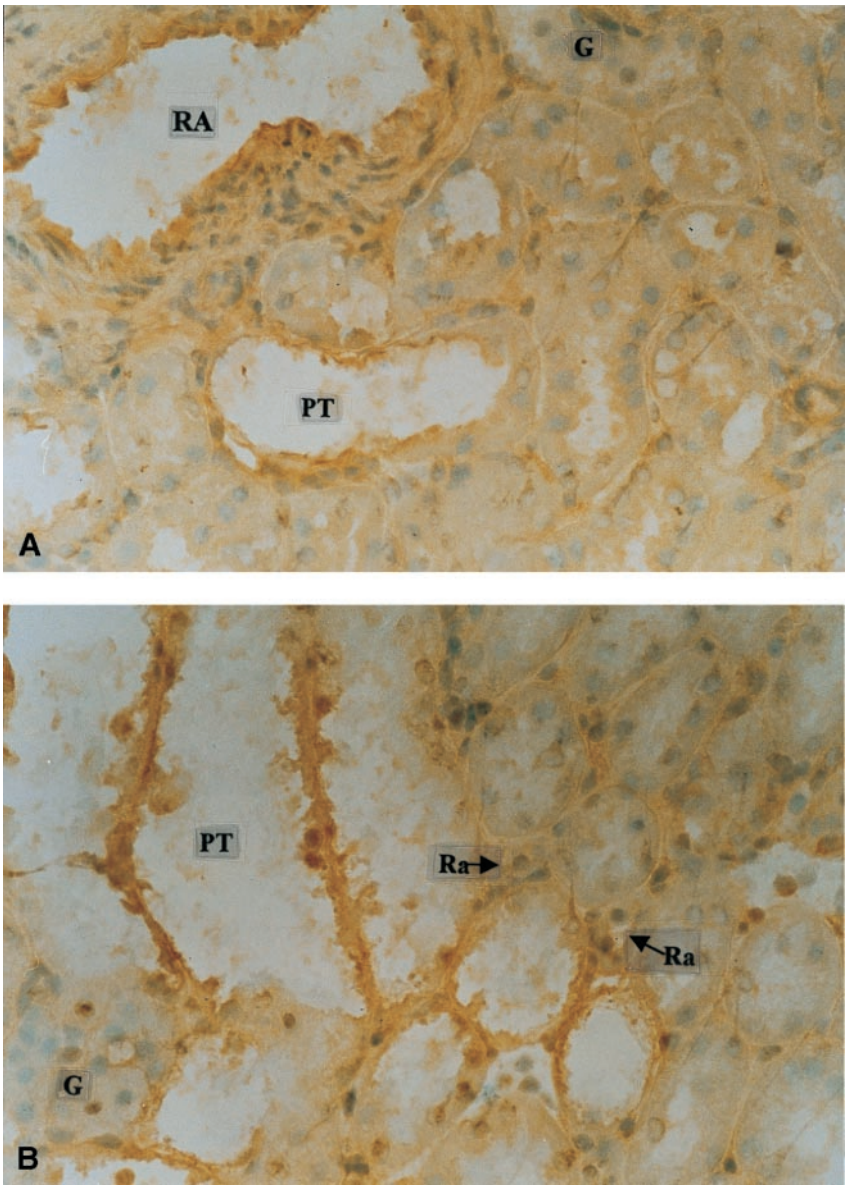

FIG. 2. Photomicrograph of ANF (A) and BNP (B) immunostaining in kidney sections: RA = renal artery: Ra = renal arteriole; g = glomerulus; and PT = proximal tubule. Nuclei counterstained with haematoxylin.

with TBS) and then with avidin (1:50 with TBS) (15 min). Slides were rinsed with TBS and incubated with biotin diluted 1:50 with TBS (15 min). Slides were rinsed in TBS and incubated overnight with either rabbit anti-alpha-ANF polyclonal antibody (1:1600 with TBS) (Chemicon) or rabbit anti-BNP polyclonal antibody (1:8000 with TBS) (Chemicon), after which they were rinsed with TBS. Goat anti-rabbit IgG biotinylated antibody with rat IgG was added to each slide and incubated for 1 hr. Slides were rinsed in TBS and incubated (30 min) with ABC–HRP (avidin–biotin complex–horseradish peroxidase, Dakopatts kit) and rinsed with TBS. Slides were incubated (20 min) with a solution containing 30% H_2O_2 and 3,3-diaminobenzidine tetrahydrochloride (Sigma) diluted in 0.05 M Tris–HCl buffer. Slides were rinsed in distilled water and the nuclei stained with haematoxylin solution, after which they were rinsed in Scots tap water. The slides were finally rinsed with distilled water and mounted with a coverslip.

RADIOIMMUNOASSAYS FOR ANF, BNP AND CNP. Atrial, brain, and C-type natriuretic peptide concentrations in cell supernatant were measured using standard radioimmunoassays (Peninsula Laboratories). Results are expressed as pg/mg protein.

ISOLATION OF TOTAL RNA FOR NORTHERN ANALYSIS. The cells were dissociated from the tissue culture flasks with 0.02% trypsin/EDTA (0.05 M), washed in cold PBS, and transferred to a microfuge tube. Total RNA was extracted using standard methods [17]. The cells were lysed with Nonidet P-40 (0.5% v/v) Tris-buffered saline (pH 8.6) in the presence of 20 mM ribonucleoside vanadyl complex

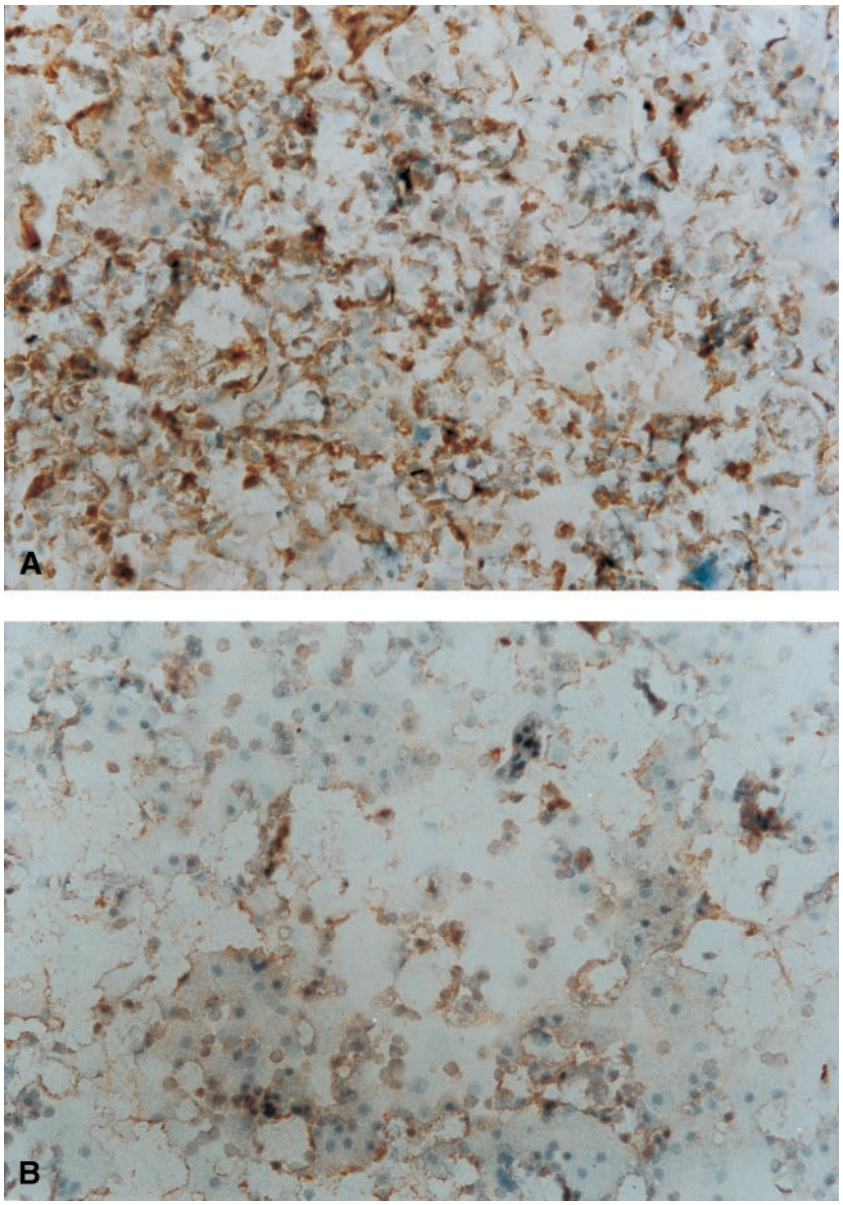

FIG. 3. Immunohistochemical localisation of ANF (A) and BNP (B) in freshly isolated RPT cell cytospins. Nuclei counterstained with haematoxylin. Staining for ANF and BNP was observed.

inhibitor (Sigma). Nuclei were removed by centrifugation (150 *g* at 4°) for 5 min and digested with proteinase K (50 μg/mL, Boehringer Mannheim) and 2% SDS Tris-buffered saline (pH 8.0). Protein was phenol:chloroform-extracted and RNA collected by precipitation of the aqueous phase in ice-cold isopropanol (Sigma). After centrifugation, the RNA pellet was washed three times in 70% ethanol, air-dried, and resuspended in water treated with diethylpyrocarbonate (Sigma). The RNA purity was measured by spectroscopy at 260 and 280 nm.

Northern Analysis

The pGEM-T Vector System (Promega) was used to clone sequenced PCR products for use in Northern analysis. Northern blot analyses of NPR-A, NPR-B, NPR-C, ANF, BNP, CNP, and GAPDH mRNA were performed using 20 μg total RNA. Probes for Northern analysis were designed from cloned and sequenced RT–PCR products of the natriuretic peptides and NPRs as previously described [18]. Total RNA was extracted and fractionated on a 1.2% agarose gel containing 0.7 M formaldehyde. The RNA was transferred to a nylon membrane (Amersham International plc) by capillary action and cross-linked using a spectrolinker. The blots were hybridised with enzyme-restricted DNA probes labelled with [α^{32}P] dCTP, using random priming (Amersham). Hybridisations were performed at 42° in the presence of 50% deionised formamide, 0.25 M NaHPO4, 0.25 M NaCl, 20% (w/v) dextran sulphate solution, and 100 μg salmon sperm DNA. Blots were washed with two washes of 2X SSC (5 min) at room temperature, two washes of 0.5X SSC/1% SDS (30 min) at

TABLE 3. Immunohistochemical staining for ANF and BNP in rat kidney sections, freshly isolated RPT cell cytospins, and primary cultures of RPT cells (day 7)

Antibody	Kidney section staining	Cell cytospin	PT cultures		
			D3	D5	D7
Anti-ANF	Renal artery, PT, and glomeruli	++	+	++	+++
Anti-BNP	Renal artery and PT	+	+	+	++

Degree of cellular staining: + = 11–50%, ++ = 51–90%, and +++ = 91–100%.

55° and two washes of 0.2X SSC (30 min) at room temperature. Blots were counted on a phosphoimager and exposed to medical x-ray film (Fuji) at −70° for 7–10 days.

RESULTS

Cell Characterisation

Throughout the duration of the experiments, RPT cells showed and maintained typical epithelial cobblestone morphology with no evidence of significant fibroblast overgrowth. Using the above methods, RPT cell preparations were previously characterised in our laboratory using phase-contrast and electron microscopy, enzyme histochemistry, and anionic and cationic transport mechanisms and found to be 96% pure, maintaining PT characteristics for at least 10 days following seeding [15]. Prior to use, freshly isolated cells in this series of experiments were characterised according to their response to parathyroid hormone, calcitonin, arginine vasopressin, and anionic and cationic trans-

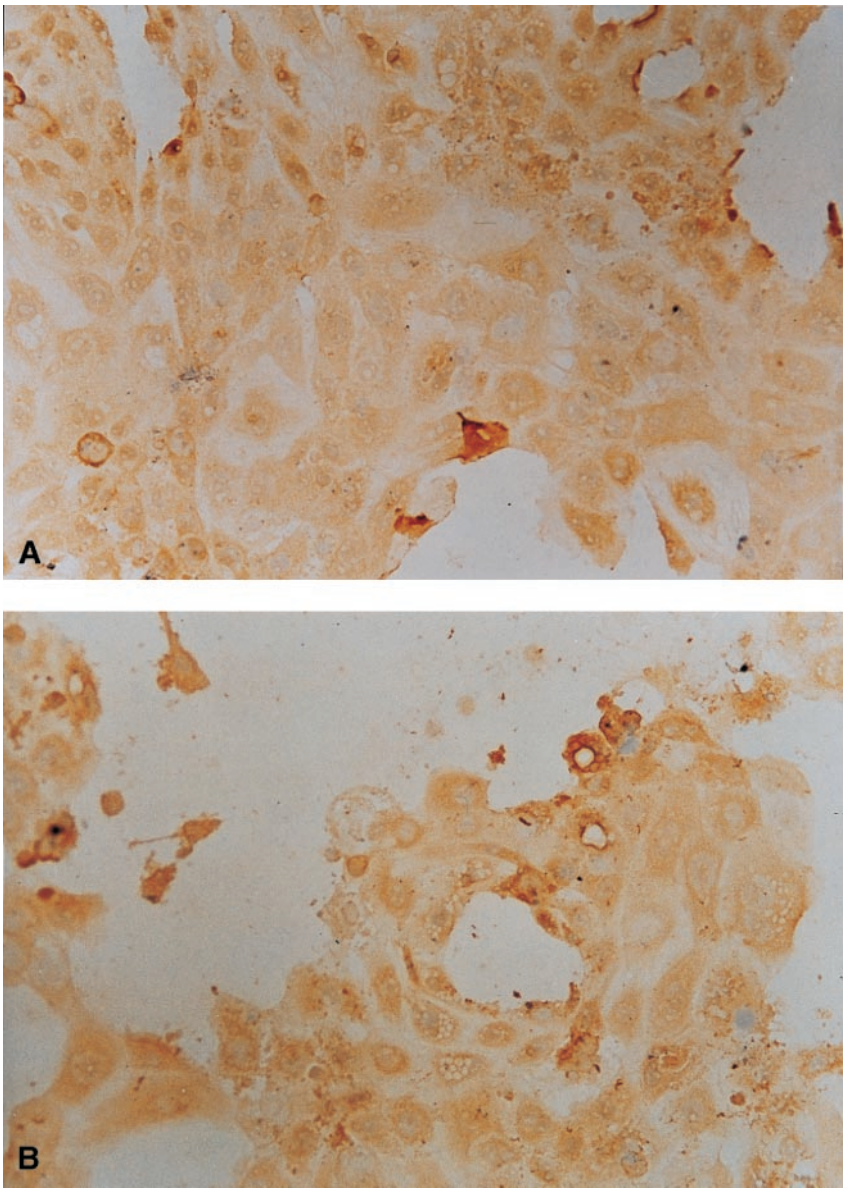

FIG. 4. Immunohistochemical localisation of ANF (A) and BNP (B) in RPT cell primary cultures (day 5) grown in lux chambers. Nuclei counterstained with haematoxylin. Intensified staining for ANF and BNP was observed at cell confluence.

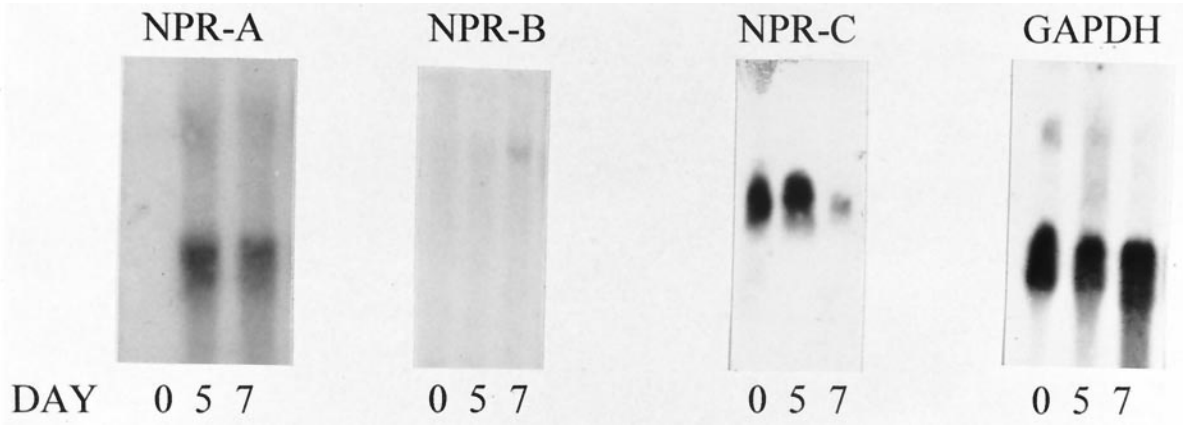

FIG. 5. NPR-A, B, and C transcript expression in freshly isolated and primary cultures of RPT cells. Total RNA was extracted from freshly isolated (day 0) and cultured (days 5 and 7) cells after plating. GAPDH RNA was used to assess the RNA load of the lanes. Product sizes: NPR-A (2.37 kb), NPR-B (7.46 kb), NPR-C (4.4 kb), and GAPDH (2.37 kb).

port, as previously described [19]. The cells retained functional proximal tubular characteristics throughout.

Natriuretic Peptide Gene Expression

ANF (1.35 kb) and BNP (2.37 kb) gene transcripts were detected in freshly isolated RPT cells by Northern analysis (Fig. 1). As cells grew to confluence, there was a marked increase in the levels of mRNA transcripts for ANF and BNP. No CNP transcript expression was detected by Northern analysis (Table 1).

ANF, BNP, and CNP Immunoreactivity in Cell Supernatant

During culture, immunoreactive (ir) ANF and BNP concentration increased in the supernatant in association with the observed increase in mRNA transcript levels (Table 2). Levels increased from 1.16 (day 0) to 661.38 pg/mg protein (day 7), 9.28 (day 0) to 14.95 pg/mg protein (day 7), and 1.02 (day 0) to 5.98 pg/mg protein (day 7) for ANF, BNP, and CNP, respectively. To avoid interassay variability, all samples were processed at the same time using the radioimmunoassay kit. The intra-assay variability for these assays in this laboratory were 11.8%, 9.9%, and 15% for ANF, BNP, and CNP, respectively.

Immunohistochemical Detection of ANF and BNP

In kidney sections, ANF and BNP immunostaining was present on the lining of the renal artery, glomerular cells, and in both the PT and distal tubules (Fig. 2, a and b). In freshly isolated RPT cell cytospins, immunostaining for ANF was denser than that for BNP (Fig. 3, a and b) (Table 3). During primary culture, immunostaining for ANF and BNP was observed in the PT cell cytoplasm throughout the culture period, with intensified staining in RPT cells at confluence (Fig. 4, a and b). These results suggested that both ANF and BNP are co-localised in RPT cells and that during cell culture, cellular natriuretic peptide production increases as RPT cells approach confluence.

Natriuretic Peptide Receptor Gene Expression

Only NPR-C (4.4 kb) expression was detected in freshly isolated RPT cells (Fig. 5, Table 4). During culture, increasing levels of gene transcripts for the NPR-A (2.37 kb) and -B (7.46 kb) (Fig. 5) were detected, while levels of NPR-C mRNA decreased. These results suggested that a shift in NPR expression from the NPR-C in freshly isolated cells to the NPR-A and -B occur as cells reach confluence at day 7.

DISCUSSION

The results of this study confirm that freshly isolated RPT cells express and secrete ANF and BNP. Furthermore, during primary culture there is an increase in the expression and secretion of both these peptides with the concurrent appearance of mRNA expression for the NPR-A and -B and decrease in expression for the NPR-C. The observed

TABLE 4. The ratios of natriuretic peptide receptor mRNA to GAPDH mRNA expression in freshly isolated (day 0) and primary cultures (days 5 and 7) of RPT cells

DAY	NPR-A (dpm)	NPR-B (dpm)	NPR-C (dpm)	GAPDH (dpm)
Freshly isolated (D0)	ND	ND	329,999 (0.59)	560,112
D5	253,500 (0.44)	137,635 (0.24)	230,964 (0.40)	578,809
D7	434,440 (0.79)	220,914 (0.39)	158,446 (0.29)	580,112

Values in brackets are ratios to GAPDH. ND not detected. The results of two separate experiments are shown.

increase in both the expression and levels of these natriuretic peptides at the same time as changes in receptor expression occur are in agreement with our previously published preliminary results based solely on RT–PCR assay [18] and suggest that these peptides may act in an autocrine manner to regulate receptor development.

In freshly isolated RPT cells, only mRNA transcripts for ANF, BNP, and the NPR-C were detected. During culture, mRNA transcript expression for both peptides increased as the cells approached and attained confluence, suggesting that the process of culture results in an increased expression of both peptides. The increase in mRNA expression for ANF and BNP occurred concurrently with an increase in the concentration of immunoreactive ANF and BNP in the cell supernatant, suggesting the endogenous release of both these peptides during primary culture. Other groups have previously reported that only ANF and CNP are expressed in the rat kidney under normal physiological conditions [20, 21]. Our observations that freshly isolated RPT cells also express and secrete BNP and that there is positive immunohistochemical staining for BNP would appear to be in contradiction. It has also been previously suggested that ANF is only synthesised in the distal convoluted tubule and that only CNP is synthesised in the proximal tubule [12, 22, 23]. However, both proANF 1–30 and proANF 31–67 have been localised in the sub-brush border of the proximal convoluted tubule (PCT) and proximal straight tubule (PST) in male Sprague–Dawley rats [13]. ANF has also been localised in the PCT and glomerular efferent and afferent arteries in the kidneys of rats with experimentally induced renal failure [24]. Our observations strengthen these previous reports and confirm that ANF is synthesised within the proximal tubule. In agreement with Terada *et al.*, who reported that mRNA for CNP could only be found in the rat glomeruli, VR bundle, and arcuate artery, a mRNA transcript for CNP was not detected in RPT cell cultures [25].

We also identified mRNA transcript for the NPR-C but not the NPR-A or -B in freshly isolated RPT cells, a finding in agreement with previous reports of NPR-C predominance *in vivo* and in freshly isolated proximal tubular cells [26]. During primary culture, the NPR profile changed as confluence was attained, with a decrease in mRNA transcript expression for the NPR-C and an increase in NPR-A and -B gene expression, in line with the concurrent increase in ANF expression and secretion. We have reported similar observations following short-term incubation of freshly isolated RPT cells with exogenous ANF [18], raising the possibility that endogenous ANF may act to down-regulate the NPR-C, while up-regulating the NPR-A and -B during RPT cell growth. Alternatively, the changes that occur during culture may reflect a change in phenotype resulting from removal of cells from a normal environment. Changes in NPR subtype expression during culture with a shift from predominantly NPR-A or -B to the NPR-C have been reported in a variety of tissues [27], but we observed a shift from the NPR-C to the NPR-A and -B as the cells approached confluence. An explanation for these differences may be the passage number or the age of the cell cultures used by other groups. Both cell passage and the age of the animal from which cells are obtained have been reported to influence changes in the observed NPR expression [28–30].

Why RPT cells should express and release ANF and BNP is unclear. It is possible that these observations reflect a loss of differentiated function in culture. However, this is unlikely, as we have previously reported similar changes in peptide and receptor expression following short-term incubation of freshly isolated RPT cells with ANF. A further explanation is that the natriuretic peptides are potent inhibitors of cellular mitogenesis and proliferation [14, 37–33], and endogenous ANF and BNP may regulate RPT cell growth acting via the NPR-C in the early stages of culture and via the NPR-A and -B as confluence is attained.

In summary, this study confirms that freshly isolated RPT cells are able to produce the components of an autocrine natriuretic peptide system, which may in turn regulate NPR expression and, subsequently, cell growth. Furthermore, it is clear that the timing of studies performed on cell cultures is crucial to any investigations on the natriuretic peptide or receptor systems.

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