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Interactions of ANP and ANG II in tubular nephron acidification

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Abstract

In order to examine the effects and the interaction of angiotensin II (ANG II, 1 pM) and atrial natriuretic peptide (ANP, 1 µM) on the kinetics of bicarbonate reabsorption in the rat middle proximal tubule, we performed *in vivo* experiments using a stopped-flow microperfusion technique with the determination of lumen pH by Sb microelectrodes. These studies confirmed that ANG II added to the luminal or peritubular capillary perfusion fluid stimulates proximal bicarbonate reabsorption and showed that ANP alone does not affect this process, but impairs the stimulation caused by ANG II. We also studied the effects and the interaction of these hormones in cortical distal nephron acidification. Bicarbonate reabsorption was evaluated by the acidification kinetic technique in early (ED) and late (LD) distal tubules in rats during *in vivo* stopped-flow microperfusion experiments. The intratubular pH was measured with a double-barreled microelectrode with H^+-sensitive resin. The results indicate that ANG II acted by stimulating Na^+/H^+ exchange in ED (81%) and LD (54%) segments via activation of AT_1 receptors, as well as vacuolar H^+-ATPase in LD segments (33%). ANP did not affect bicarbonate reabsorption in either segment and, as opposed to what was seen in the proximal tubule, did not impair the stimulation caused by ANG II. To investigate the mechanism of action of these hormones in more detail, we studied cell pH dependence on ANG II and ANP in MDCK cells using the fluorescent probe BCECF. We showed that the velocity of cell pH recovery was almost abolished in the absence of Na^+, indicating that it is dependent on Na^+/H^+ exchange. ANP (1 µM) alone had no effect on this recovery but reversed both the acceleration of H^+ extrusion at low ANG II levels (1 pM and 1 nM), and inhibition of H^+ extrusion at higher ANG II levels (100 nM). To obtain more information on the mechanism of interaction of these hormones, we also studied their effects on the regulation of intracellular free calcium concentration, $[Ca^{2+}]_i$, monitored with the fluorescent probe Fura-2 in MDCK cells in suspension. The data indicate that the addition of increasing concentrations of ANG II (1 pM to 1 µM) to the cell suspension led to a progressive increase in $[Ca^{2+}]_i$ to 2-3 times the basal level. In contrast, the addition of ANP (1 µM) to the cell suspension led to a very rapid 60% decrease in $[Ca^{2+}]_i$ and reduced the increase elicited by ANG II, thus modulating the effect of ANG II on $[Ca^{2+}]_i$. These results may indicate a role of $[Ca^{2+}]_i$ in the regulation of the H^+ extrusion process mediated by Na^+/H^+ exchange and stimulated/impaired by ANG II. The data are compatible with stimulation of Na^+/H^+ exchange by increases of $[Ca^{2+}]_i$ in the lower range, and inhibition at high $[Ca^{2+}]_i$ levels.

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The atrial natriuretic peptides (ANPs) are a family of peptides originating from mammalian heart atria which present strong natriuretic and diuretic properties. Several studies indicate that ANP acts directly on the kidney by enhancing the glomerular filtration rate (GFR) (1-3). However, it remains unclear whether ANP has a direct effect on the proximal tubule or whether the observed diuresis and natriuresis are the result of an increased fluid flow along this nephron segment caused by the increased GFR. Because several of the studies were performed *in vivo* and functional cholinergic receptors on the basolateral side of the proximal tubule can regulate bicarbonate reabsorption and fluid flux (4), systemic effects of ANP and the influence of the renal nerves may mask a direct action of ANP on the proximal nephron.

Angiotensin II (ANG II), an octapeptide naturally found in blood, is a potent regulator of acidification in the rat early proximal tubule (5). This hormone stimulates both apical Na^+/H^+ exchange and basolateral Na^+-HCO_3^- cotransport in perfused S1 segments of proximal tubules isolated from superficial nephrons of the rabbit kidney (6). Electrophysiological studies of ANG II regulation of Na^+-HCO_3^- cotransport in isolated perfused S2 segments of rabbit renal proximal tubules confirmed that picomolar concentrations of ANG II stimulate (7), while micromolar concentrations inhibit (8) basolateral Na^+-HCO_3^- cotransport.

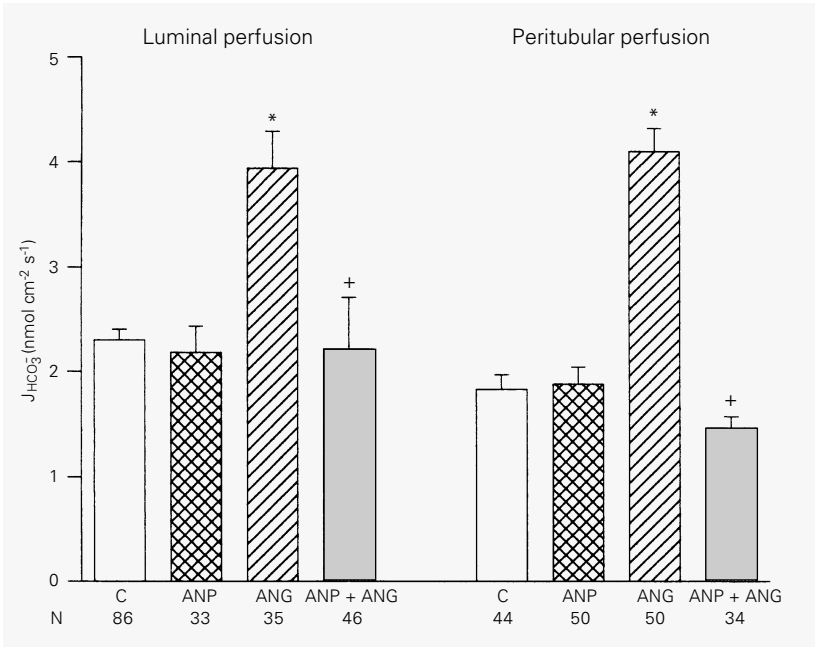

Figure 1 - Effect of atrial natriuretic peptide (ANP, 1 µM) and/or angiotensin II (ANG, 1 pM) on HCO_3^- reabsorption in proximal tubule. The experiments were performed in tubules undergoing luminal (left) or peritubular capillary (right) perfusions with the hormones. Data are reported as means ± SEM. N, Number of microperfusions; $J_{HCO_3^-}$, net HCO_3^- reabsorption. *P<0.05 compared to control (C); +P<0.05 compared to ANG II (Bonferroni test).

An interaction between ANP and ANG II has been observed in a variety of tissues. ANP inhibits the vasoconstrictor effect of ANG II on blood vessels *in vitro* (9), as well as the systemic pressor action of ANG II (10) and ANG II-stimulated aldosterone synthesis (11). Using the split-droplet technique, Harris et al. (12) found that ANP had an inhibitory effect on proximal fluid absorption stimulated by preliminary ANG II perfusion. Similar results were obtained by Garvin (13) in isolated perfused proximal straight tubules. However, the same effect was not observed by Liu and Cogan (14) during *in vivo* free-flow micropuncture experiments.

In order to examine the effects and the interaction of ANG II (1 pM) and ANP (1 µM) on the kinetics of bicarbonate reabsorption in the middle proximal tubule of rats, we performed *in vivo* experiments using a stopped-flow microperfusion technique, which is not affected by GFR (15). In this preparation, the systemic effects of both hormones were eliminated during luminal or peritubular capillary perfusion, as confirmed by the absence of changes in urine flow and Na^+ excretion under these conditions. Bicarbonate reabsorption was measured by stationary microperfusion and lumen pH was determined with Sb microelectrodes. Net bicarbonate reabsorption ($J_{HCO_3^-}$) was obtained by the following relation:

$$J_{HCO_3^-} = k([HCO_3^-]_i - [HCO_3^-]_s)\ r/2$$

where k is the rate constant of the reduction

of luminal bicarbonate [k = ln2/(t/2)], t/2 is the half-time of luminal bicarbonate disappearance, r is the tubule radius, and $[HCO_3^-]_i$ and $[HCO_3^-]_s$ are the concentrations of the injected HCO_3^- and HCO_3^- at the stationary level, respectively.

Figure 1 shows some of the results obtained in this study. It was noted that ANP perfused in the lumen did not change proximal bicarbonate reabsorption but inhibited the stimulation induced by luminal perfusion of ANG II: $J_{HCO_3^-}$ was 2.30 ± 0.10 nmol cm^{-2} s^{-1} (N = 86) with luminal control perfusion, 2.18 ± 0.25 nmol cm^{-2} s^{-1} (N = 33) during ANP luminal perfusion, increased to 3.94 ± 0.35 nmol cm^{-2} s^{-1} (N = 35) with ANG II luminal perfusion and decreased to 2.21 ± 0.15 nmol cm^{-2} s^{-1} (N = 46) during ANG II plus ANP luminal perfusion. The perfusion of ANP in the peritubular capillary also did not affect proximal bicarbonate reabsorption but caused an inhibition of the stimulatory effect of ANG II in peritubular capillary perfusion: $J_{HCO_3^-}$ was 1.83 ± 0.14 nmol cm^{-2} s^{-1} (N = 44) with peritubular capillary control perfusion, 1.88 ± 0.16 nmol cm^{-2} s^{-1} (N = 50) during ANP peritubular perfusion, increased to 4.10 ± 0.22 nmol cm^{-2} s^{-1} (N = 50) during capillary perfusion with ANG II and decreased to 1.56 ± 0.11 nmol cm^{-2} s^{-1} (N = 34) when ANG II plus ANP were added to the peritubular perfusion.

Therefore, these studies confirmed that ANG II stimulates proximal bicarbonate reabsorption and showed that ANP alone does not affect this process, but impairs the stimulation caused by ANG II.

We have recently studied the effects and interaction of these hormones also in cortical distal nephron acidification (16). Bicarbonate reabsorption was evaluated by the acidification kinetic technique in early (ED) and late (LD) distal tubules in rats during *in vivo* stopped-flow microperfusion experiments. The intratubular pH was measured with a double-barreled microelectrode with one barrel filled with H^+-sensitive resin and the other with 1 M KCl (17).

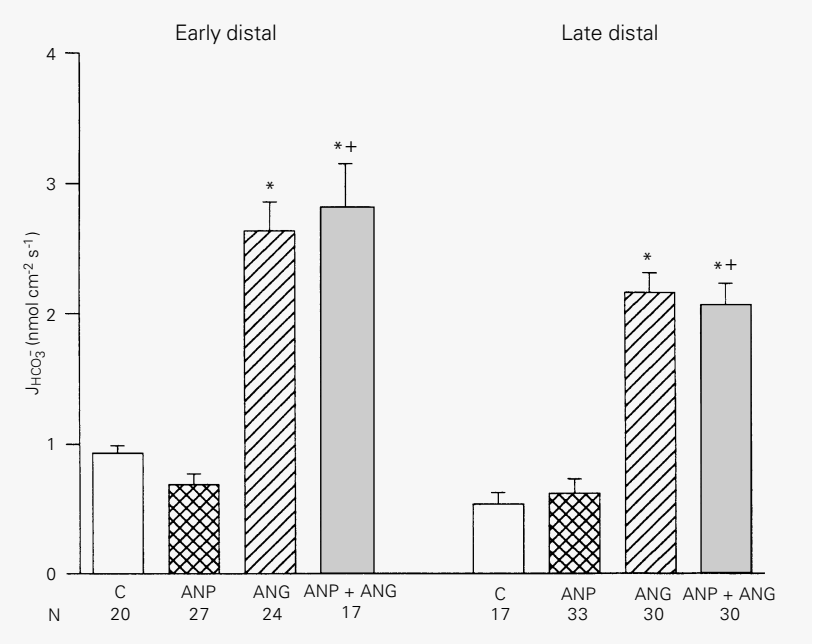

Figure 2 - Effect of atrial natriuretic peptide (ANP, 1 µM) and/or angiotensin II (ANG, 1 pM) on HCO_3^- reabsorption in early (left) and late (right) distal tubules. The experiments were performed in tubules undergoing luminal perfusions with the hormones. Data are reported as means ± SEM. N, Number of microperfusions; $J_{HCO_3^-}$, net HCO_3^- reabsorption. *P<0.05 compared to control (C); $^+$P<0.05 compared to ANP (Bonferroni test).

Figure 2 shows a significant increase in HCO_3^- reabsorption in the presence of luminal ANG II (1 pM) both in ED (from 0.93 ± 0.06, N = 20, to 2.64 ± 0.21 nmol cm^{-2} s^{-1}, N = 24) and LD segments (from 0.542 ± 0.086, N = 17, to 2.16 ± 0.151 nmol cm^{-2} s^{-1}, N = 30). ANP (1 µM) alone did not affect bicarbonate reabsorption in either the ED or LD segment nor did it impair the stimulation caused by ANG II.

The addition of the AT_1 receptor antagonist losartan (1 µM) to luminal perfusion blocked luminal ANG II-mediated stimulation in ED and LD segments.

We also examined the specific mechanisms by which luminal ANG II stimulates bicarbonate reabsorption. Figure 3 summarizes the results obtained in ED and LD segments during luminal perfusion with ANG II plus hexamethylene-amiloride (HMA, 100 µM, a specific blocker of Na^+/H^+ exchange) or with ANG II plus bafilomycin A1 (200

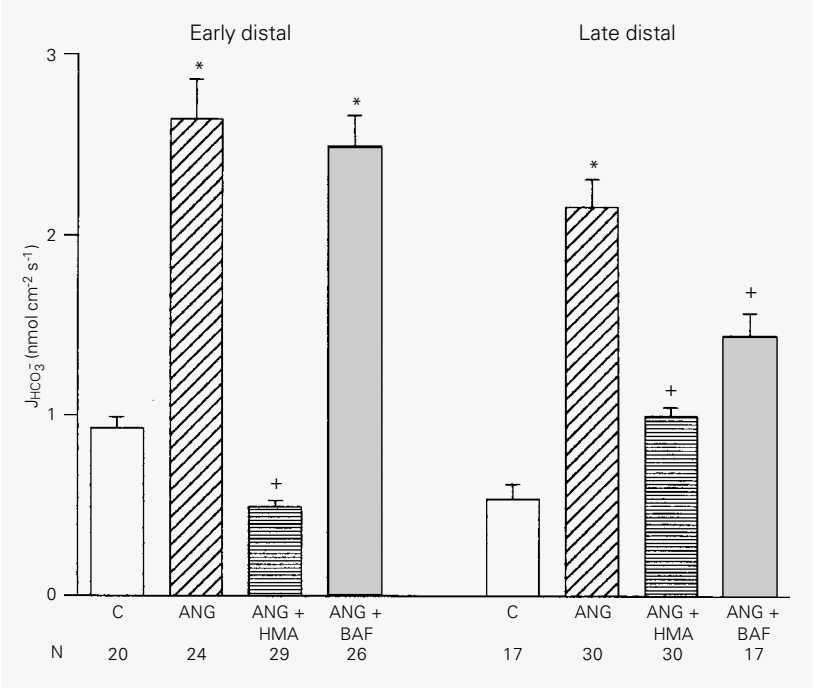

Figure 3 - Effect of luminal angiotensin II (ANG, 1 pM) plus HMA (100 µM) or bafilomycin A1 (BAF, 200 nM) on HCO_3^- reabsorption. The experiments were performed in early (left) and late (right) distal tubules. Data are reported as means ± SEM. N, Number of microperfusions; $J_{HCO_3^-}$, net HCO_3^- reabsorption. *P<0.05 compared to control (C); $^+$P<0.05 compared to ANG II (Bonferroni test).

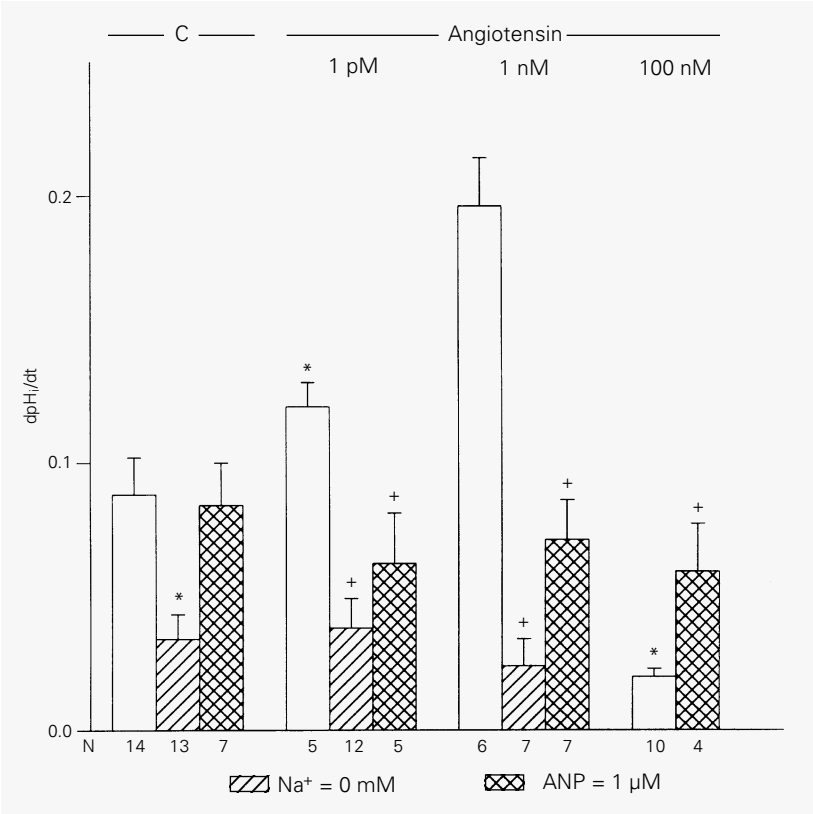

Figure 4 - Velocity of pH recovery (dpH_i/dt) in MDCK cells subjected to increasing angiotensin II concentrations (1 pM, 1 nM and 100 nM) alone or in the presence of atrial natriuretic peptide (ANP, 1 µM). The experiments were done in the presence (145 mM) or absence of Na^+. *P<0.05 compared to control (C); $^+$P<0.05 compared to ANG II (Bonferroni test).

nM, a highly specific inhibitor of vacuolar H^+-ATPase). HMA inhibited the stimulation of bicarbonate reabsorption induced by ANG II in ED ($J_{HCO_3^-}$ decreased to 0.497 ± 0.035 nmol cm^{-2} s^{-1}, N = 29) and LD segments ($J_{HCO_3^-}$ decreased to 0.998 ± 0.047 nmol cm^{-2} s^{-1}, N = 30). Bafilomycin A1 did not inhibit the stimulation of ED bicarbonate reabsorption induced by ANG II ($J_{HCO_3^-}$ was 2.50 ± 0.172 nmol cm^{-2} s^{-1}, N = 26); however, in LD segments $J_{HCO_3^-}$ was significantly decreased to 1.45 ± 0.139 nmol cm^{-2} s^{-1} (N = 17).

In conclusion, these results indicate that ANG II acts by stimulating Na^+/H^+ exchange in ED (81% of the ANG II-stimulated rate) and LD (54%) segments via activation of AT_1 receptors, as well as vacuolar H^+-ATPase in LD segments (33%). ANP does not affect bicarbonate reabsorption in either segment and, as opposed to what was seen in the proximal tubule, does not impair the stimulation caused by ANG II. This result is in accordance with data indicating that in ED segments bicarbonate reabsorption is mediated predominantly by Na^+/H^+ exchange and in LD segments by vacuolar H^+-ATPase (17,18) and with studies that indicate the absence of ANP receptors in distal segments (19). The present study, however, does not allow us to ascribe the results with ANG II to any specific cell type that might contribute to acidification in cortical distal tubules.

In order to investigate the mechanism of action of these hormones in more detail, we studied the dependence of cell pH on ANG II and ANP in MDCK cells, a permanent cell line derived from dog kidneys, with some properties of distal nephron epithelia. Cells were grown to confluence on coverslips, and cytosolic pH was determined with the fluorescent probe BCECF in spectrofluorimeter cuvettes. The cells were acid loaded using the NH_4Cl pre-pulse technique, and the rate of cell pH recovery was followed with control or hormone-containing solutions.

Some of the results obtained in these studies are illustrated in Figure 4. The velocity of cell pH recovery (dpH/dt) was almost abolished in the absence of Na^+, indicating that it is dependent on Na^+/H^+ exchange. This recovery was markedly increased when ANG II was used at concentrations from 1 pM to 1 nM. On the other hand, when concentrations of 100 nM ANG II were used this recovery rate was significantly reduced. We also showed that ANP (1 µM) alone had no effect on this recovery but reversed both the acceleration of H^+ extrusion at low ANG II levels and inhibition of H^+ extrusion at higher ANG II levels.

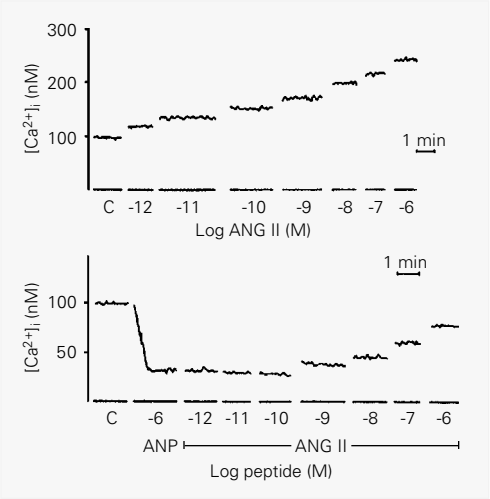

Figure 5 - Cell calcium levels in MDCK cells subjected to increasing angiotensin II (ANG II) concentrations (upper panel) alone or in the presence of atrial natriuretic peptide (ANP, 1 µM; lower panel). Data from Ref. 20, with permission.

To obtain more information on the mechanism of interaction of these hormones, we also studied their effects on the regulation of intracellular free calcium concentration, $[Ca^{2+}]_i$, monitored with the fluorescent probe Fura-2 in MDCK cells in suspension (20). The results are shown in Figure 5. $[Ca^{2+}]_i$ increased progressively from control values of 100 nM to approximately 250 nM when ANG II concentration in the cell suspension increased from 1 pM to 1 µM. On the other hand, when 1 µM ANP was added to the cell suspension, $[Ca^{2+}]_i$ decreased from control values to 35 nM. The addition of ANG II (1 pM-1 µM) in the presence of ANP increased $[Ca^{2+}]_i$, but without exceeding normal control values. These results may indicate a role of $[Ca^{2+}]_i$ in the regulation of the

process of H^+ extrusion mediated by Na^+/H^+ exchange and stimulated/impaired by ANG II. The data are compatible with stimulation of Na^+/H^+ exchange by increases of $[Ca^{2+}]_i$ in the lower range, and inhibition at high $[Ca^{2+}]_i$ levels.

At lower levels of ANG II (1 pM-1 nM), the increase in $[Ca^{2+}]_i$ may be mediated by AT_{1B} ANG II receptors which activate phospholipase C, causing the stimulation of inositol triphosphate and diacylglycerol, which elevate $[Ca^{2+}]_i$ by releasing it from cell stores. Calcium activates protein kinase C which, via phosphorylation, may stimulate the Na^+/H^+ exchanger (21). Another possible mechanism of action of low-dose ANG II is a signalling pathway involving the activation of an inhibitory G protein and inhibition of adenosylate cyclase, causing a decrease in cAMP levels and in the catalytic activity of protein kinase A, a reduction which causes the activation of Na^+/H^+ exchange (22). On the other hand, ANP has been shown to block the activity of phospholipase C, thus impairing the pathway causing the increase in cell calcium (23).

At high levels (100 nM) ANG II interacts with AT_{1A} receptors, causing the liberation of arachidonic acid, which is part of a pathway that elevates $[Ca^{2+}]_i$ by activating voltage-sensitive calcium channels in the plasma membrane (21). At high cytosolic concentrations, calcium may inhibit Na^+/H^+ exchange by activating Na^+/Ca^{2+} exchange at the cell membrane level, thereby increasing cell sodium, which in turn decreases the gradient responsible for H^+ extrusion by the exchanger. However, this mechanism is somewhat questionable considering the large discrepancy between cytosolic calcium and extracellular sodium concentrations.

On the other hand, it has been shown that the NHE1 exchanger has calmodulin-binding sites at the cytoplasmic regulatory domain, which modulate its activity. A high-affinity site, which is tonically inhibitory, binds to low calcium/calmodulin suppressing the inhibition, i.e., stimulating the exchanger at low cell Ca^{2+}/calmodulin levels. A low-affinity site, however, binds to calcium and calmodulin only at high concentrations, and under these conditions inhibits the exchanger activity (24). This behavior is compatible with our findings about the ANG II-ANP interaction in proximal nephron and MDCK cells.

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