Angiotensin II Upregulates the Expression of Vasopressin V_2 mRNA in the Inner Medullary Collecting Duct of the Rat

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Previous in vivo studies in cardiomyopathic hamsters suggested that the expression of vasopressin (AVP) V_2 mRNA is upregulated by angiotensin II. The present study was performed to determine whether angiotensin II plays a role in regulating the expression of AVP V_2 mRNA and aquaporin-2 (AQP2) mRNA in the inner medullary collecting duct (IMCD) of the male Wistar rat. The expression of AVP V_2 mRNA and AQP2 mRNA in the IMCD was measured by competitive reverse-transcriptase polymerase chain reaction (RT-PCR). Six groups of experiments were performed. In the first group, we incubated IMCD with 3 different doses of angiotensin II (10^{-11}, 10^{-9} and 10^{-7} mol/L). Angiotensin II caused a significant increase in the AVP V_2 mRNA in a dose-dependent manner but its effect on AQP2 mRNA was modest. This effect of angiotensin II was inhibited by angiotensin II receptor antagonist, [Sar^1,Ile^8]-angiotensin II. To examine the role of PKA in mediating an increase in AVP V_2 mRNA expression, we incubated IMCD with10^{-7} and 10^{-11} M of angiotensin II in the presence of a specific protein kinase A (PKA) inhibitor, Rp diasteroisomer of adenosine 3′-5′-cylic monophosphothionate (Rp-cAMPS). The angiotensin II–induced upregulation of V_2 mRNA was abolished. In the fourth group, we examined the effect of protein kinase C (PKC) inhibition on V_2 mRNA expression. The upregulation of V_2 mRNA induced by angiotensin II was greatly exaggerated when IMCD was incubated with angiotensin II and RO-31-8220 (PKC inhibitor). In the fifth and sixth groups of studies, we determined the direct effect of PKA and PKC on regulating the expression of V_2 mRNA and AQP2 mRNA in the IMCD, respectively. Dibutryl cAMP stimulated an upregulation in the expression of V_2 mRNA and AQP2 mRNA, whereas phorbol esters suppressed the expression of V_2 mRNA. These results suggested that PKA stimulates and PKC suppresses the expression of V_2 mRNA in the IMCD of the kidney.

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IMPAIRMENT IN water excretion is a cardinal feature of cardiac failure. Studies in congestive heart failure rats showed that renal water retention was associated with marked increase in aquaporin-2 (AQP2) mRNA and protein levels.[1,2] In addition, studies with vasopressin (AVP) receptor antagonists showed that AVP V_2 receptors played an important role in retaining free water in normal and heart failure animals.[3,4] Furthermore, inner medullary collecting ducts (IMCD) isolated from cardiomyopathic hamsters induced a greater accumulation of cyclic adenosine monophosphate (camp) in response to AVP stimulation.[5] These studies suggested that increase in water retention in heart failure is due to increase in AVP V_2 receptors and the water channel protein AQP2. This notion is confirmed by our recent studies in cardiomyopathic hamsters that showed upregulation of the expression of AVP V_2 and AQP2 mRNA in the IMCD.[6] Clearance experiments showed that the diuretic response to exogenous atrial natriuretic peptide (ANF) is impaired in cardiomyopathic hamsters, and after enalapril treatment, the diuretic effect of ANF is restored.[7] The normalization of the renal response to ANF was associated with a reduction in the expression of AQP2 and AVP V_2 mRNA.[6] These results suggested that either AVP V_2 receptors are upregulated, or, alternatively, there is an increased in adenyl cyclase activities or reduction in phosphodiesterase hydrolysis of cAMP in congestive heart failure. The increase in AVP V_2 mRNA seen in cardiomyopathic hamsters may be related to elevation in circulating angiotensin II, because enalapril treatment that lowered circulating angiotensin II restored AQP2 and AVP V_2 mRNA in cardiomyopathic hamsters to level seen in normal hamsters.[6] These data suggested that angiotensin might play a role in regulating the expression of AVP V_2 receptors in the IMCD.

The aim of the present study is to determine if angiotensin II plays a role in upregulating the expression of AVP V_2 mRNA in the IMCD. Studies were performed in the IMCD isolated from the kidney of male Wistar rat. Our results from these experiments suggested that angiotensin II induced increased expression of AQP2 and AVP V_2 mRNA via the protein kinase A (PKA) pathway.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing between 200 and 250 g were purchased from the University of British Columbia Animal Center. They had free access to standard lab chow.

IMCD Preparation

Rats were anesthetized with phenobarbital (50 mg/kg intraperitoneally) and kidneys were removed and cut into half in phosphate-buffered saline (PBS). The IMCD was isolated according to a method previously described.[5] The renal papilla was minced and digested in RPMI 1640 medium (Sigma-Aldrich Canada Ltd, Oakville, Ontario, Canada) containing 1.5 mg /mL of collagenase (United States Biochemical, Cleveland, OH) for 30 minutes at 37°C. An equal volume of RPMI 1640 containing 10% fetal calf serum was added to the mixture to stop the digestion. The mixture was then centrifuged at 300 × *g* and the resulting pellet was resuspended in 10 mL of RPMI 1640 containing 10% fetal calf serum and fractionated in percoll (specific gravity [sp. gr.], 1.07) for 20 minutes at 15,000 × *g*. The papillary collecting ducts

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were located on the top of the percoll layer. The IMCD cells were examined by histological and biochemical methods to confirm that these cells were of collecting duct origin.[5]

AVP V_2 Receptor and AQP2 mRNA Quantitation

Total cellular RNA was isolated by extraction using TRIZOL (Life Technologies, Burlington, Ontario, Canada). Competitive reverse-transcriptase polymerase chain reaction (RT-PCR) method was used to quantify the expression of AQP2 and AVP V_2 mRNA in the IMCD of the kidney. For RNA quantification by competitive RT-PCR, RT reactions were done with a fix amount of target RNA and the corresponding RNA competitor. A single set of primers was used to amplify both target and added competitor of known concentration. Competitors for AVP V_2 mRNA were synthesized with the sense primer 5′-AGC AAC AGC AGC CAG GAG GAA C-3′ and the antisense primer 5′-GGC CCA GCA ATC AAA CAC CCG CCA GGA TCA TGT AGG AGG AGG-3′ resulting in a amplification product of 355 bp length. The competitive PCR for AVP V_2 mRNA was done with the sense primer 5′-AGC AAC AGC CAG GAG GAG GAA-3′ and the antisense primer 5′-GGC CCA GCA ATC AAA CAC CC-3′ resulting in a PCR fragment of 522 bp. Competitors for AQP2 were synthesized with the sense primer 5′TCC TTC CTT CGA GCT GCC TT-3′ and the antisense primer 5′-ACG TTC CTC CCA GTC GGT GTC AGG GGT CCG ATC CAG AAG A-3′ producing a 404-bp fragment. The competitive PCR for AQP2 was done with the sense primer 5′-TCC TTC CTT CGA GCT GCC TT-3′ and the antisense primer 5°-ACG TTC CTC CCA GTC GGT GT-3° resulting in PCR product of 504 bp length. PCR was performed in a Perkin Elmer GeneAMP PCR system (Norwalk, CT). Amplification was performed in a total volume of 50 μL containing 2 μL of cDNA, 2 μL of competitor, 20 pmol of each primer, 100 μmol/L dNTPs, 10 mmol/L Tris HCl, 0.75 mmol/L $MgCl_2$, and 1.25 U of Taq DNA polymerase (GIBCO-BRL, Gaithersburg, MD). The amplification profile AVP V_2 and its competitor was as follows: 25 cycles of 95°C × 30 seconds, 65°C × 1 minute, 72°C × 1 minute followed by strand extension at 72°C × 7 minutes. The following condition was used for the amplification of AQP2: 30 cycles of 95°c × 30 seconds, 55°C × 45 seconds, 72°C × 1minute followed by strand extension at 72°C × 7 minutes. The PCR products were analyzed in 1.5% agarose gel followed by staining with ethidium bromide. The bands were scanned and analyzed by computer densitometry (Alphalmager, 1200 Alpha Innotech Corp, San Leandro, CA).

Experimental Protocol

To determine the optimal experimental conditions, different incubation times (2, 4, 6, and 17 hours) and different concentrations of angiotensin II (10^{-7}, 10^{-9}, and 10^{-11} mol/L) purchased from Sigma were tested. We found that IMCD in RPMI-1640 medium containing 10% fetal calf serum incubated in a 37°C incubator containing 5% CO_2 for 17 hours gave the best results. Thus, all subsequent studies were done with 17 hours of incubation. Time control and angiotensin II receptor antagonist ([Sar^1,Ile^8]-Angiotensin II, Sigma-Aldrich) experiments were performed to make sure any changes detected following incubation were due to the action of the agonist and antagonist. Studies were done to determine a possible role of PKA in upregulating the expression of AVP V_2 and AQP2 mRNA in the IMCD. In these experiments, IMCD were incubated with a specific inhibitor of PKA Rp diastereoisomer of adenosine 3′, 5′-cyclic monophosphothionate (Rp-cAMPS 10^{-6} mol/L) for 2 hours at 37°C before overnight incubation with angiotensin II (10^{-7} mol/L). Additional studies were done to examine the effect of incubating IMCD with dibutryl cAMP (10^{-4} mol/L) on the expression of AVP V_2 and AQP2 mRNA. Further studies were performed to determine the role of protein kinase C (PKC) in regulating the expression of AVP V_2 and AQP2 mRNA in the presence of angiotensin II. In these experiments, the PKC inhibitor R0-31-8220 (10^{-6} mol/L) was used in conjunction with angiotensin II (10^{-7} mol/L) for incubating the IMCD. The effect of PKC was examined by incubating IMCD with PMA (10^{-6}mol/L) for 17 hours. Following incubation, the IMCD cells were harvested for competitive RT-PCR analysis.

For Western blot analysis, lysates was prepared, subjected to sodium dodecyl sulfate–polyacylamide gel electrophoresis, and transferred to nitrocellulose membrane by using the Semi-dry Transfer Cell (Bio-Rad, Hercules, CA). All incubations were done at room temperature with gentle rocking. Membranes were blocked in phosphate buffter containing 0.1% Tween-20 and 5% nonfat dry milk at room temperature for 2 hours. After blocking procedure, membranes were incubated in the primary antibody for 1 hour. Anti-rat V_2 receptor antibody purchased from Alpha Diagnostic International (San Antonio, TX) detect a band at 62 kd in membranes from rat inner medulla and N-glycanase reduced the AVP V_2 size to 36 kd.[8] The bound antibodies were detected with horseradish peroxidase–conjugated antibody (Amersham, Baie d'Urfé, Quebec, Canada) and enhanced chemiluminescence (ECL) according to the manufacturer's protocol. The bands were quantitated by densitometry using NIH image-analysis software. The membrane was stripped and reprobed with β-actin to ensure equal loading of protein.

Table 1. The Effect of Incubation Time on the Expression of AVP V_2 and AQP2 mRNA in the Presence of 10^7 mol/L of Angiotensin II

			amol/μg of Total RNA		
	Time	n	Control	Angiotensin II	*P* Value
AVP V_2 RNA	1 h	6	185 ± 33	175 ± 40	NS
	2 h	6	175 ± 35	186 ± 49	NS
	4 h	6	160 ± 35	178 ± 35	NS
	17 h	12	164 ± 14	351 ± 41	<.01
AQP_2 RNA	1 h	6	5.9 ± 0.7	5.7 ± 0.6	NS
	2 h	6	4.9 ± 0.5	5.2 ± 0.8	NS
	4 h	6	5.3 ± 0.6	5.8 ± 0.5	NS
	17 h	12	4.0 ± 0.3	4.9 ± 0.4	<.01

Statistics

The results are shown as mean ± SEM and "n" indicates the number of rats. The differences between groups were tested using *t* test. A *P* value less than .05 was considered statistically significant.

RESULTS

Effects of Incubation Time

The effect of incubation time on angiotensin II–induced upregulation of vasopressin V_2 mRNA and AQP2 is shown in Table 1. No significant changes were noted in AVP V_2 and AQP2 mRNA expressions when IMCDs were incubated with angiotensin II for 1, 2, or 4 hours. However, following 17 hours of incubation with angiotensin II, there was a significant increase in both AVP V_2 (164 ± 14 10^{-18} mol attomoles [amol]/μg of total RNA to 351 ± 41 amol/μg of total RNA, $P < .01$, n = 12) and AQP2 mRNA (4.0 ± 0.3 amol/μg of total RNA to 4.9 ± 0.4 amol/μg of total RNA, $P < .01$, n = 12). Thus, all subsequent studies were performed with 17 hours of incubation.

Effect of Angiotensin II Incubation on AVP V_2 and AQP2 mRNA Expression in the IMCD of the Kidney

The IMCD was incubated with different concentrations of angiotensin II (10^{-7}, 10^{-9}, and 10^{-11}mol/L) and the effects on

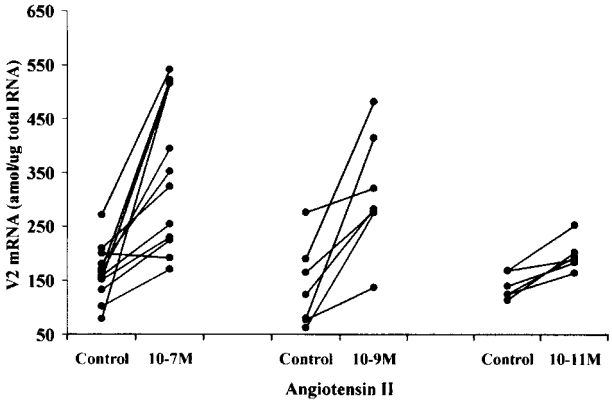

Fig 1. The effect of different concentrations of angiotensin II on the expression of AVP V_2 mRNA expression in the IMCD of the rat.

the expression of AVP V_2 and AQP2 mRNA are shown in Figs 1 and 2. IMCD incubated with 10^{-7} and 10^{-9} mol/L of angiotensin II had a significant increased in the expression of AVP V_2 mRNA (164 ± 14 to 351 ± 41 amol/μg of total RNA, $P < .01$, n = 12; 125 ± 34 to 284 ± 54 amol/μg of total RNA, $P < .015$, n = 7) and its effect on AQP2 mRNA expression is modest (4.0 ± 0.3 to 4.9 ± 0.3 amol/μg of total RNA, $P < .01$, n = 12; 4.5 ± 0.2 to 5.3 ± 0.2 amol/μg of total RNA, $P < .01$, n = 7). Low concentration of angiotensin II (10^{-11} mol/L) induced a modest increase in the expression of AVP V_2 mRNA (141 ± 10 to 199 ± 12 amol/μg of total RNA, $P < .01$, n = 6) but caused an insignificant increase in AQP2 mRNA (5.2 ± 0.5 to 5.9 ± 0.3 amol/μg of total RNA, difference not significant [NS], n = 6).

When IMCDs were incubated with angiotensin II (10^{-7} mol/L) and an angiotensin II receptor antagonist, [Sar^1,Ile^8]-angiotensin II, the upregulation of AVP V_2 (336 ± 40 *v* 206 ± 40 amol/μg of total RNA, $P < .05$, n = 6) expression was inhibited and had no effect on AQP2mRNA expression (3.9 ± 0.19 *v* 3.5 ± 0.18 amol/μg of total RNA, NS, n = 6). Figure 3 shows PCR products of representative gel for AVP V_2 in the

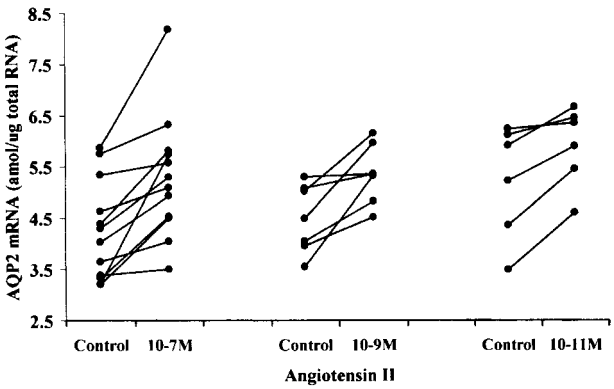

Fig 2. The effect of different concentrations of angiotensin II on the expression of AQP2 mRNA in the IMCD of the rat.

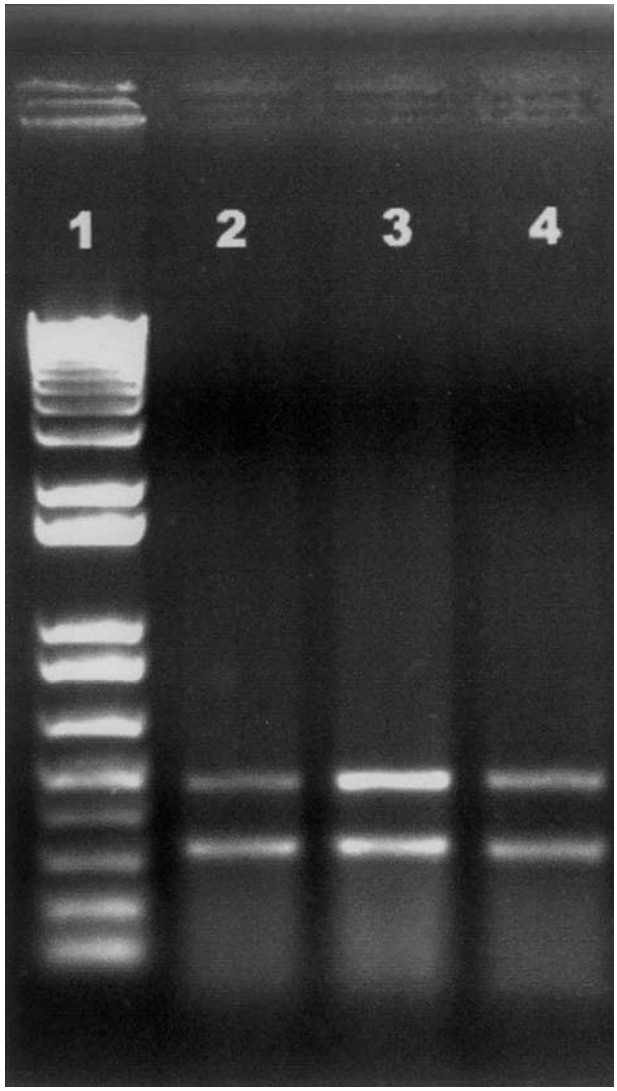

Fig 3. Ethidium bromide–stained agarose gel of a RT-PCR assay for AVP V_2 mRNA is shown. The top band is the specific RT-PCR product of AVP V_2 mRNA and the bottom band is the internal control standard for the competitive PCR. The molecular weight marker shown in lane 1 and control in lane 2. Angiotensin-induced change is shown in lane 3 and in the presence of angiotensin II receptor antagonist in lane 4.

untreated IMCD (lane 2) and IMCD incubated with angiotensin II with (lane 4) and without (lane 3) angiotensin II receptor antagonist ([Sar^1,Ile^8]-angiotensin II). The levels of AVP V_2 mRNA were increased with angiotensin II and then normalized with angiotensin II receptor antagonist. Western blot analysis showed the AVP V_2 receptors increased significantly (25% ± 3%, $P < .01$, n = 5) after 17 hours of incubation with angiotensin II and in the presence of [Sar^1,Ile^8]-angiotensin II there was no increase (−2.4% ± 1.1%, NS, n = 3). Figure 4 illustrates the changes in AVP V_2 receptors in the IMCD induced by angiotensin II in the presence and absence of [Sar^1,Ile^8]-angiotensin II.

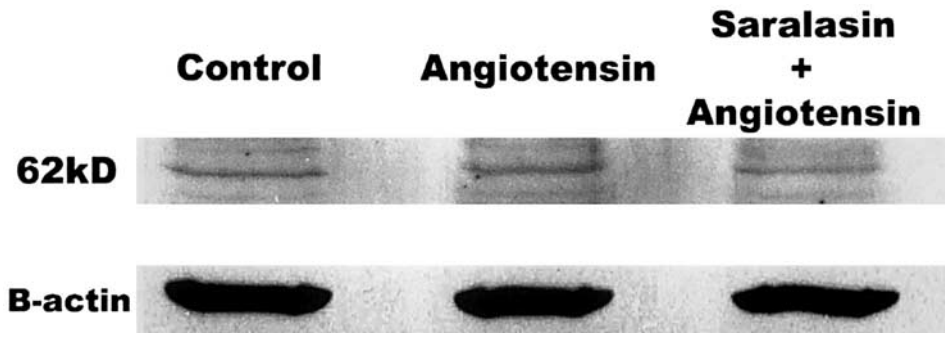

Fig 4. Western blot of AVP V_2 receptor from the IMCD of rats induced by angiotensin II in the presence and absence of angiotensin II receptor antagonist.

Effect of Rp-cAMPS on AVP V_2 and AQP2 mRNA Expression in the IMCD of the Kidney

The possible involvement of PKA in causing an increase in AVP V_2 and AQP2 mRNA expression in the IMCD was examined in this set of experiment. The results are shown in Figs 5 and 6. The increase in expression of AVP V_2 mRNA (151 ± 12 to 266 ± 19 amol/μg of total RNA, $P < .01$, n = 6) was abolished in the presence of Rp-cAMPS (266 ± 19 to 168 ± 18 amol/μg of total RNA, $P < .03$, n = 6). Similar results were obtained in AQP2 mRNA expression (control *v* angiotensin II *v* angiotensin II + Rp-cAMPS: 4.7 ± 0.4 *v* 5.5 ± 0.5 *v* 4.7 ± 0.4 amol/μg of total RNA, $P < .05$, n = 6).

Effect of PKC Inhibitor (RO-31-8220) on AVP V_2 and AQP2 mRNA Expression in the IMCD of the Kidney

The effect of PKC inhibition on the expression of AVP V_2 and AQP2 mRNA in the IMCD is shown in Figs 7 and 8. The increase in AVP V_2 mRNA expression induced by angiotensin II was exaggerated with PKC inhibitor RO-31-8220 in the culture medium. The mean AVP V_2 mRNA expression rose from 169 ± 17 amol/μg of total RNA to 411 ± 59 amol/μg of total RNA ($P < .01$, n = 6) with angiotensin II and increased further with PKC inhibition to 680 ± 116 amol/μg of total RNA ($P < .03$, n = 6). Angiotensin II induced a modest rise in the expression of AQP2 mRNA in the IMCD (3.2 ± 0.1 to 3.7 ± 0.2 amol/μg of total RNA, $P < .03$, n = 6) that increased further with PKC inhibition (4.1 ± 0.2 amol/μg of total RNA, n = 6).

Effect of PKC and PKA Agonists on AVP V_2 and AQP2 mRNA Expression in the IMCD of the Kidney

The effect of dibutryl cAMP and phorbol myristate acetate (PMA) on the expression of AVP V_2 and AQP2 mRNA in the IMCD is shown in Figs 9 and 10. Dibutryl cAMP caused a significant increase in AVP V_2 (157 ± 41 to 352 ± 92 amol/μg of total RNA, $P < .01$, n = 6) and AQP2 mRNA (4.4 ± 0.4 to 5.8 ± 0.5 amol/μg of total RNA, $P < .01$,n = 6) expression. In contrast, PMA induced a significant decrease in the AVP V_2 (172 ± 27 to 76 ± 30 amol/μg of total RNA, $P < .02$, n = 6) and AQP2 mRNA (4.8 ± 0.2 to 4.2 ± 0.2 amol/μg of total RNA, $P < .01$, n = 6) expression in the IMCD.

DISCUSSION

Our results showed that IMCD incubated with angiotensin II for 17 hours induced a significant increased in the expression of AVP V_2 and AQP2 mRNA. The effects are more pronounced with V_2 mRNA. These data confirmed the results of the previously reported in vivo experiments.[6] The in vivo studies were done in cardiomyopathic hamsters. These animals had an ele-

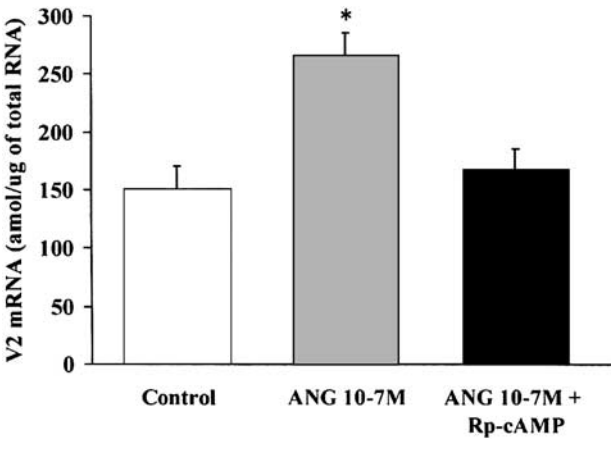

Fig 5. The increase in the expression of AVP V_2 mRNA in the IMCD induced by angiotensin II is attenuated by Rp-cAMP (10^{-6} mol/L). *$P < .01$.

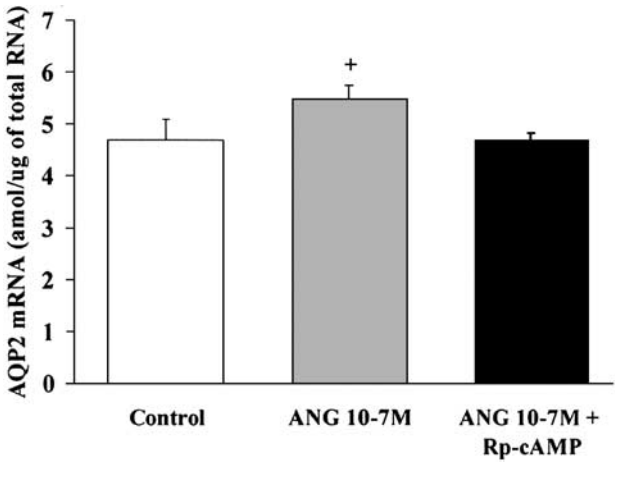

Fig 6. The increase in the expression of AQP2 mRNA in the IMCD induced by angiotensin II is attenuated by Rp-cAMP (10^{-6} mol/L). $^{+}P < .05$.

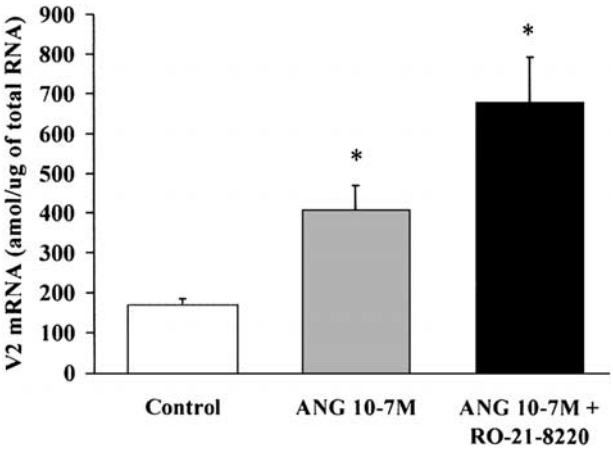

Fig 7. The increase in the expression of AVP V_2 mRNA in the IMCD induced by angiotensin II is amplified by PKC inhibitor RO-31-8220 (10^{-6} mol/L). *$P < .01$.

vated circulating angiotensin II levels that were associated with an increased in expression of V_2 mRNA. When these cardiomyopathic hamsters were treated with enalapril, an angiotensin-converting enzyme inhibitor, there was significant fall in circulating angiotensin II levels accompanied by a decline in V_2 mRNA expression in the IMCD. These results provide indirect evidence that angiotensin II is involved in upregulating the expression of V_2 mRNA in the IMCD of the kidney. In the present study, when IMCD cells were incubated with different concentrations of angiotesin II, we saw dose-dependent effects. These results provide direct evidence that angiotensin II affects V_2 mRNA expression in the IMCD and confirm the in vivo observations.

The physiological effect of angiotensin II is initiated by the binding of the angiotensin II to the angiotensin I receptor. The cellular responses to angiotensin II include activation of ty-

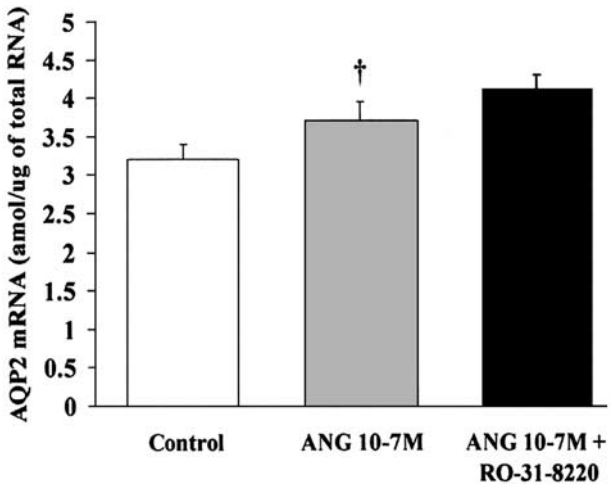

Fig 8. The increase in the expression of AQP2 mRNA in the IMCD induced by angiotensin II is amplified by PKC inhibitor RO-31-8220 (10^{-6} mol/L). †$P < .03$.

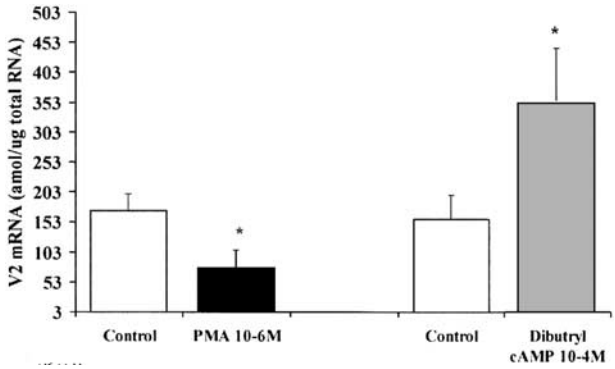

Fig 9. The effect of phorbol esters and dibutryl cAMP on the expression of AVP V_2 mRNA in the IMCD of the rat kidney. *$P < .01$.

rosine kinases, increases in diacylglycerol and intracellular calcium, thereby activating PKC.[9] In vascular smooth muscle, angiotensin II increases the production of prostaglandin leads to increase in cAMP, and stimulating PKA formation.[9] In the present study, we determine whether the upregulation of V_2 mRNA in the IMCD of the rat kidney induced by angiotensin II is mediated by PKC and PKA. PKA inhibitor abolished the angiotensin II–induced upregulation of V2 mRNA in the IMCD. In addition, when IMCD was incubated with dibutryl cAMP, there was a significant upregulation of V_2 mRNA. These results suggested that the upregulation of V_2 mRNA is mediated by PKA. The origin of PKA can be from prostaglandin production induced by angiotensin II. There is abundant evidence from in vivo and in vitro studies to show that angiotensin II stimulates renal prostaglandin production.[10-13] Other studies have shown that proximal and distal tubules synthesize very little prostaglandin, and most synthesis occurs at the thin descending limb of Henle, the medullary thick ascending limb of Henle and the medullary collecting tubules.[14,15] Hence, in the present study, we conclude that IMCD was stimulated by angiotensin II to produce prostaglandin that in turn led to cAMP accumulation and activation of PKA. PKA then translocates into the nucleus, where it induces transcription.[16]

We showed that PKC inhibitor exaggerates the upregulation

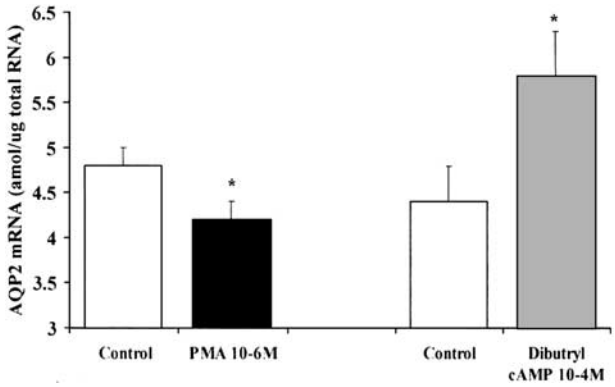

Fig 10. The effect of phorbol esters and dibutryl cAMP on the expression of AQP2 mRNA in the IMCD of the rat kidney. *$P < .01$.

of V2 mRNA expression induced by angiotensin II. These results suggested that following angiotensin II stimulation both PKA and PKC are activated. The former induced upregulation of V2 mRNA and the latter suppressed the expression of V2 mRNA. Thus, when PKC was inhibited with RO-31-8220 the upregulation of V2 mRNA induced by angiotensin II was enhanced. PKC is known to regulate transcription of other receptor genes[17,18] and could be responsible for the down-regulation of V2 mRNA expression following angiotensin II. To be certain that PKC plays a role in the down-regulation of V2 mRNA expression, additional experiments were done with PMA, an activator of PKC. Our results showed a reduction in the expression of V2 mRNA when IMCD was incubated with PMA. These data are in agreement with the suggestions that PKC suppressed the expression of V2 mRNA in the IMCD of the kidney. This is not a unique observation because the effect of agonist-induced down-regulation of mRNA has been reported for a number of G-protein couple receptors. For example, PKC has been shown to decrease the expression of thyrotropin-releasing hormone receptor mRNA.[19,20]

In the present study, we also examined the effect of angiotensin II on the expression of AQP2 mRNA in the IMCD. The response to angiotensin II induction of AQP2 mRNA expression in the IMCD is very modest. When incubated with a low concentration of angiotensin II (10^{-11} mol/L) there was no change in AQP2 expression. The effect of angiotensin II on V2 mRNA expression in the IMCD is more intense when compared to AQP2. These observations suggested that angiotensin II has a mild effect on upregulating the expression of AQP2. However, the upregulation of AQP2 mRNA expression was more pronounced when IMCD was incubated with dibutryl cAMP. These data suggested that cAMP-PKA signal plays an important part in upregulating the expression of AQP2. These is in agreement with report by Yasui et al suggesting cAMP-PKA plays a significant role in stimulating AQP2 mRNA accumulation in the kidney.[21]

In summary, the present study showed that angiotensin II stimulates an increase in the expression of AVP V_2 mRNA in the IMCD of the rat. cAMP-PKA plays an important part in mediating the angiotensin II–induced increase in AVP V_2 mRNA expression in the IMCD whereas PKC suppresses the expression. The effect of angiotensin II on the expression of AQP2 is modest and cAMP-PKA plays an important part in stimulating AQP2 mRNA accumulation in the kidney.

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