Calmodulin Is Required for Vasopressin-stimulated Increase in Cyclic AMP Production in Inner Medullary Collecting Duct*

Received for publication, January 3, 2005, and in revised form, February 9, 2005
Published, JBC Papers in Press, February 14, 2005, DOI 10.1074/jbc.M500040200

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Calmodulin plays a critical role in regulation of renal collecting duct water permeability by vasopressin. However, specific targets for calmodulin action have not been thoroughly addressed. In the present study, we investigated whether Ca\(^{2+}\)/calmodulin regulates adenylyl cyclase activity in the renal inner medullary collecting duct. Rat inner medullary collecting duct suspensions were incubated in the presence or absence of 0.1 nm vasopressin and the calmodulin inhibitors, monodansylcadaverine, W-7, and trifluoperazine, followed by measurement of cAMP. Vasopressin-stimulated cAMP elevation was significantly attenuated in the presence of calmodulin inhibitors. Analysis of transglutaminase 2 knock-out mice confirmed that these compounds were not acting through inhibition of transglutaminase 2 activity. Calmodulin inhibitors also blocked both cholera toxin- and forskolin-stimulated cAMP accumulation. In isolated perfused tubules, W-7 reversibly blocked vasopressin-stimulated urea permeability, a process that requires a rise in intracellular cAMP but does not appear to involve protein trafficking to the apical plasma membrane. These results suggest that calmodulin is required for vasopressin-stimulated adenylyl cyclase activity in the intact inner medullary collecting duct. Reverse transcription-PCR, immunoblotting, and immunohistochemistry revealed the presence of the calmodulin-sensitive adenylyl cyclase type 3 in the rat collecting duct, an isoform previously not known to be expressed in the collecting duct. Long-term treatment of Brattleboro rats with a vasopressin analog markedly decreased adenylyl cyclase type 3 protein abundance, providing an explanation for long-term down-regulation of vasopressin response in the collecting duct. These studies demonstrate the importance of calmodulin in the regulation of collecting duct adenylyl cyclase activity and transport function.

The collecting duct portion of the mammalian renal tubule regulates water and solute transport via the action of the antidiuretic hormone arginine vasopressin (AVP). AVP is released from the posterior pituitary in response to elevated plasma osmolality and binds to V2 receptors on the basolateral surface of the collecting duct epithelium, triggering a G-protein-linked signaling cascade, which leads to an elevation of cAMP and water channel aquaporin-2 (AQP2) vesicle insertion into the apical plasma membrane (1). Recently we demonstrated that calmodulin (CaM), a ubiquitous Ca\(^{2+}\)-binding protein, is required for AQP2 vesicle trafficking in response to vasopressin stimulation (2). Preincubation of isolated perfused rat inner medullary collecting duct (IMCD) with the CaM inhibitors W-7 and trifluoperazine (TFP) blocked AVP-stimulated water permeability. Further investigation revealed that CaM activates myosin light chain kinase and subsequent non-muscle myosin II-dependent vesicle trafficking of AQP2 (3).

In this paper, we sought to identify a role for CaM in regulating more proximal events in the collecting duct response to vasopressin, which could have an effect on other collecting duct functions including urea and Na\(^{+}\) transport. Given that CaM is known to regulate a wide range of cellular processes, it is reasonable to assume that this protein could act at multiple levels in the vasopressin-signaling pathway. One of the major secondary messengers that is increased in response to AVP is cAMP. Elevation of cAMP is required for AQP2 vesicle exocytosis (4) as well as the corresponding increase in collecting duct water permeability (5). Other collecting duct proteins regulated by cAMP include urea transporter UT-A1 (6) and the epithelial sodium channel (7).

Measuring cAMP in enriched IMCD fractions, we found that elevation of cAMP in response to AVP requires CaM. Further analysis suggested that CaM is acting at the level of adenylyl cyclase. This is the first demonstration of CaM-dependent cAMP accumulation in response to AVP in intact IMCD tubules, which supports prior conclusions from studies in cultured LLC-PK1 cells (8) and mouse outer medulla (9). In addition, we present evidence showing that CaM is required for AVP-mediated urea permeability in isolated perfused IMCD, another process that is cAMP-dependent (10), suggesting that CaM may play a broader regulatory role in the collecting duct than initially thought.

We utilized RT-PCR, immunoblotting, and immunohistochemistry to look for the presence of a CaM-sensitive adenylyl cyclase (AC) isoform in IMCD cells. Of the nine mammalian AC isoforms identified, three have been shown to be calmodulin-sensitive: AC1, -3, and -8 (11). AC1 and -8 are expressed mainly in tissues of the central nervous system, whereas AC3 has a broader profile, having been found in olfactory neuroepithelium (12), testes (13), brown adipose tissue (14), and uterus (15). Our studies demonstrated the presence of a single CaM-sensitive adenylyl cyclase isoform in IMCD, namely AC3. In the collecting duct, AC3 may act as the target cyclase for Ca\(^{2+}\)/CaM-dependent cAMP accumulation in response to vasopressin.

* This work was supported by the intramural budget of the NHLBI, National Institutes of Health, Project Z01-HL-01282-KE (to M. A. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: AVP, arginine vasopressin; AQP, aquaporin; CaM, calmodulin; IMCD, inner medullary collecting duct; TFP, trifluoperazine; RT, reverse transcription; AC, adenylyl cyclase; TG, transglutaminase; MDC, monodansylcadaverine; CTX, cholera toxin; dDAVP, [deamino-Cys-1,n-Arg-8]vasopressin.
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EXPERIMENTAL PROCEDURES

Animals—Pathogen-free male Sprague-Dawley rats (Taconic Farm Inc., Germantown, NY) were maintained on an autoclaved pelleted rodent chow (3141100-75-56, Zeigler Bros., Gardners, PA) and ad libitum drinking water. All experiments were conducted in accordance with an animal protocol approved by the Animal Care and Use Committee of the NHLBI, National Institutes of Health (ACUC protocol number 2-K3-E). Transglutaminase 2 (TG2) knock-out mice and wild-type mixed background mice, a kind gift of Dr. Gerry Melino (University of Roma, Italy) (16), were maintained on the same autoclaved pelleted rodent chow and ad libitum drinking water. Immunoblotting as well as PCR amplification of tail genomic DNA was used to distinguish knock-out from wild-type mice. All experiments were conducted in accordance with an animal protocol H-0047 approved by the Animal Care and Use Committee of the NHLBI.

Materials—Monodansylcadaverine (MDC), TFP, forskolin, and cholera toxin (CTX) were from Sigma. W-7 was from Calbiochem. Cyclic AMP enzyme immunoassay kit was from Cayman Chemical. Adenylyl cyclase antibodies, AC3 (20-2, sc-588) and AC5/6 (C-17, sc-590), were from Santa Cruz Biotechnology and were used at the manufacturer’s recommended dilutions. Both affinity-purified aquaporin-1 (L266) (17) and aquaporin-2 (L127) (18) antibodies have been described previously. IMCD Suspensions—IMCD suspensions were prepared from inner medulla of rat kidney using the method of Stokes et al. (19) with some modifications (20). Briefly, rats were killed by decapitation, and whole inner medullas were removed and finely minced with a razor blade. Minced tissue was incubated for 90 min at 37 °C with gentle agitation in a collagenase/hyaluronidase solution to dissociate individual tubule segments. After incubation, the sample was centrifuged at 80 × g for 30 s to enrich for heavier IMCD structures followed by centrifugation of the supernatant at 1500 × g for 5 min to pellet the lighter non-IMCD fragments. Pellets were resuspended in either bicarbonate buffer (118 mM NaCl, 25 mM NaHCO3, 5 mM KCl, 4 mM Na2HPO4, 1.2 mM MgSO4, 2 mM CaCl2, 5.5 mM glucose) for measurement of cAMP or Laemmli buffer for immunoblotting.

Measurement of cAMP—Fifty-microliter aliquots of IMCD suspensions were preincubated at 37 °C for 10 min with 0.5 mM isobutyl methylxanthine in the presence or absence of various CaM inhibitors followed by incubation with 0.1 nM AVP for 5 min. Samples were pelleted at 1000 × g for 1 min, and the supernatant was discarded. Tissue pellets were lysed by adding 0.2 N HCl and incubating for 20 min at room temperature followed by centrifugation at >10,000 × g for 10 min. Supernatants were saved for measuring cAMP, and pellets were used to measure protein content (BCA assay, Pierce). cAMP content was measured using a non-radioactive enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) based on competitive binding between endogenous cAMP and an exogenous cAMP-tagged acetylcholinesterase tracer. Samples were run in 96-well microtiter plate format and measured fluorometrically on a plate reader (Labsystems Multiskan MCC/ 340). Absorbance data were analyzed using a spreadsheet program provided by Cayman Chemical, which calculated cAMP content in pmol/μl. This value was then normalized to total protein, which had been measured previously using the BCA assay. The final value was expressed as fmol of cAMP/μg of protein.

Isolated Perfused IMCD Tubules—IMCD segments were microdissected from the mid-region of the inner medulla (40–70% of the distance from the inner-outer medullary junction to the papillary tip of the rat kidney). The tubules were transferred to a perfusion chamber, mounted on an inverted microscope, cannulated by concentric pipettes, and perfused in vitro. The perfusate and the perfusate bath solutions were identical to the dissection solution, except that in the bath solution, 5 mM creatinine was replaced by 5 mM urea. The urea permeability was determined by measuring the urea flux resulting from the transepithelial urea gradient. The urea concentrations in the perfusate, bath, and collected fluid were measured fluorometrically using a continuous flow ultramicrofluorometer and an enzymatic assay (Infinity urea nitrogen reagent, catalogue number TR12321, ThermoTrace).

RT-PCR—Total RNA was isolated from rat IMCD and brain using the guanidinium thiocyanate/cesium-trifluoroacetic acid method. Potentiating cDNA from the transcriptome was amplified with 20 cycles by a 30-min incubation with DNsase I (DNA-free, Ambion). Total RNA (1 μg) was reverse transcribed using oligo(dT) and Superscript II RT (Invitrogen) following the manufacturer’s recommended protocol. RT-negative controls were performed to assess the presence of possible genomic DNA contamination of RNA samples. PCR primers were designed against the corresponding cDNAs of rat adenyl cyclase isoforms 1–9 to generate products of 200–500 bp in size. All primers were designed to span at least one intronic region to distinguish possible amplification of genomic DNA. All amplified products were confirmed by sequencing.

DDAVP Infusion—Under methoxyflurane anesthesia (Metofane, Pitman-Moore, Mundelein, IL), osmotic minipumps (model 2002, Alzet, Palo Alto, CA) were implanted subcutaneously in Brattleboro rats to deliver 20 ng/h DDAVP (Rhone-Poulenc Rorer, Collegeville, PA), a V2 vasopressin receptor-selective agonist. Brattleboro rats used as controls were implanted with minipumps containing vehicle (saline) alone. After 7 days of DDAVP or vehicle infusion, during which time rats received water and pelleted chow ad libitum, all rats were killed by decapitation. Immunoblotting and Immunohistochemistry—Tissue samples were homogenized in isolation solution (10 mM triethanolamine, 250 mM sucrose, pH adjusted to 7.6, Roche protease inhibitor tablet) using a mechanical tissue grinder (Omni International), and total protein concentration was determined by the BCA assay (Fierce) using bovine serum albumin as the standard. Samples were then solubilized in Laemmli buffer (10 mM Tris, pH 6.8, 1.5% SDS, 6% glycerol, 0.05% bromphenol blue, and 40 mM dithiothreitol). 15–50 μg of protein was subjected to SDS-PAGE (21) and immunoblotting as described previously (22). Rat kidneys were perfusion fixed, paraffin-embedded, and processed for immunostaining via horseradish peroxidase as described previously (23).

Statistics—Quantitation of changes in cAMP as well as densitometric analysis of protein immunoblots are expressed as the mean ± S.E. (n ≥ 3) for each group. Unpaired t tests or analysis of variance were performed as appropriate for the given data set.

RESULTS

Effect of CaM Inhibition on AVP-stimulated cAMP Accumulation in Rat IMCD—To address the role of CaM in regulating cAMP production in response to vasopressin, we incubated rat IMCD cell suspensions with three different CaM inhibitors: MDC (24), W-7 (25), and TFP (26). Following a 10-min preincubation period with these compounds in the presence of the phosphodiesterase inhibitor isobutyl methylxanthine (0.5 mM), 0.1 nM AVP or vehicle (bicarbonate buffer) was added to the tubules for 5 min. cAMP content was subsequently measured by a non-radioactive enzyme immunoassay as described under “Experimental Procedures.” cAMP levels were significantly increased 4–5-fold in IMCD suspensions incubated with AVP alone (Fig. 1). Preincubation of tubules with MDC (200 μM) completely abolished AVP-stimulated cAMP accumulation in IMCD cells (61.2 ± 17.5 versus 529.3 ± 52.7 (AVP alone) fmol cAMP/μg of protein) (Fig. 1A). Preincubation of tubules with two other CaM inhibitors, W-7 (25 μM) (165.4 ± 33.1 versus 378.5 ± 19.2 (AVP alone) fmol/μg) and TFP (30 μM) (174.5 ± 37.4 versus 378.5 ± 19.2 (AVP alone) fmol/μg), also significantly reduced the increase in cAMP because of AVP treatment. Increasing the concentration of these drugs to 100 μM gave an even greater inhibition (Fig. 1B), demonstrating the dose dependence of this phenomenon.

CaM Inhibitors Act at the Level of Adenylyl Cyclase—Although our results indicated that CaM was required for elevation of cAMP by vasopressin, it was unclear at what level CaM was affecting the signaling pathway (e.g. V2 receptor, Gαo, or adenyl cyclase). Incubation of IMCD suspensions with 1 μg/ml CTX, a potent ADP-ribosetransferase that causes persistent activation of Gαo, produced a 4.6-fold increase in cAMP that was blocked by either W-7 (25 μM) or TFP (30 μM) (Fig. 2A). The fact that CaM inhibitors block CTX-mediated cAMP elevation rules out the V2 receptor as the site of action of Ca2+/CaM, and suggests that CaM is probably acting either at the level of Gαo or of the adenyl cyclase responsible for cAMP production in the IMCD.

Forskolin is a direct activator of nearly all known adenylyl cyclase isoforms (11). Treatment of IMCD cells with forskolin (1 μM) resulted in a nearly 10-fold increase in cAMP, which was significantly decreased by preincubation with MDC (638.3 ± 45.3 versus 2076 ± 390.4 (forskolin alone) fmol/μg) (Fig. 2B). Similar results were obtained when tubules were preincubated...
with W-7 or TFP prior to stimulation with forskolin (data not shown). These results indicate that CaM is acting beyond the level of G_{o1}, viz. on the adenylyl cyclase responsible for most cellular cAMP production itself.

Effect of MDC on cAMP Accumulation in Wild-type and TG2 Knock-out Mice—Previous studies have shown that both MDC and W-7 can inhibit transglutaminase in addition to calmodulin (24, 27). Both MDC and W-7 possess a high degree of structural similarity (Fig. 3) and can act as primary amine substrates for cross-linking by transglutaminase. To determine whether these inhibitors are acting through transglutaminase, we obtained TG2 knock-out mice (Dr. Gerry Melino (16)), which lack both transcript and protein for the major isoform expressed in IMCD (2). IMCD from wild-type mixed background lack both transcript and protein for the major isoform expressed in IMCD.2 IMCD from wild-type mixed background were obtained TG2 knock-out mice (Dr. Gerry Melino (16)), which

Effect of CaM inhibition on AVP-stimulated cAMP accumulation in isolated IMCD suspensions. A, preincubation of tubules with MDC (200 μM) completely blocked the elevation of cAMP by vasopressin (0.1 nM). MDC also significantly reduced base-line cAMP levels in the absence of AVP. Levels of cAMP are expressed as fmol of cAMP/mg of total protein (n ≥ 3; *p < 0.05). B, preincubation of tubules with either TFP or W-7 resulted in a dose-dependent inhibition of AVP-stimulated cAMP (n ≥ 3; *p < 0.01).

Effect of MDC on cAMP Accumulation in Wild-type and TG2 Knock-out Mice—Previous studies have shown that both MDC and W-7 can inhibit transglutaminase in addition to calmodulin (24, 27). Both MDC and W-7 possess a high degree of structural similarity (Fig. 3) and can act as primary amine substrates for cross-linking by transglutaminase. To determine whether these inhibitors are acting through transglutaminase, we obtained TG2 knock-out mice (Dr. Gerry Melino (16)), which lack both transcript and protein for the major isoform expressed in IMCD (2). IMCD from wild-type mixed background and TG2(−/−) mice produced similarly elevated levels of cAMP in response to AVP (Fig. 4). Preincubation with MDC blocked AVP-induced cAMP responses in both wild-type and TG2(−/−) mice to a similar extent, suggesting that these inhibitors are not acting through promiscuous inhibition of transglutaminase and that transglutaminase activity is not required for elevation of cAMP by vasopressin.

Effect of CaM Inhibition on AVP-stimulated Urea Permeability in Isolated Perfused Rat IMCD—Both vasopressin-stimu-

lated water and urea permeability in IMCD require elevation of intracellular cAMP. Our previous studies have demonstrated a clear role of calmodulin in regulation of water permeability at the level of aquaporin trafficking (2, 3); however, no clear role for calmodulin had been identified for AVP-stimulated urea permeability. To address this, we isolated rat IMCD segments by microdissection and utilized the perfused tubule method described under “Experimental Procedures” to measure changes in collecting duct urea permeability in response to AVP. The transepithelial urea permeability of isolated tubules was increased following the addition of 0.1 nM AVP to the bath solution (45.7 ± 0.9 (basal) versus 116.7 ± 19.2 (AVP) × 10^−5 cm/s) (Fig. 5). The subsequent addition of W-7 (25 μM) dramatically reduced urea permeability to basal levels (AVP + W-7 = 26.3 ± 11.3 × 10^−5 cm/s). The addition of W-7 prior to stimulation with AVP also reduced urea permeability (14.7 ± 3.8 (W-7) versus 21.7 ± 5.8 (W-7 + AVP) × 10^−5 cm/s). More importantly, urea permeability increased upon washout of W-7 with fresh AVP solution (AVP washout = 92.0 ± 7.8), demonstrating the reversibility of this process.

Identification of Adenylyl Cyclase Isoforms in Rat IMCD by RT-PCR—To determine which isoforms of adenylyl cyclase are present in rat IMCD, specific primer sets were generated for each AC isoform (1–9) using sequences obtained from the full-length cDNA of the corresponding gene. Total RNA was extracted from tissue and used for reverse transcription reactions

TABLE 1

| Time (min) | Water Permeability (cm/s) | Urea Permeability (cm/s) |
|-----------|---------------------------|--------------------------|
| 0         | 23.4 ± 3.2                | 116.7 ± 19.2             |
| 15        | 26.3 ± 11.3 × 10^-5       | 21.7 ± 5.8 × 10^-5       |
| 45        | 26.3 ± 11.3 × 10^-5       | 92.0 ± 7.8               |

FIG. 2. Effect of CaM inhibitors on CTX and forskolin-stimulated cAMP accumulation. A, IMCD suspensions were preincubated for 10 min in the presence or absence of two different CaM inhibitors, W-7 (25 μM) or TFP (30 μM), and then incubated with CTX (1 μg/ml) for 45 min after which cAMP was measured (n = 4; *, p < 0.01 versus CTX alone). CaM inhibitors blocked elevation of cAMP produced by CTX. B, IMCD suspensions were preincubated with either vehicle (−) or MDC (+) (200 μM) for 10 min followed by a 5-min incubation in the presence or absence of forskolin (1 μM). MDC blocked forskolin-stimulated cAMP accumulation (n = 3; p < 0.01).

2 J. D. Hoffert, C.-L. Chou, R. A. Fenton, and M. A. Knepper, unpublished observations.
in the presence or absence of reverse transcriptase (Fig. 6, \(RT^+\) and \(RT^-\), respectively) followed by amplification by PCR. Rat brain was utilized as a positive control for all primer sets, and bands of the appropriate size were present for each AC isoform (Fig. 6, top panel). In all reactions without RT, the band is absent. This eliminates the possibility that bands in \(RT^+\) reactions represent amplified contaminating genomic DNA. Analysis of rat IMCD revealed the presence of the majority of AC isoforms except AC1 and -8 (Fig. 6, bottom panel). Most importantly, the only CaM-stimulated isoform of adenylyl cyclase detectable by RT-PCR was AC3.

**AC3 Is Enriched in IMCD Cells**—Rat inner medullas were processed as described under "Experimental Procedures" to generate two fractions: IMCD cells and non-IMCD cells. The former is enriched in collecting duct fragments, and the latter generate two fractions: IMCD cells and non-IMCD cells. The IMCD pellet is devoid of a large amount of contaminating glia (28). In addition, these two bands, along with a smaller band under 160 kDa, were present in a sample of whole brain homogenate (Fig. 7B). Both bands were absent with preadsorption of the AC3 antibody with its corresponding blocking peptide (Fig. 8C). We have demonstrated previously that AC6 protein is present in the IMCD by immunocytochemistry (30). Interestingly, both AC3 and -6 appear to have similar distributions. Collecting duct staining was confirmed using an antibody to AC6 in AC3 protein expression in the collecting duct fraction of the inner medulla.

To further address the presence of AC3 in IMCD, we performed immunohistochemistry on rat inner medullary sections. AC3 was found in all IMCD cells, with a lower level of staining present in thin limb cells (Fig. 8A). This staining was largely ablated by preadsorption of the AC3 antibody with its corresponding blocking peptide (Fig. 8B). As expected, AC6, the major isoform identified previously by RT-PCR in more proximal portions of the collecting duct (29), was also found in IMCD (Fig. 8C). We have demonstrated previously that AC6 protein is present in the IMCD by immunocytochemistry (30). Interestingly, both AC3 and -6 appear to have similar distributions. Collecting duct staining was confirmed using an antibody to the collecting duct-speciﬁc marker protein AQP2 (Fig. 8D). The presence of AC3 in IMCD directly demonstrates the presence of a Ca\(^{2+}\)/CaM-stimulated isoform in the IMCD and supports our conclusion that Ca\(^{2+}\)/CaM may act directly on adenylyl cyclase to enhance AVP-stimulated cAMP production.

**Effect of Long-term dDAVP Administration on AC3 Expression**—A prior study has shown that AC6 expression in the...
collecting duct is reduced during long-term dDAVP treatment, thought to be the result of a conditioned “negative feedback” response (30). To address whether AC3 is similarly affected by dDAVP, Brattleboro rats were given dDAVP (20 ng/h) or saline (control) via osmotic minipumps for 7 days followed by isolation of inner medulla and immunoblotting. AQP2 was used as a positive control for the effect of dDAVP in collecting duct. AQP2 protein abundance was significantly increased 2.5-fold with dDAVP treatment (100\% control versus 247.7\% dDAVP; p < 0.001) (Fig. 9, bottom panel). AC3 protein abundance decreased 2.9-fold (12.3 control versus 34.5\% dDAVP; p < 0.01) during long-term dDAVP treatment (Fig. 9, top panel). AC6 expression was reduced 5-fold (11.7 control versus 19.8\% dDAVP; p < 0.0002) (Fig. 9, middle panel). This result suggests that AC3 and -6 expression may be subject to similar long-term regulatory influences in IMCD cells.

**DISCUSSION**

Vasopressin acts via the V2 receptor to increase water and urea permeability in the inner medullary collecting duct of the kidney. Water and urea are transported via different channels (31). Water transport occurs via AQP2 in the apical plasma membrane and aquaporins 3 and 4 in the basolateral plasma membrane (32). Regulation of water transport occurs via vasopressin-mediated AQP2 trafficking to the apical plasma membrane (33). Urea transport in the IMCD occurs via two urea channels, UT-A1 and -A3, present in the apical and basolateral plasma membrane, respectively. The mechanism of urea transport regulation by vasopressin is not known, although it is believed that urea channels do not traffic to the apical plasma membrane together with AQP2 (34, 35).

The regulation of both water and urea transport in the IMCD depends on activation of adenylyl cyclase activity via the het-
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rhotrimmeric GTP-binding protein $G_s$ (1). The molecular identity of the adenylyl cyclase responsible for vasopressin-mediated increases in cyclic AMP in IMCD has not been addressed previously, although it has been widely assumed that AC6 is responsible for vasopressin-stimulated cAMP increases because it has been demonstrated to be relatively abundant in collecting duct principal cells (29, 36). This isoform is inhibited by Ca$^2^+$ in the micromolar range (37) and is not calmodulin-sensitive (38). AC6 has been localized in the collecting duct by RT-PCR (29) and in situ hybridization (36). Furthermore, increasing intracellular calcium appears to inhibit AVP-stimulated cAMP in the outer medullary part of the collecting duct (29), supporting the conclusion that AC6 is responsible for vasopressin-dependent cAMP production by outer medullary collecting duct principal cells. However, earlier reports strongly suggest that vasopressin-sensitive renal epithelia possess CaM-sensitive AC activity. First, Ausiello and Hall (8) described CaM-stimulated adenylyl cyclase activity in a cell line sensitive to vasopressin (LLC-PK1). A subsequent study in microdissected outer medullary collecting ducts by Takaichi and Kurokawa (9) reported that AVP-sensitive cAMP production was CaM-dependent. Despite these findings, a candidate CaM-sensitive AC has not been found in the renal collecting duct.

In this study, we have identified a role for CaM in regulating the IMCD response to vasopressin at the level of adenylyl cyclase. Utilizing various CaM inhibitors, we were able to block AVP-dependent cAMP accumulation in both rat and mouse IMCD. Isolated perfused tubule experiments demonstrated that CaM is required for AVP-stimulated urea permeability in the collecting duct, a process known to be cAMP-dependent. A recent paper demonstrated that CaM binds to the COOH terminus of the V2 receptor and mediates some of the actions of vasopressin (39). However, in the present study, CaM inhibitors also blocked the rise in cAMP in the presence of either choleratoxin or forskolin, providing strong evidence that CaM acts directly on adenylyl cyclase to increase cAMP production.

What AC isoform could be responsible for CaM-dependent cAMP production in the IMCD? Three isoforms have been reported to be CaM-sensitive, namely AC1, -3, and -8 (11). Among these, we found evidence for the expression of only AC3 in the IMCD. Specifically, we have identified AC3 mRNA by RT-PCR in IMCD suspensions. Neither AC1 nor -8 transcripts were detectable in IMCD cells, even though these transcripts were readily detectable in brain with the same loading and expression data, we propose that AC3 is a target of CaM in IMCD cells and is, at least in part, responsible for the rise in cAMP in response to AVP.

Interestingly, our data indicate that both AC3 and the Ca$^{2+}$-inhibited isoform AC6 are expressed in the inner medullary collecting duct. Based on immunocytochemical labeling, it appears that both isoforms are expressed in the same cells. As of yet, it remains unclear what the relative contribution of each isoform is to the overall rise in cAMP during the stimulation of the collecting duct with AVP. The dramatic decrease in AVP-stimulated cAMP in the presence of the CaM inhibitors in our study suggests that the contribution of AC3 is quite significant under the conditions of the measurements. Previously, it was demonstrated that stimulation of inner medullary collecting ducts with AVP produces an increase in intracellular Ca$^{2+}$ (10) via the V2 vasopressin receptor (42, 43). The increase in intracellular Ca$^{2+}$ is oscillatory in nature (44) and is dependent on Ca$^{2+}$ release via ryanodine-sensitive stores (2). AC3 likely contributes to cAMP accumulation during periods of elevated intracellular Ca$^{2+}$ when AC6, the Ca$^{2+}$-inhibited isoform, is probably in an inactive state. Conversely, AC6 may be the predominant producer of cyclic AMP when Ca$^{2+}$ is low in the cell. Overall, the two isoforms in combination may provide a "smoothed" cAMP signal in the face of variable intracellular Ca$^{2+}$.

There is also evidence that Ca$^{2+}$/CaM can indirectly inhibit AC3 via activation of a specific calmodulin-dependent kinase, CaM kinase II, which phosphorylates AC3, thereby inhibiting its cyclase activity (46, 47). Inhibition of AC3 activity through CaM kinase II-mediated phosphorylation may provide a critical switch in terminating cAMP-mediated signaling in the collecting duct. For instance, inhibition of AC3 may hold relevance in the phenomenon of escape from vasopressin-induced antidiuresis seen in the clinical syndrome of inappropriate antidiuresis, in which cyclic AMP activity and aquaporin-2 expression undergo marked decreases despite high levels of circulating vasopressin (48).

Our RT-PCR studies indicate that other AC isoforms aside from AC3 and -6 are expressed in the IMCD, namely AC2, -4, -5, -7, and -9. Further studies would be required to pinpoint the functional role of these isoforms.

We have identified at least two sites of calmodulin action in the regulation of AQP2 in the IMCD, viz. myosin light chain kinase (3) and AC3 (this study). Given the multiplicity of its actions in cells, calmodulin likely plays other physiologically significant roles in the IMCD. One such role is stimulation of phosphodiesterase activity, which has been demonstrated in prior studies (49, 50). The potential attenuation of the cAMP response via CaM-sensitive phosphodiesterase-1 has not been addressed in this study. Inclusion of the phosphodiesterase inhibitor isobutyl methylxanthine prior to measurement of cAMP precluded evaluation of CaM-sensitive phosphodiesterase activity.

In conclusion, we have demonstrated CaM-dependent cAMP accumulation in response to AVP in IMCD and have provided evidence for AC3 as the adenylyl cyclase isoform that is responsible for this activity. Calmodulin-stimulated adenylyl cyclase activity may play a critical role in the fine regulation of water and urea transport in the IMCD.

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