Vasopressin-induced nitric oxide production in rat inner medullary collecting duct is dependent on V$_2$ receptor activation of the phosphoinositide pathway

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O’Connor PM, Cowley AW Jr. Vasopressin-induced nitric oxide production in rat inner medullary collecting duct is dependent on V$_2$ receptor activation of the phosphoinositide pathway. Am J Physiol Renal Physiol 293: F526–F532, 2007. First published May 16, 2007; doi:10.1152/ajprenal.00052.2007.—We previously reported that arginine vasopressin (AVP) stimulates the production of nitric oxide (NO) in inner medullary collecting duct (IMCD) via activation of V$_2$ receptors (V$_2$R) and the mobilization of intracellular Ca$^{2+}$. The aim of this study was to determine the pathway(s) through which this response is mediated. IMCDs were dissected from male Sprague-Dawley rats and intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) and NO production were measured using a fluorescence imaging system. AVP (100 nmol/l) produced a rapid increase [Ca$^{2+}$]$_i$ of 381 ± 78 nmol/l that was followed by a significant increase of NO production (166 ± 61%). The specific nonpeptide V$_2$R antagonist OPC31260 (1 μM), but not the V$_1$R antagonist OPC21268 (1 μM), inhibited the increase in [Ca$^{2+}$]$_i$ (up to 91 ± 5%) and abolished the NO response to AVP. Both the phospholipase C inhibitor U73112 (3 μM) and the inositol (1,4,5) tri-phosphate 3 receptor blocker 2-APB (75 μM) reduced the peak [Ca$^{2+}$]$_i$ response to AVP (by 65 ± 9 and 59 ± 15%, respectively) and abolished the NO response. Although forskolin (100 μM; an activator of adenyl cyclase) elicited a moderate increase in [Ca$^{2+}$]$_i$, neither preincubation with the adenyl cyclase inhibitor PKA14-22 (100 μM) nor the protein kinase A (PKA) inhibitor thapsigargin. We conclude that AVP-induced NO production in IMCD is dependent on V$_2$R activation of the phosphoinositide pathway and the mobilization of Ca$^{2+}$ from both intracellular and extracellular pools.

SUSTAINED PHYSIOLOGICAL INCREASES in arginine vasopressin (AVP) result in only a transient reduction of medullary blood flow (MBF) (6), expansion of extracellular fluid volume (26), and elevation of arterial pressure (1, 6, 21, 26, 28). When renal medullary nitric oxide synthase (NOS) activity is reduced, however, even small elevations in circulating AVP produce sustained reductions of MBF and persistent hypertension (29). Medullary NO production may help prevent medullary ischemia and protect from AVP-induced hypertension when circulating levels of AVP are high. Nephron NOS activity is highest in inner medullary collecting duct segments (IMCD) (33) and the mRNA for V$_2$R is present only in tubules such as IMCD but not in the renal vasculature (19), indicating that renal medullary NO production in response to AVP may be primarily mediated by IMCD (18). There is evidence that administration of the V$_2$ receptor (V$_2$R)-selective peptide agonist dDAVP alone results in increased medullary NO production while administration of the selective V$_1$R agonist [Phe$_2$Ile$_3$Orn$_3$]-vasopressin does not (20). These observations indicate that AVP stimulates medullary NO production via activation of vasopressin V$_2$-like receptors.

We recently reported that AVP stimulates the production of NO in IMCD and that this is dependent on the mobilization of intracellular Ca$^{2+}$ (18). The classical pathway by which AVP stimulates Ca$^{2+}$ mobilization in most cell types is via V$_1$R activation of phospholipase C (PLC) and subsequent release of Ca$^{2+}$ from inositol (1,4,5) tri-phosphate (IP$_3$)-sensitive intracellular stores (10). IMCD, however, may not express V$_1$R protein (7) and the V$_2$R peptide agonist dDAVP is equipotent to AVP in eliciting calcium mobilization in IMCD (18) suggesting V$_2$R-mediated Ca$^{2+}$ mobilization in these cells. V$_2$R activation of adenyl cyclase and cAMP has been reported to increase intracellular Ca$^{2+}$ in IMCD, a response that appears to depend in part on influx of extracellular Ca$^{2+}$ secondary to activation of EPAC (exchange protein directly activated by cAMP) by cAMP and the opening of as yet unidentified membrane ion/Ca$^{2+}$ channels (36). A second pathway through which V$_2$R activation may mobilize Ca$^{2+}$ in IMCD has also been suggested by observations that when highly expressed in cell lines, V$_2$R are capable of coupling to PLC which could stimulate release of Ca$^{2+}$ from IP$_3$-sensitive intracellular stores (38). Furthermore, some evidence indicates that AVP may stimulate IP$_3$ hydrolysis by activation of the oxytocin or V$_2$R-like V1bR in IMCD (17, 30). Therefore, multiple signaling pathways may contribute to V$_2$R-mediated NO production in IMCD. However, to date the intracellular signaling cascades by which AVP stimulates NO production in IMCD remain unclear. The present study was designed to determine whether multiple signaling pathways capable of mobilizing Ca$^{2+}$ are active within IMCD and to determine the importance of each of these pathways toward AVP-induced NO production.

MATERIALS AND METHODS

Experimental animals. Studies used male Sprague-Dawley rats weighing 150–300 g (Harlan, Madison, WI) maintained ad libitum on water and a standard pellet diet (Purina Mills, St. Louis, MO) in the Animal Resource Center of the Medical College of Wisconsin. All protocols were approved by the institutional animal care and use committee.

Preparation of IMCD. Isolation of IMCDs was performed as described previously (18). Briefly, rats were anesthetized with pentobarbital sodium (50 mg/kg ip) and the left kidney was perfused with 10 ml of HBSS (Invitrogen, Grand Island, NY) with 20 mmol/l...
HEPES buffer adjusted to pH 7.40 (Sigma, St. Louis, MO) at 3 ml/min. The left kidney was then excised and cut sagitally for the removal of the inner medulla, which was microdissected under a Leica M3Z stereomicroscope to remove a single layer of collecting ducts. The thin layer of tissue containing IMCD was placed on a glass coverslip coated with the tissue adhesive Cell-tak (BD Biosciences, Bedford, MA) for fluorescence imaging. The experimental buffer was HBSS with 20 mmol/l HEPES and 1 mmol/l L-arginine (Sigma) adjusted to pH 7.40.

**Fluorescence detection.** Fluorescence measurements were made using a Nikon TE2000 inverted microscope with a ×60 water immersion (numerical aperture 1.2) objective lens. The signal was detected using a high-resolution digital camera (Photometrics Cascade 512B Roper Scientific, Tucson, AZ). Excitation was provided by a Sutter DG-4 175-W xenon arc lamp (Sutter Instruments, Novato, CA) that allowed high-speed excitation wavelength switching. For the experiments, coverslips were placed in an imaging chamber (maintained at 37°C) mounted on the stage of the inverted microscope that allowed the superfusion of the experimental buffer and buffer containing agonists/antagonists. Five to fifteen cells were selected within each IMCD to quantify changes in fluorescent intensity of Fura-2 AM and DAF-FM dyes using Metafluor imaging software (Universal Imaging, Downingtown, PA).

**Intracellular \( \text{Ca}^{2+} \) measurement in IMCD.** The IMCDs isolated on coverslips were incubated in 5 μmol/l fura-2 AM (Molecular Probes, Eugene, OR) for 60 min at room temperature and then washed to remove excess dye. Pluronic F127 (Molecular Probes) was used to dissolve the fura-2 AM dye to prevent dye compartmentalization upon loading. The coverslips were again incubated in the experimental buffer for 15 min before the experiments. Fura-2 fluorescent signal was stimulated by dual-wavelength excitation at 340 and 380 nm. A 510/40-nm band pass emission filter was used to collect fura-2 signals at 1-s intervals. Ratios between the fluorescence intensity stimulated by 340/380-nm excitation were calculated and the excitation intensity was adjusted on the DG-4 to minimize fura-2 fluorescence bleaching and to balance 340/380 excitation intensities.

**Intracellular \( \text{Ca}^{2+} \) concentration ([\( \text{Ca}^{2+} \)])** was calibrated from maximum and minimum fura-2 signals at the end of each experiment. Specifically, the tissue bath solution was exchanged to 5 μmol/l of the Ca2+ ionophore 4-bromo-A23187 (Molecular Probes) with 2.5 mmol/l of the NO donor DETA-NONOate (Cayman Chemical, Ann Arbor, MI) to act as a positive control and to ensure the DAF-2 dye was not saturated at the end of the experiment. NO measurement in IMCD. IMCDs were incubated on coverslips in 10 μmol/l of the NO-donor DAF-FM (Molecular Probes) for 60 min at room temperature and then washed and incubated for another 15 min in experimental buffer. DAF-2 was excited at 480 nm and collected through a 535/40-nm band pass emission filter at 3-s intervals. At the end of the experimental protocol, the superfusion solution was exchanged with 20 μmol/l of the NO donor DETA-NONOate (Cayman Chemical, Ann Arbor, MI) to act as a positive control and to ensure the DAF-2 dye was not saturated at the end of the experiment.

**Solutions.** OPC21268 and OPC31260 were obtained from Otsuka (Otsuka Pharmaceutical, Tokyo, Japan). AVP, 2’-5’-dideoxycytidine, PKA14-22, U73122, 2-aminoethyl-diphenylborinate (2-APB), forskolin, ryanodine, thapsigargin, EGTA, and L-arginine were obtained from Sigma. 2’-5’-Dideoxyadenosine, dibutyryl-cAMP, PKA14-22, U73122, 2-APB, ryanodine, thapsigargin, and DAF-2FM were dissolved in DMSO (Sigma) before being added to experimental buffer.

**Data analysis.** Data are expressed as means ± SE. Peak intracellular \( \text{Ca}^{2+} \) responses to agonists (peak \( [\text{Ca}^{2+}] \)) were calculated as equation 1

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[\text{Ca}^{2+}] = \frac{K_d}{R - R_{\text{max}}} \left( R_{\text{max}} - R \right) F \cdots \cdots (1)
\]

where \( K_d \) is the dissociation constant of fura-2, \( R \) is the actual ratio of intensities at excitation wavelengths 340 and 380 nm, \( R_{\text{max}} \) and \( R_{\text{min}} \) are the maximal and minimal fura-2 ratios in the presence and absence of \( \text{Ca}^{2+} \), and \( F \) is the ratio of fura-2 intensities at 380 nm in the presence and absence of \( \text{Ca}^{2+} \). \( K_d \) of fura-2 at 37°C was 224 nM as reported (11).

**RESULTS**

**Measurement of intracellular \( [\text{Ca}^{2+}] \) in IMCD in response to AVP.** Baseline \( [\text{Ca}^{2+}] \) in IMCD averaged 85 ± 24 nM before stimulation with AVP. During the period before AVP stimulation, rhythmic fluctuations in \( [\text{Ca}^{2+}] \) were commonly observed (Fig. 1). Stimulation with 100 nmol/l AVP produced a statistically significant peak increase in \( [\text{Ca}^{2+}] \) of 381 ± 78 nmol/l (n = 11) − 25 s after stimulation. Following this peak increase, \( [\text{Ca}^{2+}] \) returned to baseline levels over the next 150–200 s.
Effect of the specific nonpeptide V1 receptor antagonist OPC31260 and V2 receptor antagonist OPC21268 on peak [Ca^{2+}]_{i} in response to 100 nM AVP. Preincubation of IMCD with the specific nonpeptide V2R antagonist OPC31260 [pK_i 140 nM (35); 300 and 1,000 nmol/l] significantly inhibited peak [Ca^{2+}]_{i} in response to 100 nmol/l AVP (−54 ± 9 and −91 ± 5%, respectively; n = 5) in a dose-dependent manner (Fig. 2). In contrast, incubation of IMCD with the specific nonpeptide V1 receptor antagonist OPC21268 [pK_i 140 nM (35); 300 and 1,000 nmol/l] had no effect on peak [Ca^{2+}]_{i} responses to AVP (n = 5; Fig. 2).

Effect of inhibitors/activators of known V1R/V2R receptor signaling pathways on peak AVP response. To determine the AVP-induced signaling pathways involved in Ca^{2+} mobilization in IMCD (see Fig. 3), a number of inhibitors/activators of known V1R and V2R intracellular signaling pathways were tested.

**Known V1R signaling pathway inhibitors.** Preincubation of inner medullary tissue strips with the PLC inhibitor U73112 (3 mM) significantly inhibited peak [Ca^{2+}]_{i} responses to AVP (100 nmol/l) by 65 ± 6% compared with AVP alone (n = 5; Fig. 3A). Blockade of the inositol 1,4,5-triphosphate (IP3) receptor with 2-APB (75 mM) also reduced AVP (100 nmol/l)-stimulated peak [Ca^{2+}]_{i}, responses by 59 ± 15% (n = 5) compared with AVP alone (Fig. 3A).

**Known V2R signaling pathway activators/inhibitors.** The addition of the membrane-permeable cAMP analog dibutyryl-cAMP (100 mM) did not increase [Ca^{2+}]_{i}, responses to AVP (100 nmol/l) (n = 5; Fig. 3A). Forskolin (30 μM; an activator of adenylyl cyclase activity) resulted in only a small increase in [Ca^{2+}]_{i}, equivalent to 14 ± 3% of that of the response to 100 nmol/l AVP alone (n = 5; Fig. 3A). Inhibition of adenylyl cyclase with 2’-5’-dideoxyadenosine (50 μM) had no effect on either baseline [Ca^{2+}]_{i} or the peak [Ca^{2+}]_{i} response to 100 nmol/l AVP (Fig. 3A). Preincubation of IMCD with PKA_{14-22}, an inhibitor of protein kinase A, did not significantly affect peak [Ca^{2+}]_{i} responses to 100 nmol/l AVP (Fig. 3). Incubation of IMCD tissue strips in Ca^{2+}-free media (n = 5) or with ryanodine (100 mM; n = 5), which inhibits sarcoplasmic Ca^{2+} release from ryanodine-sensitive stores, reduced peak [Ca^{2+}]_{i}, responses to 100 nmol/l AVP (by 72 ± 8 and 42 ± 16%, respectively; Fig. 3B). Depletion of intracellular Ca^{2+} stores with the Ca^{2+}-ATPase inhibitor thapsigargin (5 mM; n = 5) completely abolished the [Ca^{2+}]_{i} responses to AVP (Fig. 3B).

Production of NO by IMCD in response to AVP. NO production by IMCD was measured using the intensity of

![Graph](image-url)

**Fig. 2.** Effect of the specific nonpeptide V2 receptor antagonist OPC31260 and V1 receptor antagonist OPC21268 on peak [Ca^{2+}]_{i} in response to 100 nM AVP. Data are means ± SE. Y-axis, peak response to 100 nmol/l AVP [% of peak response to 100 nmol/l AVP in the absence of inhibitors (381 ± 78 nmol/l)]; x-axis, dose of inhibitor (nmol/l). Horizontal dotted line represents 100% response; open bars represent OPC21268 (pK_i 140 nM); filled bars represent OPC31260 (pK_i 6.36 nM). *Significant difference from peak response to 100 nmol/l AVP (P < 0.05).
with the IP3 receptor blocker 2-APB (75 mM) also completely abolished the NO response to 100 nmol/l AVP compared with vehicle (HBBS-H-L-Arg; the NO response to 100 nmol/l AVP). There was no significant difference in the basal rate of NO production or baseline fluorescent intensity measured before administration of AVP between untreated IMCD and IMCD incubated with inhibitors of the phosphoinositide pathway and V2R. Data are means ± SE. Y-axis, response to agent (% of control period); x-axis, agent. *Significant difference from vehicle response (P < 0.05). OPC31260, V2R receptor inhibitor; U73122, inhibitor of phospholipase C; 2-APB, inhibitor of IP3R activation.

Fig. 4. Changes in NO production (DAF-FM flourescents intensity) in IMCD in response to AVP alone or in the presence of inhibitors of the phosphoinositide pathway and V2R. Data are means ± SE. Y-axis, response to agent (% of control period); x-axis, agent. *Significant difference from vehicle response (P < 0.05). OPC31260, V2R receptor inhibitor; U73122, inhibitor of phospholipase C; 2-APB, inhibitor of IP3R activation.

DAF-FM fluorescence. Exchange of the control buffer solution with buffer solution containing 100 nmol/l AVP resulted in an increase in NO production in IMCD of 166 ± 6% [indicated by an increase in the slope of DAF-2 fluorescence intensity/time (equation 3); Fig. 4; n = 6] compared with baseline. The increase in NO production in IMCD following addition of 100 nmol/l AVP was sustained for the length of recording (400 s; Fig. 1). No change in the rate of NO production by IMCD was observed following exchange of the buffer solution alone (vehicle) or with buffer containing 1 nmol/l AVP (n = 6; Fig. 4). Preincubation of tissue strips with the V2R antagonist OPC31260 (1,000 nmol/l) had no effect on baseline NO production but inhibited the NO response to AVP (100 nmol/l; Fig. 4).

Effect of inhibitors of the phosphoinositide signaling pathway on NO responses to AVP in IMCD. To determine whether the phosphoinositide pathway may be involved in AVP-stimulated production of NO by IMCD, tubules were incubated with inhibitors of PLC and IP3 for 400 s before administration of 100 nmol/l AVP. There was no significant difference in the basal rate of NO production or baseline fluorescent intensity measured before administration of AVP between untreated IMCD and IMCD incubated with inhibitors of the phosphoinositide signaling pathway. Preincubation of IMCD with the PLC inhibitor U73122 (3 μM), however, completely abolished the NO response to 100 nmol/l AVP (n = 6; Fig. 4). Similarly, incubation of IMCD with the IP3 receptor blocker 2-APB (75 mM) also completely abolished the NO response to 100 nmol/l AVP (n = 6; Fig. 4).

DISCUSSION

The primary findings of this study are that multiple pathways appear to be capable of stimulating Ca2+ mobilization in IMCD following V2R activation, but activation of the phosphoinositide pathway is specifically required for AVP-stimulated NO production (Fig. 5).

Role of the V2R in AVP-stimulated NO production. We used in vivo microdialysis techniques to determine that administration of the V2R-selective peptide agonist dDAVP alone stimulates an increased medullary NO production in the rat kidney (20). Since the mRNA for V2R is present only in tubules such as IMCD but not in the renal vasculature (19), and NOS activity is also highest in IMCD (33), we proposed that the increased rate of renal medullary NO production observed in response to AVP is mediated primarily by IMCD (18). In a subsequent in vitro study using isolated IMCD, we were able to demonstrate that NO production increased in these tubular segments in response to activation of V2R and that this response was dependent on the mobilization of intracellular Ca2+ (18). One novel observation of the present study is that inhibition of V2R inhibited both Ca2+ mobilization and NO production in IMCD (Figs. 2 and 4).

Evidence that the phosphoinositide pathway contributes to V2R activation of Ca2+ mobilization in IMCD. It is well-known that AVP activates adenylyl cyclase via V2Rs (5, 10, 31) and V2R activation of adenylyl cyclase has been reported to increase intracellular Ca2+ in IMCD (7), a process that has recently been reported to be dependent on cAMP-mediated activation of Epac (36). In addition to activation of adenylyl cyclase, when expressed in high concentration in cell lines, V2R have also been demonstrated to be capable of coupling to PLC (38). In the current study, the specific PLC inhibitor U73122 (13, 16, 22, 37) and the IP3R blocker 2-APB (15, 25) were used to determine the possible contribution of phosphoinositide signaling to the AVP-induced intracellular calcium increase [Ca2+]i then activates NOS [presumably via calmodulin (24)] stimulating medullary NO production observed in vivo microdialysis techniques to determine that administration of the V2R-selective peptide agonist dDAVP alone stimulates an increased medullary NO production in the rat kidney (20). Since the mRNA for V2R is present only in tubules such as IMCD but not in the renal vasculature (19), and NOS activity is also highest in IMCD (33), we proposed that the increased rate of renal medullary NO production observed in response to AVP is mediated primarily by IMCD (18). In a subsequent in vitro study using isolated IMCD, we were able to demonstrate that NO production increased in these tubular segments in response to activation of V2R and that this response was dependent on the mobilization of intracellular Ca2+ (18). One novel observation of the present study is that inhibition of V2R inhibited both Ca2+ mobilization and NO production in IMCD (Figs. 2 and 4).

Fig. 5. Diagrammatic representation of proposed pathway whereby AVP stimulates NO release in IMCD. AVP binds to the V2R activating both adenylyl cyclase (AC) and phospholipase C (PLC). PLC hydrolyzes inositol triphosphate (IP3) stimulating release of Ca2+ from IP3-sensitive sacroplasmic reticulum (SR) stores. Ca2+ influx from as yet unidentified membrane Ca2+ channels and from ryanodine (RyR)-sensitive stores also appears to contribute to the AVP-induced intracellular calcium increase [Ca2+]i. Increased [Ca2+]i then activates NOS [presumably via calmodulin (24)] stimulating medullary NO production.

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inositide pathway signaling to V2R-mediated Ca\(^{2+}\) mobilization and NO production in IMCD. While previous studies suggested the possibility that multiple signaling pathways may be linked to V2R in IMCD (3), there is no direct evidence for this (4, 9). The current study provides the first direct evidence that activation of the phosphoinositide pathway participates in V2R-mediated Ca\(^{2+}\) mobilization in these nephron segments.

Measuring IP\(_3\) in cultured rat IMCD cells, Teitelbaum (30) was unable to demonstrate any evidence that AVP can elicit IP\(_3\) production in IMCD via activation of V2R. The current study is the first to use the selective PLC inhibitor U73122 to determine the role of the phosphoinositide pathway in AVP-stimulated Ca\(^{2+}\) mobilization in freshly isolated IMCD. AVP-stimulated Ca\(^{2+}\) mobilization in IMCD was inhibited by both compounds indicating that under the conditions used in the current study, phosphoinositide pathway activation is a major contributor to V2R-induced Ca\(^{2+}\) mobilization in IMCD. The differences in our findings compared with others could involve the dose of AVP used, the use of freshly isolated vs. cultured cells, the sensitivity of the IP\(_3\) assay, or specificity of receptor antagonists used.

\(\text{Ca}^{2+}\) pools contributing to AVP-stimulated Ca\(^{2+}\) mobilization in IMCD. Release of Ca\(^{2+}\) from intracellular stores and influx of Ca\(^{2+}\) across the basolateral membrane both appear to contribute to the increase in \([\text{Ca}^{2+}]_i\), observed in IMCD in response to stimulation by AVP. Ca\(^{2+}\) responses to AVP could be completely abolished by preincubation of the tissue with the Ca\(^{2+}\)-ATPase inhibitor thapsigargin indicating that release of Ca\(^{2+}\) from intracellular stores was required for AVP-induced Ca\(^{2+}\) mobilization (Fig. 3B). Interestingly, however, in Ca\(^{2+}\)-free media the peak \([\text{Ca}^{2+}]_i\), response to AVP was reduced by 72 \pm 8% suggesting influx of extracellular Ca\(^{2+}\) also contributed to the response to AVP (Fig. 3B). These results are in agreement with those of Champigneulle et al. (3) who demonstrated an apparent increase in permeability of the basolateral membrane of rat IMCD to extracellular Ca\(^{2+}\) in response to 10 nM AVP.

Role of cAMP in V2R-mediated Ca\(^{2+}\) mobilization and NO production in IMCD. Our data indicate that activation of adenyl cyclase is unlikely to be the primary pathway by which Ca\(^{2+}\) is mobilized in response to activation of the V2R in IMCD (Fig. 3A). An increase in \([\text{Ca}^{2+}]_i\), was observed, however, following addition of a selective activator of adenyl cyclase (forskolin) supporting previous observations that cAMP is capable of eliciting Ca\(^{2+}\) mobilization in IMCD. However, peak \([\text{Ca}^{2+}]_i\) was only 14 \pm 3% of that elicited by AVP alone (Fig. 3A).

Our finding that cAMP is unlikely to be responsible for the increase in \([\text{Ca}^{2+}]_i\), observed in IMCD following stimulation of AVP is in agreement with several published reports (3, 12, 27) with a notable exception. Chou et al. (5) reported that addition of 100 \(\mu\)M of the cAMP analog dibutyryl-cAMP elicited Ca\(^{2+}\) mobilization in freshly isolated IMCD and that this response was proportional to that induced by AVP alone. They also reported that responses to AVP could be completely abolished by preincubation with ryanodine and that responses to AVP were unaffected by extracellular Ca\(^{2+}\) concentration (5), findings not supported by the results of the current study. An explanation for our contrasting results may be related to the dose of AVP used to elicit Ca\(^{2+}\) mobilization in IMCD, 100 nM AVP being used to elicit maximal Ca\(^{2+}\) mobilization in IMCD in the current study, whereas concentrations between 0.1 and 1 nM were used by Chou et al. (5). PLC activation and Ca\(^{2+}\) mobilization may inhibit intracellular cAMP production in IMCD (2). Since our results demonstrate that stimulation of V2-like receptors can activate at least 2-s messenger signaling pathways involved in calcium mobilization in IMCD, it is possible that the phosphoinositide pathway was activated in the current study and that this inhibited cAMP-mediated Ca\(^{2+}\) mobilization normally observed at lower levels of AVP stimulation. In support of this concept, Champigneulle et al. (3) demonstrated that the magnitude of the Ca\(^{2+}\) response of IMCD to AVP is dose dependent with maximal stimulation occurring at agonist concentrations of \(\sim 5\) nM.

V2R-mediated NO production in IMCD. While in preliminary studies we were able to observe increased IMCD \([\text{Ca}^{2+}]_i\), in response to 1 nM AVP, we chose a dose of 100 nM AVP as we found that maximal stimulation of IMCD Ca\(^{2+}\) mobilization was required to stimulate reproduceable and detectable increases in the rate of increase of DAF-FM fluorescent intensity in response to AVP. It remains to be determined whether the pathways that act to stimulate NO production in IMCD in vitro underlie the in vivo production of NO in response to AVP. It is clear, however, that infusion of AVP at nonpressor doses, which produced plasma AVP levels within the physiological range, stimulate NO production in the renal medulla of conscious rats (6, 8, 29).

While our data indicate that activation of phosphoinositide signaling is required to stimulate detectable increases in NO production in IMCD in response to V2R activation, we cannot exclude the possibility that smaller increases in peak \([\text{Ca}^{2+}]_i\), stimulated by other signal transduction pathways such as activation of cAMP are also capable of stimulating NO production in IMCD. Although no detectable increase in DAF-FM was observed in response to maximal stimulation by AVP during blockade of phosphoinositide signaling, it is possible a small increase in NO production occurred that was below the detection limits of the fluorescent indicator used. However, we believe that this is unlikely since DAF-FM is a highly sensitive indicator of cellular NO production and is capable of detecting NO levels down to 3 nM (14). Additionally, large increases in \([\text{Ca}^{2+}]_i\), are required to fully activate calmodulin-dependent NOS, so small Ca\(^{2+}\) transients elicited by nonphosphoinositide pathways may not have been sufficient to increase IMCD NO production significantly (24).

Possible role of non-V2R-mediated phosphoinositide signaling toward AVP-stimulated NO production in IMCD. Both oxytocin receptors and V1bR have been reported to activate the PLC/IP\(_3\) cascade and mobilize Ca\(^{2+}\) in IMCD in response to stimulation with AVP (23, 30), so it is possible that activation of one or both of these receptors could mediate NO production in IMCD in response to stimulation by AVP rather than activation of V2R per se. However, the specific nonpeptide V2 antagonist OPC31260 has both a poor affinity for V1bR (\(pK_a < 10000,000\) nM)}34) and is a poor antagonist of the oxytocin receptor (32). Thus the observation that OPC31260 inhibited peak \([\text{Ca}^{2+}]_i\), in IMCD in the current study is not consistent with significant V1bR or oxytocin receptor activation of phosphoinositide signaling in IMCD. While the exact nature of the receptor remains unclear, the results of the current study indicate that the specific nonpeptide V2R antagonist OPC31260 was able to inhibit more than 90% of the increase
in $[Ca^{2+}]_i$, observed following administration of AVP. These observations are consistent with previous reports obtained using peptide agonists/antagonist indicating V2R or some as yet unidentified subtype of the V2R underlies AVP-induced $Ca^{2+}$ mobilization in IMCD (3, 17, 18).

**Physiological significance.** In conclusion, the results of the current study provide the first direct evidence that V2R-like receptors are capable of activating phosphoinositide second messenger signaling pathways in IMCD to mobilize $Ca^{2+}$ and that activation of this pathway is required to stimulate NO production in isolated IMCD. We speculate that this signaling pathway may provide a mechanism by which the vasoconstrictive effects of AVP on the medullary circulation may be offset by paracrine signaling of NO between IMCD and the medullary vasa recta, thereby protecting the renal medulla from reductions in MBF and preventing the development of hypertension when circulating levels of AVP are high. Further studies will be required to fully elucidate the biological significance of this pathway in vivo.

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