Mechanisms of vasopressin-induced intracellular Ca\(^{2+}\) oscillations in rat inner medullary collecting duct

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Yip KP, Sham JS. Mechanisms of vasopressin-induced intracellular Ca\(^{2+}\) oscillations in rat inner medullary collecting duct. Am J Physiol Renal Physiol 300: F540–F548, 2011. First published December 8, 2010; doi:10.1152/ajprenal.00544.2009.—Arginine vasopressin (AVP) causes increase in intracellular Ca\(^{2+}\) concentration with an oscillatory pattern. Ca\(^{2+}\) mobilization is required for AVP-stimulated apical exocytosis in inner medullary collecting duct (IMCD). The mechanistic basis of these Ca\(^{2+}\) oscillations was investigated by confocal fluorescence microscopy and flash photolysis of caged molecules in perfused IMCD. Photorelease of caged cyclic ADP-ribose (cADPR) both mimicked the AVP-induced Ca\(^{2+}\) oscillations. Preincubation of IMCD with 100 \(\mu\)M 8-bromo-cADPR (a competitive inhibitor of cADPR) delayed the onset and attenuated the magnitude of AVP-induced Ca\(^{2+}\) oscillations. These observations indicate that the cADPR/RyR pathway is capable of supporting Ca\(^{2+}\) oscillations and endogenous cADPR plays a major role in the AVP-induced Ca\(^{2+}\) oscillations in IMCD. In contrast, photorelease of caged inositol 1,4,5-trisphosphate (IP\(_3\)) induced Ca\(^{2+}\) release but did not maintain sustained Ca\(^{2+}\) oscillations. Removal of extracellular Ca\(^{2+}\) halted ongoing AVP-mediated Ca\(^{2+}\)-oscillation, suggesting that it requires extracellular Ca\(^{2+}\) entry. AVP-induced Ca\(^{2+}\) oscillation was unaffected by nifedipine. Intracellular Ca\(^{2+}\) store depletion induced by 20 \(\mu\)M thapsigargin in Ca\(^{2+}\)-free medium triggered store-operated Ca\(^{2+}\) entry (SOCE) in IMCD, which was attenuated by 1 \(\mu\)M GdCl\(_3\) and 50 \(\mu\)M SKF-96365. After incubation of IMCD with 1 nM AVP in Ca\(^{2+}\)-free medium, application of extracellular Ca\(^{2+}\) also triggered Ca\(^{2+}\) influx, which was sensitive to GdCl\(_3\) and SKF-96365. In summary, our observations are consistent with the notion that AVP-induced Ca\(^{2+}\) oscillations in IMCD are mediated by the interplay of Ca\(^{2+}\) release from RyRs and a Ca\(^{2+}\) influx mechanism involving nonselective cation channels that resembles SOCE.

caged cyclic ADP-ribose; caged inositol 1,4,5-trisphosphate; store-operated calcium entry; laser scanning confocal microscopy

OCCILLATION of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) serves as the signal transduction mechanism for many physiological stimuli in both excitable and nonexcitable cells, with information being encoded in both the frequency and the amplitude of the Ca\(^{2+}\) signal (2). There is emerging evidence that Ca\(^{2+}\) oscillation in renal epithelium is an integral part of the signaling transduction process for regulation of water reabsorption (8, 45). A physiological dose of AVP has been shown to trigger intracellular Ca\(^{2+}\) mobilization and Ca\(^{2+}\) oscillations in inner medullary collecting duct (IMCD) (32, 45), and the Ca\(^{2+}\) mobilization is necessary for AVP-stimulated apical exocytosis and osmotic water permeability (8, 45). AVP also induced Ca\(^{2+}\) oscillations in mouse thick ascending limb, which was associated with AVP-stimulated secretion of nucleotides (30). Ca\(^{2+}\) oscillations were also reported recently in tubular epithelial cells near the macula densa (23). They are modulated by luminal NaCl concentration and luminal flow, and are possibly related to juxtaglomerular signaling. Furthermore, angiotensin II-triggered Ca\(^{2+}\) oscillations have been recorded in descending vasa recta pericytes (11, 51). It has been suggested that Ca\(^{2+}\) oscillations in pericytes are due to repetitive cycles of ryanodine-sensitive sarcoplasmic reticulum (SR) Ca\(^{2+}\) release and SKF-96365-sensitive refilling of SR Ca\(^{2+}\) stores.

Our previous studies (8, 45) have provided the initial characterization of AVP-induced Ca\(^{2+}\) oscillation in IMCD. With the use of confocal fluorescence microscopy to monitor [Ca\(^{2+}\)], and apical exocytosis in individual cells, AVP was found to trigger a rapid increase in [Ca\(^{2+}\)], followed by sustained repetitive Ca\(^{2+}\) oscillations. These Ca\(^{2+}\) responses were mediated through a cAMP-dependent mechanism and mimicked by an agonist of exchange protein directly activated by cAMP (Epac) (46). Removal of extracellular Ca\(^{2+}\) did not prevent the initial rise of [Ca\(^{2+}\)] induced by AVP but abolished the sustained Ca\(^{2+}\) oscillation (45). In the absence of extracellular Ca\(^{2+}\), ryanodine completely obliterated AVP-induced Ca\(^{2+}\) mobilization and AVP-stimulated increase of osmotic water permeability (8). These results suggested that both extracellular Ca\(^{2+}\) influx and intracellular Ca\(^{2+}\) release contribute to the AVP-induced Ca\(^{2+}\) response.

Ca\(^{2+}\) oscillation can be driven by influx of extracellular Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) from intracellular Ca\(^{2+}\) stores, or both (33). Ryanodine receptors (RyRs) and inositol 1,4,5-trisphosphate (IP\(_3\)) receptors (IP\(_3\)Rs) are expressed in endoplasmic reticulum (ER) of IMCD (8, 19, 44). Both receptors are capable of supporting CICR and Ca\(^{2+}\) oscillations (13, 14). Ca\(^{2+}\) release through RyRs or IP\(_3\)Rs may deplete intracellular Ca\(^{2+}\) stores and trigger store-operated Ca\(^{2+}\) entry (SOCE), which can serve as a mechanism to replenish the depleted Ca\(^{2+}\) stores and sustain Ca\(^{2+}\) oscillations (33, 34). To capture the dynamics of Ca\(^{2+}\) release from RyRs and IP\(_3\)Rs from the ER of IMCD, flash photolysis of caged cyclic ADP-ribose (cADPR) and caged IP\(_3\) was used in the present study to directly activate RyRs and IP\(_3\)Rs, respectively. The specific contributions of RyRs, IP\(_3\)Rs, and SOCE in the AVP-induced Ca\(^{2+}\) oscillations were evaluated. Our results indicate that AVP-induced Ca\(^{2+}\) oscillation in IMCD is mediated in part by the endogenous production of cADPR and is maintained through the interplay of Ca\(^{2+}\) release from RyRs and Ca\(^{2+}\) influx through SOCE.
Ca$^{2+}$ OSCILLATIONS IN COLLECTING DUCT

MATERIALS AND METHODS

Isolation and perfusion of single IMCD segment. Experiments were carried out in accordance with guidelines for the care and use of research animals. All experiments were performed under protocols approved by the Institutional Animal Care and Use Committee at the University of South Florida, in accordance with Public Health Service Policy on Humane Care and Use of Laboratory Animals. Experiments were conducted in IMCD isolated from male Sprague-Dawley rats (60–100 g body wt; Harlan). Rats were treated with furosemide (5 mg) intraperitoneally for 30 min and then killed with an overdose of 5% halothane through a Fluotec Mark-3 vaporizer (Ohmeda). Furosemide treatment was used to wash out the medullary osmotic gradient, in order to minimize osmotic shock when the medulla is placed in isotonic dissection solution (5). The kidneys were rapidly removed through a midline abdominal incision and placed in an ice-cold dissecting solution. The dissection solution consisted of (in mM) 120 NaCl, 25 NaHCO3, 2 K2HPO4, 1.2 MgSO4, 2 CaCl2, 5.5 glucose, and 5 sodium acetate. Terminal IMCD segments were dissected from the inner half of the inner medulla (5). The isolated piece of IMCD was then transferred to a temperature-controlled perfusion chamber (Vestavia) mounted on a Leica DMI6000 inverted microscope, which was coupled to a Leica TCS SP5 confocal scanning unit. The IMCD was then cannulated and perfused with glass microcapillaries by the method developed by Burg (3). Luminal and bath perfusates were identical to the dissecting solution. All solutions were gassed with 95% O2 and 5% CO2 before use, and pH was adjusted to 7.4. For Ca$^{2+}$-free perfusate, CaCl2 was replaced by 2 mM EGTA. For the sulfate- and phosphate-free solution, K2HPO4 and MgSO4 were replaced by equimolar KCl and MgCl2, respectively.

Measurement of intracellular calcium. The changes of [Ca$^{2+}$]i in IMCD cells induced by AVP and other pharmacological agents were determined from confocal fluorescence images of the perfused tubules as described previously (8, 45). In brief, 5 μM fluo-4 AM (Molecular Probes) was loaded into the IMCD from the peritubular solution at room temperature for 30 min. The tubule was then washed and incubated at 37°C for another 30 min for deesterification before measurements were begun. Confocal fluorescence images were acquired from the lower surface of the perfused IMCD with the 488-nm laser line. All images were collected with a Leica ×63 plan-apochromat objective (numerical aperture 1.2, water immersion). Emission was collected with a spectral window of 495–540 nm at 0.5 or 1 Hz and stored digitally. The spatial and temporal variations of [Ca$^{2+}$]i in individual IMCD cells were measured from the stored images with Leica Application Suite Advanced Fluorescence software.

Flash photolysis of caged compounds in IMCD. Cell-permeant caged Ca$^{2+}$ (DMNP-EDTA-AM, 200 μM), caged cAMP (DMNB-caged cAMP, 200 μM), and caged IP3 [β-2,3-O-isopropyliden-6-O-(2-nitro-4,5-dimethoxy)benzyl-myophosvitin 1,4,5-trisphosphate-hexakis(propionoxy)methyl]ester (IP3/PM), 1 μM] were dissolved in DMSO and loaded into IMCD together with fluo-4 AM for 30 min. Caged cADPR (NPE-caged cADPR, 40 μM) is cell impermeant. It was loaded into IMCD with a reversible permeabilization procedure (12, 40). In brief, the cannulated IMCD was washed with regular IMCD perfusate at 37°C for 15 min, and then the peritubular permease was switched to a Ca$^{2+}$-free permeabilization solution containing 40 μM caged cADPR for 2 min. The permeabilization solution was then removed, and IMCD was incubated at 37°C with regular IMCD perfusate for 60 min for membrane recovery. Fluo-4 AM was then loaded into IMCD after the recovery period. Permeabilization solution contained (in mM) 137 NaCl, 3 KCl, 5 glucose, and 20 piperoxan-N,N’-bis(2-ethanesulfonic acid) (PIPES), with 1 mg/ml bovine serum albumin and 0.4 IU/ml streptolysin-O. This permeabilization procedure did not affect AVP-induced Ca$^{2+}$ oscillation in IMCD cells (see Fig. 5B), indicating that it did not compromise this signaling process.

Flash photolysis of the caged compound was induced by an UV nitrogen-pulse laser (Laser Science, 337 nm, 4 ns/pulse, 300 μJ/pulse) coupled to a quartz optical fiber of 200-μm diameter, which was mounted on a micromanipulator. To visualize and to align the laser spot on the IMCD before UV flash photolysis, the UV laser pulses were directed through a tunable-dye control module that converted UV laser pulses into visible laser pulses centered at 640 nm (48). The firing frequency (30 Hz) and number of laser pulses in each burst were controlled with an analog-to-digital board (Data Translation DT-2801A) driven by a computer. The duration of photorelease was controlled by the number of laser pulses as described previously (48). The caging moiety in DMNB-caged cAMP and caged IP3 has the maximum of reabsorption at 370 nm, while that of NPE-caged cADPR is at 270 nm. UV laser at 337 nm is more efficient to uncage cAMP and IP3 than cADPR. Thirty and sixty pulses of UV laser were used to uncage cAMP and cADPR, respectively.

Flash photolysis of caged signaling molecules allowed rapid manipulation of the intracellular environment with minimal disturbance in confocal image acquisition. The use of consecutive short UV laser pulses (300 μJ/4 ns) to induce flash photolysis conferred more control to minimize the potential tissue damage due to UV radiation than the use of a conventional flash lamp. A single flash from a xenon flash lamp has energy on the order of 50–100 nJ/pulse and lasts for a few microseconds.

Chemicals. AVP was purchased from Bachem. SKF-93635 and xestospongin C were purchased from Calbiochem. DMNP-EDTA-AM, DMNB-caged cAMP, NPE-caged cADPR, and fluo-4 AM were purchased from Molecular Probes. IP3/PM was purchased from SiChem. Thapsigargin and streptolysin-O were from Sigma.

Data analysis. Time series of fluo-4 emission variations in individual IMCD cells were sampled at 0.25 or 0.5 Hz for spectral analysis. Each time series was normalized with respect to the baseline before AVP stimulation or photolysis and was subjected to linear trend removal. Two hundred fifty-six data points were used to calculate the power spectrum with an algorithm based on fast Fourier transform (47). Results are reported as means ± SE. Statistical significance was calculated by using Student’s t-tests for paired or unpaired data. Only one IMCD was studied in each rat.

RESULTS

Flash photolysis of caged Ca$^{2+}$ and cAMP induced Ca$^{2+}$ mobilization in IMCD. The efficacy of the UV pulsed laser system and the sensitivity of the detection system were first evaluated by using a photosensitive calcium chelator in IMCD. In IMCD loaded with DMNP-EDTA and fluo-4, a single UV laser pulse (4 ns) was sufficient to induce a detectable increase in fluo-4 emission (Fig. 1). A graded accumulative increase of fluo-4 emission was observed when the number of laser pulses was increased in each successive laser burst. The mean normalized fluo-4 emission after one, two, and three UV laser pulses was 1.12 ± 0.05, 1.32 ± 0.08, and 1.57 ± 0.06 (29 cells/2 tubules), respectively. These observations indicated that a single UV laser pulse provides sufficient energy to initiate photolysis of in perfused IMCD, and that the extent of photolysis can be controlled by the number of laser pulses used.

We have shown previously (8, 45) that AVP-induced Ca$^{2+}$ oscillations are mediated by cAMP. To examine whether cAMP-mediated Ca$^{2+}$ oscillation is intact in IMCD after exposure to the UV laser illumination, DMNP-caged cAMP was introduced into perfused IMCD. A burst of 30 UV laser pulses delivered over 1 s triggered a rapid increase in cytosolic Ca$^{2+}$, which was followed by repetitive [Ca$^{2+}$]i oscillations (Fig. 2A). The time delay between the uncaging of cAMP and the onset of rise in cytosolic [Ca$^{2+}$], ranged from 4 to 10 s. The mean normalized fluo-4 emission of the initial spike was 1.49 ± 0.06 (49 cells/5 tubules). The corresponding mean power spectrum

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had multiple peaks (see Fig. 4B). This means that the oscillatory frequency is time varying. Most of the power of oscillation was found in the frequencies below 0.1 Hz. A log scale was used to enhance the resolution of oscillatory frequencies in the lower frequency range. Each of these frequencies was associated with a different power level. A similar pattern was found in oscillations triggered by AVP (45). These observations confirmed that cAMP alone is sufficient to induce Ca\textsuperscript{2+} oscillations.

Fig. 1. Flash photolysis of Ca\textsuperscript{2+} chelator in inner medullary collecting duct (IMCD). A: mean normalized time course of changes in fluo-4 emission from intact IMCD cells induced by photolysis of DMNP-EDTA. Photorelease of Ca\textsuperscript{2+} was triggered by UV laser pulse. Each arrow indicates a burst of laser pulses; 1, 2, and 3 pulses of UV laser were applied sequentially. Dashed lines are SE (29 cells/2 tubules). B: 3 consecutive images (sampling at 1 Hz) showing the changes of fluo-4 emission induced by photolysis of DMNP-EDTA with 2 UV laser pulses before (a), during (b), and after (c) photolysis.

Fig. 2. Flash photolysis of caged cAMP and caged cyclic ADP-ribose (cADPR) in IMCD. Mean normalized time courses of changes in fluo-4 emission from intact IMCD cells induced by photolysis of caged cAMP (49 cells/5 tubules; A) and caged cADPR (26 cells/3 tubules; B) are shown. A burst of 30 and 60 UV laser pulses (at 30 Hz) were used for uncaging cAMP and cADPR, respectively. Insets: time courses collected from 4 IMCD cells of the same tubule (red, blue, green, and black). Arrow indicates the laser burst. Filled square indicates that mean value is significantly different from the prephotolysis baseline ($P < 0.05$). Dashed lines are SE.
lation in IMCD cells, and UV flash photolysis did not compromise the ability of IMCD cells in generating Ca\(^{2+}\) response.

Effects of photoreleased cyclic ADP-ribose and IP\(_3\) in IMCD. We have shown previously (8, 45) that AVP-induced Ca\(^{2+}\) mobilization is dependent on ryanodine-sensitive Ca\(^{2+}\) stores. To further investigate the RyR-dependent Ca\(^{2+}\) oscillations, IMCDs were loaded with NPE-caged cADPR, an endogenous agonist of RyR, by a reversible permeabilization protocol using streptolysin-O (50). Photorelease of cADPR with 60 UV laser pulses delivered over 2 s triggered a rapid increase in cytosolic Ca\(^{2+}\) followed by [Ca\(^{2+}\)]\(_i\) oscillations. The mean normalized time course for fluo-4 emission is shown in Fig. 2B. The time delay between the uncaging of cADPR and the rise in cytosolic [Ca\(^{2+}\)]\(_i\) was 10–20 s. The mean normalized fluo-4 emission at the peak of oscillation was 1.18 ± 0.04 (26 cells/3 tubules). The corresponding mean power spectrum is shown in Fig. 4B. The distribution of power spectral density was similar to that triggered by uncaging cAMP, but with less power in all oscillatory frequencies.

To test whether cADPR is involved in AVP-induced Ca\(^{2+}\) oscillations, cell-permeant 8-bromo-cADPR (100 μM) was used as a competitive inhibitor to suppress the endogenous activity of cADPR. Preincubation of IMCD with 8-bromo-cADPR for 25 min suppressed the amplitude and delayed the onset of the AVP-induced Ca\(^{2+}\) oscillations by 200 s (Fig. 3B). The mean normalized fluo-4 emission at the peaks of Ca\(^{2+}\) oscillations was reduced from 1.61 ± 0.17 (45 cells/3 tubules) to 1.25 ± 0.05 (42 cells/4 tubules) in the presence of 8-bromo-cADPR. The mean power spectra of AVP-induced oscillations in the absence and presence of 8-bromo-cADPR are shown in Fig. 3C. Both had multiple peaks and a broad distribution of spectral density below 0.1 Hz. The continuous presence of AVP seemed to partially overcome the inhibition of 8-bromo-cADPR. Ca\(^{2+}\) oscillations developed gradually after the initial 200 s. These observations suggest that endogenous cADPR may play a crucial role in the initiation of AVP-induced Ca\(^{2+}\) oscillations.

To investigate the involvement of IP\(_3\)-sensitive Ca\(^{2+}\) stores in mediating Ca\(^{2+}\) oscillations, the effects of photolytic release of IP\(_3\) in fluo-4 emission were examined in IMCD. The mean normalized time courses of changes in fluo-4 emission in responding to flash photolysis of caged IP\(_3\) are shown in Fig. 4A. Three UV pulses induced a small and slow transient increase of cytosolic Ca\(^{2+}\) after a time delay. Ten UV pulses triggered an initial jump in cytosolic Ca\(^{2+}\), followed by slow decay. The time delay between the laser pulses and initial increase of cytosolic Ca\(^{2+}\) was reduced compared with photolysis induced by three UV pulses. There was an upward trend in the cytosolic Ca\(^{2+}\) after the initial decay. Thirty UV pulses triggered a larger and more synchronized initial Ca\(^{2+}\) release. Cytosolic Ca\(^{2+}\) then remained steady above the baseline after a short decay. No consistent periodic spikes of cytosolic Ca\(^{2+}\) similar to those induced by cADPR were observed in all three levels of IP\(_3\) uncaging. The mean power spectra of

Fig. 3. Inhibition of AVP-induced Ca\(^{2+}\) oscillations by 8-bromo-cADP-ribose in IMCD. A and B: AVP-induced Ca\(^{2+}\) oscillations (45 cells/3 tubules; A) and AVP-induced Ca\(^{2+}\) oscillations after 25-min incubation with 100 μM 8-bromo-cADP-ribose (42 cells/4 tubules; B). C: corresponding mean power spectra of oscillations in A and B. Filled square indicates that mean value is significantly different from the baseline before AVP exposure (P < 0.05). Dashed lines are SE.
Caged cAMP and caged cADPR from Fig. 2. Dashed lines are SE. From the prephotolysis baseline (P laser burst. Filled symbols indicate that mean values are significantly different from the controls. 

Fig. 4A: mean normalized time course of changes in fluo-4 emission from intact IMCD cells induced by uncaging of IP$_3$ (37 cells/3 tubules). Photolysis of caged IP$_3$ was induced by 3, 10, and 30 UV laser pulses. Arrow indicates the mobilization triggered by photolysis of caged IP$_3$ (red line, 26 cells/3 tubules). 

Effect of photolytic release of caged IP$_3$ in Ca$^{2+}$ mobilization. Mean normalized fluo-4 emission of the peak-to-peak oscillation and oscillations. However, the amplitude of the initial Ca$^{2+}$ spike triggered by AVP was attenuated and delayed by xestospongin C. The mean normalized fluo-4 emission of the initial spike was reduced from 1.61 ± 0.17 (45 cells/3 tubules; Fig. 3A) to 1.21 ± 0.05 (39 cells/4 tubules, P < 0.05), which suggested that IP$_3$ may contribute in part to the initial spike of the AVP-induced intracellular Ca$^{2+}$ release or xestospongin C may have other effects in the intracellular Ca$^{2+}$ stores in addition to blocking IP$_3$Rs (9).

Store-operated Ca$^{2+}$ entry in IMCD. Our previous study (45) showed that removal of extracellular Ca$^{2+}$ did not prevent the initial rise of [Ca$^{2+}$], but inhibited the sustained oscillations induced by AVP in IMCD. This suggested that entry of extracellular Ca$^{2+}$ was required to maintain the AVP-induced Ca$^{2+}$ oscillations. Ca$^{2+}$ entry was not mediated by L-type voltage-gated Ca$^{2+}$ channel, as nifedipine (10 μM) did not inhibit AVP-induced Ca$^{2+}$ oscillations (Fig. 5, A and D).

Treatment of IMCD with thapsigargin (20 μM) to inhibit the ER Ca$^{2+}$-ATPase in Ca$^{2+}$-free medium caused an increase in intracellular Ca$^{2+}$ due to Ca$^{2+}$ leak from intracellular stores (data not shown). Readdition of 2 mM Ca$^{2+}$ to the peritubular perfusate induced a rapid influx of Ca$^{2+}$ in IMCD (Fig. 6), which was absent without the use of thapsigargin to deplete the intracellular Ca$^{2+}$ stores. The latter observations are consistent with the report that changes of peritubular [Ca$^{2+}$] alone induced only slight or no change in [Ca$^{2+}$] in rat IMCD (4). The mean normalized fluo-4 fluorescence at the peaks of Ca$^{2+}$ entry was significantly reduced from 2.64 ± 0.08 (126 cells/7 tubules) to 1.41 ± 0.14 (33 cells/3 tubules, P < 0.05) in the presence of 50 μM SKF-96365 and to 1.61 ± 0.06 (88 cells/5 tubules, P < 0.05) in the presence of 1 μM GdCl$_3$. SKF-96365 and a low concentration of Gd$^{3+}$ are two commonly used antagonists for SOCE and nonselective cation channels. These results indicated that depletion of intracellular Ca$^{2+}$ stores triggered SOCE in IMCD.

To determine whether AVP could trigger extracellular Ca$^{2+}$ influx similar to that induced by thapsigargin, IMCD was incubated with 1 nM AVP in Ca$^{2+}$-free medium for 30 min. Readdition of 2 mM Ca$^{2+}$ to the peritubular perfusate produced a rapid Ca$^{2+}$ entry (Fig. 7). The mean normalized fluo-4 fluorescence at the peaks of Ca$^{2+}$ entry was significantly reduced from 2.72 ± 0.09 (135 cells/8 tubules) to 1.32 ± 0.11 (35 cells/3 tubules, P < 0.05) in the presence of 50 μM SKF-96365 and to 1.89 ± 0.07 (54 cells/4 tubules, P < 0.05) in the presence of 1 μM GdCl$_3$.

To determine whether AVP-induced Ca$^{2+}$ oscillation required calcium entry, Ca$^{2+}$ oscillation in IMCD was first induced by 1 nM AVP. Removal of extracellular Ca$^{2+}$ immediately halted the Ca$^{2+}$ oscillation. Readdition of 2 mM Ca$^{2+}$ to the peritubular perfusate triggered a large and rapid Ca$^{2+}$ entry, which was followed by Ca$^{2+}$ oscillation (Fig. 8). These results together with the aforementioned data are consistent with the notion that extracellular Ca$^{2+}$ entry activated subsequent to Ca$^{2+}$ release from ryanodine-sensitive Ca$^{2+}$ stores
participates in the AVP-induced Ca\(^{2+}\) oscillations in rat IMCD.

**DISCUSSION**

Previous studies have shown that AVP at a physiological concentration elicits robust Ca\(^{2+}\) mobilization and oscillations through a cAMP-dependent pathway and the Ca\(^{2+}\) mobilization is essential for AVP-induced apical incorporation of aquaporin-2 (AQP2) and water permeability changes in IMCD (8, 45, 46). Here we used UV laser-induced photorelease of cAMP to demonstrate that cAMP alone is sufficient to mimic AVP in initiating Ca\(^{2+}\) oscillation. We furthermore provide novel evidence suggesting that AVP-induced Ca\(^{2+}\) response is activated by the endogenous RyR agonist cADPR and maintained by Ca\(^{2+}\) influx via SOCE. However, AVP-induced Ca\(^{2+}\) oscillation is independent of Ca\(^{2+}\) release stimulated by IP\(_3\) and Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels. Hence this cAMP-cADPR-RyR-SOCE pathway may represent an integrated Ca\(^{2+}\) signaling mechanism for the regulation of water permeability in IMCD.

AVP enhances renal water reabsorption through the activation of heteromeric G\(_s\) protein-coupled V\(_2\) receptor, causing a rise in cAMP concentration, PKA-dependent phosphorylation of AQP2, and translocation of AQP2 to plasma membrane of IMCD cells (29). Increasing evidence suggests that Ca\(^{2+}\) signaling plays a pivotal role in this regulatory process. AVP has been shown to both increase cAMP and induce Ca\(^{2+}\) mobilization in IMCD (4, 8, 10, 22, 27, 38, 45). Chelation of intracellular Ca\(^{2+}\) with BAPTA completely abolished the AVP-induced apical exocytosis and increase in osmotic water permeability without affecting cAMP production (8, 45). AVP-induced Ca\(^{2+}\) mobilization appears to be a downstream effect following activation of adenylyl cyclase. It is mimicked by the membrane-permeant cAMP analog 8-(4-chlorophenylthio)-cAMP (8) and by photorelease of caged cAMP as observed in the present study. The time delay of 4–10 s in the onset of caged cAMP-induced Ca\(^{2+}\) response is congruent with the delay in the increase of membrane capacitance observed after photorelease of cAMP in cultured IMCD cells (26), suggesting that Ca\(^{2+}\) response could be a rate-limiting step for cAMP-induced exocytosis.

AVP-induced Ca\(^{2+}\) mobilization is dependent on RyR-gated Ca\(^{2+}\) stores. Inhibition of RyR completely obliterates AVP-induced Ca\(^{2+}\) response and water permeability change in IMCD (8, 45). Immunofluorescence, immunoblotting, and RT-PCR experiments showed that RyR1 is predominantly expressed and colocalized with AQP2 in IMCD cells, supporting a cooperative association between the two types of
channels (6, 8). The mechanism by which AVP activates RyR is unclear. Ca$^{2+}$/H$^{+}$ influx through voltage-gated or non-voltage-gated Ca$^{2+}$/H$^{+}$ channels is known to activate RyRs through CICR. However, AVP has been shown to trigger ryanodine-sensitive Ca$^{2+}$/H$^{+}$ release in IMCD cells in the absence of extracellular Ca$^{2+}$ (45), suggesting that the process is independent of Ca$^{2+}$/H$^{+}$ influx-mediated CICR. Studies in other cell systems have shown that agonists including angiotensin II, endothelin-1, and acetylcholine can activate RyRs through stimulation of ADP-ribosyl cyclase to generate the endogenous RyR agonist cADPR (1, 18, 49), which increases the open probability of RyRs perhaps through interactions with FK506-binding protein (39, 41). Our observations strongly suggest that the AVP-induced Ca$^{2+}$/H$^{+}$ oscillations in IMCD involve endogenous cADPR, because the Ca$^{2+}$/H$^{+}$ response was delayed and suppressed by cADPR antagonist 8-bromo-cADPR and could be mimicked by photorelease of caged cADPR.

AVP may stimulate cADPR production through the cAMP signaling pathway. It has been reported in cardiac myocytes and chromaffin cells that application of cAMP analogs or activation of adenylate cyclase enhanced cADPR synthesis (20, 28, 43), an effect that could be blocked by the PKA inhibitor H-89 or Rp-adenosine 3′,5′-cyclic monophosphothioate (Rp-cAMP[S]), an antagonist of cAMP for PKA binding (39, 41). Our observations strongly suggest that the AVP-induced Ca$^{2+}$/H$^{+}$ oscillations in IMCD involve endogenous cADPR, because the Ca$^{2+}$/H$^{+}$ response was delayed and suppressed by cADPR antagonist 8-bromo-cADPR and could be mimicked by photorelease of caged cADPR.

Multiple IP$_{3}$R subtypes have been identified in IMCD cells (19, 44), but their role in the AVP-induced Ca$^{2+}$/H$^{+}$ mobilization is not well defined. Photorelease of IP$_{3}$ in IMCD activated a robust increase of [Ca$^{2+}$], which was blocked by xestospongin C. However, xestospongin C did not abolish AVP-induced Ca$^{2+}$/H$^{+}$ mobilizations and oscillations. These observations are consistent with the previous report that AVP at physiological concentration...
does not activate the phosphoinositide signaling pathway in IMCD (7). A closer examination of the data revealed that the initial spike of AVP-induced Ca^{2+} release was attenuated and delayed by xestospongin C, suggesting that IP_{3} might still contribute in part to the initial Ca^{2+} release. However, xestospongin C is also known to inhibit ER Ca^{2+} pump in addition to IP_{3}Rs (9). If this occurs in IMCD cells, xestospongin C might reduce the calcium load in the ER and alter the dynamic of intracellular Ca^{2+} release.

In addition to RyR-gated Ca^{2+} release, AVP-induced Ca^{2+} oscillation is maintained by Ca^{2+} influx in IMCD cells. Removal of extracellular Ca^{2+} abbreviates the sustained repetitive AVP-induced Ca^{2+} oscillation to a transient Ca^{2+} release (45). We found that AVP activates a nifedipine-insensitive Ca^{2+} entry pathway, which is similar to the Ca^{2+} entry activated by thapsigargin in IMCD. It is blocked by the nonselective cation channel blocker SKF-96365 and is sensitive to a low concentration of Gd^{3+}, bearing resemblance to SOCE. However, 1 μM Gd^{3+} could only reduce Ca^{2+} influx induced by thapsigargin and AVP, while the same concentration of Gd^{3+} abolishes SOCE in other cell types (17, 36). These results suggest that the SOCE in IMCD could be different from the classical SOCE, which is mediated by Ca^{2+}-release-activated Ca^{2+} (CRAC) channels (36).

Previous studies showed that depletion of intracellular Ca^{2+} stores activates capacitative Ca^{2+} entry to replenish the Ca^{2+} content of ER/SR. Both RyR- and IP_{3}R-gated Ca^{2+} stores have been implicated in SOCE. Recent studies demonstrated that the thiol interaction molecule 1 (STIM1) operates as the endoplasmic Ca^{2+} sensor (24, 35, 37). Upon Ca^{2+} depletion of ER, STIM1 proteins aggregate, translocate, and couple to ORAI1 as well as other associated proteins including transient receptor potential (TRP) channels in the plasma membrane to activate Ca^{2+} entry (21, 25, 31, 42). TRPC3 and TRPC6 are expressed in rat IMCD (15). TRPC3 is found in apical membrane and is colocalized with AQP2 in vesicles (16). However, SOCE and its molecular counterparts have not been characterized in IMCD. Nevertheless, the prominent Ca^{2+} entry signal elicited by thapsigargin and AVP with similar sensitivity to SKF-96365 and Gd^{3+} suggests that a Ca^{2+} entry mechanism resembling SOCE is an important Ca^{2+} pathway in IMCD cells. Our observations, however, did not exclude the possibility that other nonselective cation channels may also be involved in the AVP-induced Ca^{2+} entry. In conclusion, our present observations in conjunction with those in previous studies have unraveled a cAMP-cADPR-RyR-Ca^{2+} influx signaling system that may play a central role in the regulation of water permeability in IMCD.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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