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Ryanodine receptor and capacitative Ca^{2+} entry in fresh preglomerular vascular smooth muscle cells

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Ryanodine receptor and capacitative Ca^{2+} entry in fresh preglomerular vascular smooth muscle cells.

Background. A multiplicity of hormonal, neural, and paracrine factors regulates preglomerular arterial tone by stimulating calcium entry or mobilization. We have previously provided evidence for capacitative (store-operated) Ca^{2+} entry in fresh renal vascular smooth muscle cells (VSMCs). Ryanodine-sensitive receptors (RyRs) have recently been identified in a variety of nonrenal vascular beds.

Methods. We isolated fresh rat preglomerular VSMCs with a magnetized microsphere/sieving technique; cytosolic Ca^{2+} ($[Ca^{2+}]_i$) was measured with fura-2 ratiometric fluorescence.

Results. Ryanodine (3 μmol/L) increased $[Ca^{2+}]_i$ from 79 to 138 nmol/L ($P = 0.01$). Nifedipine (Nif), given before or after ryanodine, was without effect. The addition of calcium (1 mmol/L) to VSMCs in calcium-free buffer did not alter resting $[Ca^{2+}]_i$. In Ca-free buffer containing Nif, $[Ca^{2+}]_i$ rose from 61 to 88 nmol/L after the addition of the Ca^{2+}-ATPase inhibitor cyclopiazonic acid and to 159 nmol/L after the addition of Ca^{2+} (1 mmol/L). Mn^{2+} quenched the Ca/fura signal, confirming divalent cation entry. In Ca-free buffer with Nif, $[Ca^{2+}]_i$ increased from 80 to 94 nmol/L with the addition of ryanodine and further to 166 nmol/L after the addition of Ca^{2+} (1 mmol/L). Mn^{2+} quenching was again shown. Thus, emptying of the sarcoplasmic reticulum (SR) with ryanodine stimulated capacitative Ca^{2+} entry.

Conclusion. Preglomerular VSMCs have functional RyR, and a capacitative (store-operated) entry mechanism is activated by the depletion of SR Ca^{2+} with ryanodine, as is the case with inhibitors of SR Ca^{2+}-ATPase.

A multiplicity of hormonal, neural, and paracrine factors regulates arterial tone in preglomerular resistance vessels ultimately controlling the glomerular filtration rate, sodium reabsorption, and blood pressure. Calcium entry via voltage-gated L-type channels has been thought to be the predominant mechanism for increasing cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in intact vessels or vascular smooth muscle cells (VSMCs) derived from afferent arterioles [1–5]. More recently, calcium mobilization from intracellular stores has been well documented in renal resistance vessels [3, 6–10]. Furthermore, we have shown that capacitative (store-operated) calcium entry is present in fresh VSMCs derived from rat preglomerular vessels [6].

Physiologic Ca^{2+} release from the sarcoplasmic reticulum (SR) can be stimulated by two mechanisms: (*1*) an inositol triphosphate (IP_3) receptor that is inhibited by heparin, and (*2*) a ryanodine-sensitive receptor that is stimulated by Ca^{2+} and is sensitive to the plant alkaloid ryanodine, to caffeine, and to adenosine triphosphate (ATP) [11]. Ryanodine-sensitive receptors (RyR) have been studied extensively in skeletal and cardiac muscle. More recently, investigators have identified functional RyR in a wide variety of large conduit and small resistance arteries, namely, aortic, cerebral, femoral, coronary, pulmonary, and mesenteric [12–22]. RyRs have not been identified either functionally or anatomically in the renal vasculature. Because preliminary work in our laboratory led us to believe that intracellular pathways for calcium mobilization other than the classic IP_3 receptor model might be operative in fresh preglomerular VSMCs, we tested the hypothesis that the RyR is present. Our goal was to identify functional RyR and to determine whether stimulation of RyR with caffeine or ryanodine leads to emptying of the SR and activation of capacitative calcium entry.

Key words: store-operated calcium entry, afferent arteriole, kidney, sarcoplasmic reticulum, manganese, nifedipine, glomerular filtration rate, L-type channels.

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METHODS

Preparation of preglomerular resistance vessels

We used a magnetized polystyrene microsphere-sieving technique previously described in our laboratory [6, 23] to isolate preglomerular vessels (<50 μmol/L in diameter) from six- to seven-week-old Wistar Kyoto rats maintained in the Chapel Hill Colony. After intraperitoneal administration of sodium pentobarbital, a midline incision exposed the abdominal aorta, which was cannulated below the renal arteries. The aorta was occluded

above the renal arteries. The left renal vein was incised, and the kidneys were perfused slowly with 20 to 40 mL of cold phosphate-buffered saline (PBS), with the following composition in mmol/L: 137 NaCl, 2.7 KCl, 0.88 KH_2PO_4, 6.4 Na_2HPO_4, 1.0 $MgCl_2$, adjusted to pH 7.4 at 4°C. Three milliliters of a suspension of magnetized microspheres (Spherotech, Libertyville, IL, USA) in cold PBS were then infused. Thin cortical slices were minced and homogenized, and preglomerular arterioles were separated from the crude homogenate with a magnet. Passage of the suspension through needles of decreasing size disrupted vessels from their glomeruli. Application of the suspension to a 120 μm sieve retained vessel segments; the glomeruli and other debris were washed through the sieve and were discarded. The microvessels were washed from the inverted sieve, further purified with another magnet separation, and finally treated with collagenase (type 1-A, 0.03 to 0.04%; Sigma, St. Louis, MO, USA) for 20 minutes at 37°C. The suspension was chilled and then shaken vigorously to disrupt the vessels. Cells were incubated in Hank's buffered salt solution (HBSS; in mmol/L: 137 NaCl, 5.4 KCl, 0.33 Na_2HPO_4, 0.44 KH_2PO_4, 5 glucose, 1.0 $MgCl_2$) containing 2 μmol/L fura-2-AM for 45 to 60 minutes at room temperature in the dark. After washing twice with HBSS, cells were kept on ice either in HBSS with 1 mmol/L $MgCl_2$ and 0.5 mmol/L EGTA (Ca^{2+} free, <20 nmol/L, measured) or HBSS with 1.0 mmol/L $MgCl_2$ and 1.0 mmol/L $CaCl_2$.

Measurement of cytosolic free calcium concentration

We measured cytosolic free calcium concentration ($[Ca^{2+}]_i$) as previously described [6, 23, 24]. A suspension of VSMCs (10 μL) was gently aspirated from the surface of the cell pellet and spread on a cover slip, which was placed in the optical field of a ×40 oil-immersion fluorescence objective of an inverted microscope (Olympus IX70). VSMCs, identified morphologically by their spindle or crescent shape, have been shown previously to stain with smooth muscle specific α-actin and heavy chain myosin SM-1 and SM-2 [23]. Care was taken to focus on and record from one to three VSMCs. Not infrequently, two VSMCs were adherent to each other. HBSS (5 μL) was then added to ensure that the cells were adhering to the cover slip and would not be washed away by additions of drugs. The VSMCs were excited alternately with light of 340 and 380 nm wavelength from a dual-excitation wavelength Delta-Scan equipped with dual monochronometers and a chopper [Photon Technology International (PTI), South Brunswick, NJ, USA]. After passing signals through a barrier filter (510 nm), fluorescence was detected by a photomultiplier tube. Signal intensity was acquired, stored, and processed by an IBM-compatible Pentium II computer and Felix software (PTI). The calibration of $[Ca^{2+}]_i$ was based on the signal ratio at 340/380 nm and known concentrations of calcium. The $[Ca^{2+}]_i$ was calculated according to this formula:

$$[Ca^{2+}]_i = [(R - R_{min})/(R_{max} - R)] \cdot (S_f/S_b) \cdot Kd$$

where R is the ratio of the 340/380 nm fluorescence signal. R_{max} is the 340/380 ratio in the presence of saturation calcium. R_{min} is the 340/380 ratio in calcium free buffer containing 10 nmol/L EGTA, and S_f/S_b is the ratio of the 380 nm fluorescence measured in a calcium-free buffer to that measured in a calcium-replete solution [24]. For Mn^{2+} quenching experiments, we quantitated the fluorescence signal at 360 nm to document that Mn^{2+} did indeed quench the signal [25].

All drugs and chemicals were added in a 5 μL volume to the droplet of cells on the surface of a cover slip. To study the identical cell(s) with sequential drug additions, we did not wash the cells between additions in order not to dislodge them from their position on the glass cover slip. Calculations of drug concentration were based on the changing volumes of the droplet on the cover slip. To minimize problems with evaporation, we limited our observation time to 250 seconds or less. During this observation period, control studies showed that there was no change in counts at 340 and 380 nm as well as the ratio of the two.

Protocol

Unless otherwise stated, VSMCs were prepared in nominally calcium-free buffer (approximately 3.5 h from harvesting of the kidneys to measuring Ca^{2+}). The $[Ca^{2+}]$ of the buffer was measured as approximately 200 nmol/L, whereas that of buffer containing EGTA was <20 nmol/L. To demonstrate capacitative calcium entry, emptying of the SR was achieved either by stimulating with angiotensin II (Ang II) to activate the IP_3 pathway or by inhibiting the sarcolemmal Ca^{2+}-ATPase with cyclopiazonic acid (CPA). To prevent calcium entry via voltage-gated L-type channels, cells were treated with nifedipine (Nif). Calcium was added to the bathing buffer to restore extracellular Ca^{2+} to normal (1 mmol/L) to permit Ca^{2+} entry to occur. Alternatively, Mn^{2+} replaced Ca^{2+} or was added subsequent to Ca^{2+} to document divalent cation entry. Because Mn^{2+} quenches the Ca/fura signal, it serves as a marker for the entry of the divalent cations.

Ryanodine closes the RyR at high concentrations (>100 μmol/L); therefore, we employed concentrations of <5 μmol/L to stimulate the RyR. The experimental design for identifying the activation of the capacitative entry pathway by ryanodine was identical to that described previously in this article.

Reagents

Collagenase, Ang II, EGTA, caffeine, and Nif came from Sigma. CPA and ryanodine came from Cal Biochem (La Jolla, CA, USA). Vasopressin-1 receptor ago-

nist ([Phe^2, Ile^3, Orn^8]-Vasopressin) (V1R) was from Peninsula Laboratories (Belmont, CA, USA), and Fura-2-AM came from Molecular Probes (Eugene, OR, USA) or Teflab (Austin, TX, USA).

Statistics

The data are presented as means ± SEM. Each data set is derived from VSMCs originating from at least three separate experimental days, two to three rats (4 to 6 kidneys) per experiment. Individual cells were studied only once and were then discarded. The data sets were tested with Student's paired *t*-test. Multiple comparisons were analyzed using one-way analysis of variance for repeated measures followed by the Student–Neuman–Kuels post hoc test. $P < 0.05$ was considered statistically significant.

RESULTS

Stimulation of VSMCs with the peptide agonists Ang II or V1R typically produced a nearly square wave morphology of the $[Ca^{2+}]_i$ response, characterized by an immediate step increase that was generally somewhat sustained during the period of stimulation. This was particularly apparent in time-averaged group plots, which persisted during the 50-second period of observation. In a minority of cases, an initial sharp peak of a few seconds' duration was seen. We and others have noted this square-shaped pattern of response in healthy fresh VSMCs and vessel segments, microdissected afferent arterioles, and mesenteric artery of the rat and rabbit in response to Ang II, vasopressin, norepinephrine as well as KCl [6, 9, 23, 26–33].

Because both caffeine (10 mmol/L) and ryanodine (1 to 10 μmol/L) are known to stimulate the RyR in skeletal muscle and nonrenal vascular smooth muscle by opening the RyR channel and permitting passage of Ca^{2+} from the SR to the cytosol [34–36], we conducted experiments to test their effect on mobilization of Ca^{2+} from the SR in preglomerular VSMCs. Since high concentrations of ryanodine (1 mmol/L) close the RyR, choice of an experimental concentration of ryanodine is important. In control studies, comparing the effects of 3 and 30 μmol/L concentrations of ryanodine, the stimulatory dose of ryanodine (3 μmol/L) increased $[Ca^{2+}]_i$ from 57 ± 8 to 109 ± 10 nmol/L ($N = 6$, $P < 0.01$). In contrast, 30 μmol/L ryanodine were without effect; baseline $[Ca^{2+}]_i$ was 59 ± 8 nmol/L, and following the addition of 30 μmol/L, ryanodine was 64 ± 7 nmol/L ($N = 6$, $P = 0.4$). Figure 1A shows the group-averaged response of fresh preglomerular VSMCs to ryanodine (3 μmol/L) in calcium-containing buffer (1 mmol/L). Cytosolic calcium rose from 79 ± 10 to 138 ± 18 nmol/L ($N = 8$, $P < 0.01$). Because both caffeine (10 mmol/L) and ryanodine (1 to 10 μmol/L) maximally activate a common RyR, their effects

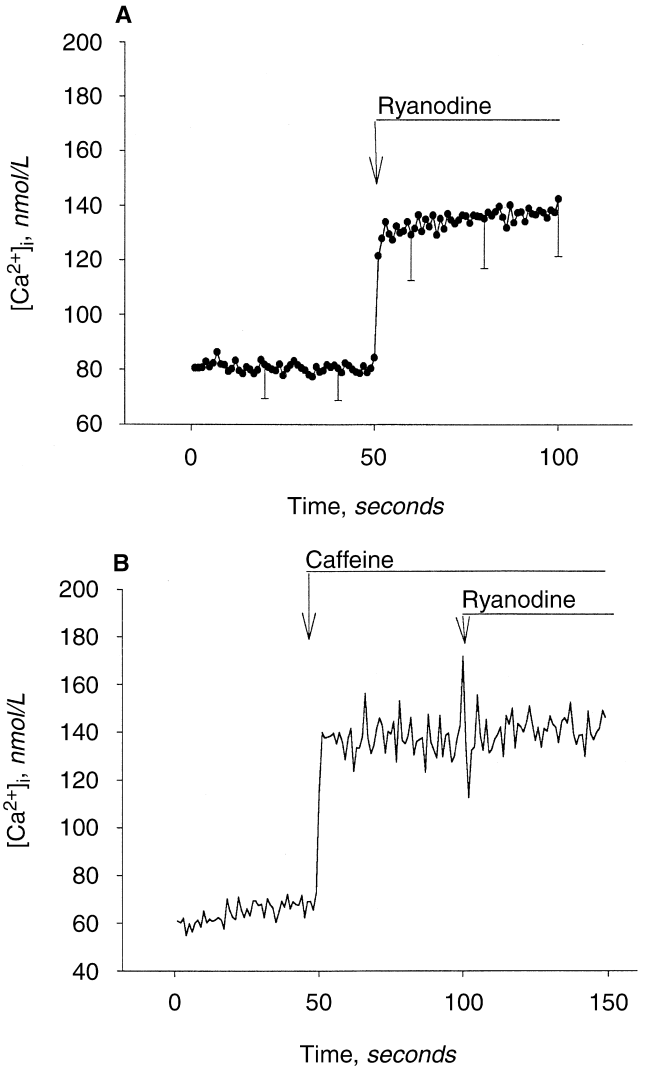

Fig. 1. Response of preglomerular vascular smooth muscle cells (VSMCs) to ryanodine (3 μmol/L). (*A*) Group averages for stimulatory effect of ryanodine on cytosolic calcium in VSMCs ($N = 8$, $P < 0.01$). Means ± SEM are shown. (*B*) Representative original tracing showing the lack of additive effect of ryanodine on VSMCs first stimulated with caffeine (10 mmol/L).

should not be additive. To demonstrate further the specificity of ryanodine for the RyR, we treated cells first with caffeine, followed by the addition of ryanodine. In our preglomerular VSMCs, caffeine (10 mmol/L) increased $[Ca^{2+}]_i$ from 78 ± 6 to 134 ± 12 nmol/L ($N = 10$, $P < 0.01$). The subsequent addition of ryanodine (2 μmol/L) did not change $[Ca^{2+}]_i$ (124 ± 10 nmol/L, $P > 0.4$). A representative tracing is shown in Figure 1B.

The ability of the RyR to increase arterial wall $[Ca^{2+}]_i$ has been shown to be blocked by inhibition of L-type channels with nimodipine in pressurized carotid arteries [36]. To evaluate whether this is the case in renal VSMCs, we studied the effect of ryanodine on preglomerular VSMCs when L-type Ca^{2+} channels were blocked by Nif

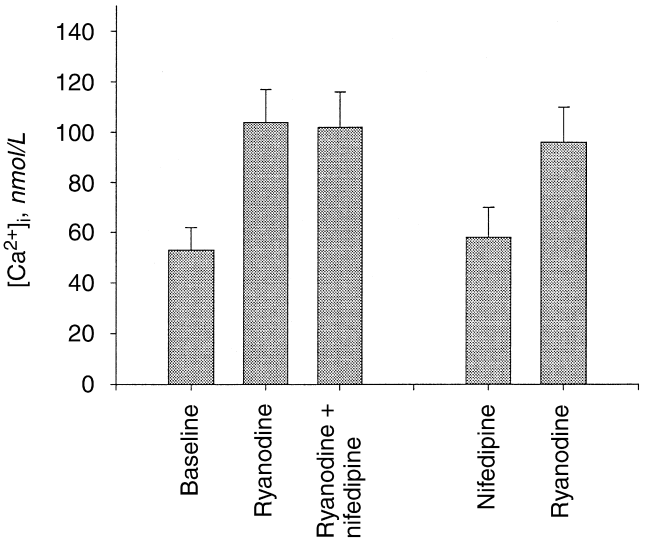

Fig. 2. Two sets of experiments showing the lack of effect of nifedipine (Nif, 10^{-7} mol/L) on the cytosolic calcium response of VSMCs to ryanodine. Neither Nif given after stimulation of VSMCs with ryanodine (left bars) nor pretreatment with Nif (right bars) altered the response to ryanodine ($N = 6$ in both groups).

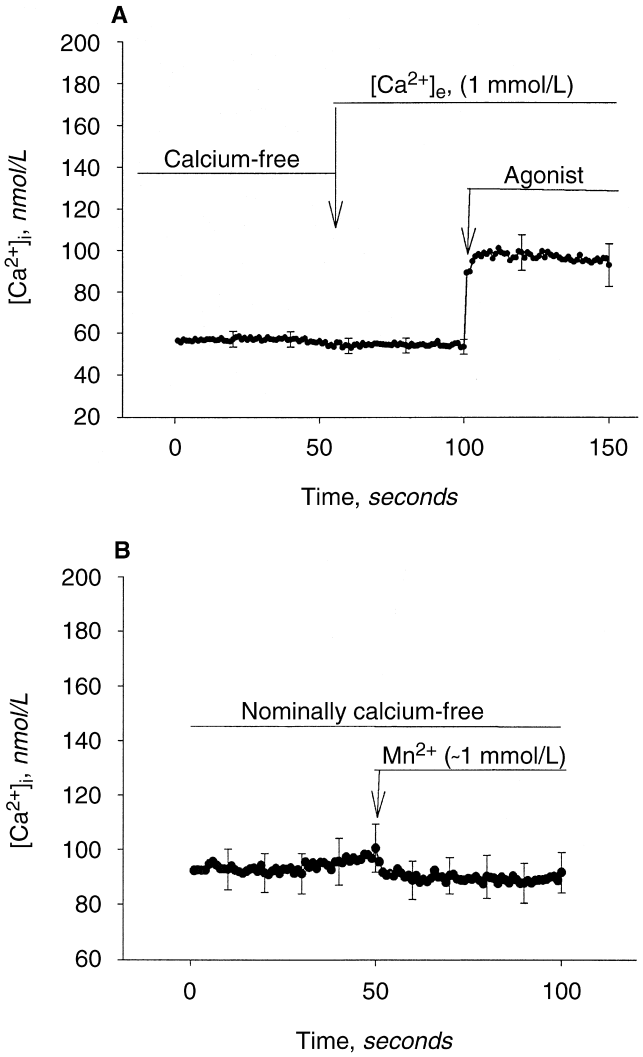

Fig. 3. Control experiments showing the lack of effect of external Ca^{2+} (1 mmol/L) and Mn^{2+} (1 mmol/L) on resting $[Ca^{2+}]_i$ in VSMCs previously maintained in a nominally calcium-free buffer. (*A*) Sequential addition of Ca^{2+} and agonist ($N = 9$, $P > 0.8$ for Ca^{2+} vs. calcium-free, $P < 0.01$ for Ca^{2+} vs. agonist). (*B*) Addition of external Mn^{2+} ($N = 12$, $P > 0.6$).

(10^{-7} mol/L). Dihydropyridine calcium channel blockers (CCBs) at a concentration of 10^{-7} mol/L have been shown to completely inhibit potassium-stimulated ^{45}Ca uptake and to reverse potassium-mediated reductions in renal plasma flow [36, 37]. We have previously demonstrated that CCBs at this concentration inhibit by 50% the global response of renal VSMCs to arginine vasopressin [6]. Figure 2 shows that neither pretreatment nor post-treatment with Nif (10^{-7} mol/L) influenced the stimulatory effect of ryanodine on renal VSMCs. In the presence of Nif, ryanodine increased $[Ca^{2+}]_i$ from 58 ± 12 to 96 ± 14 ($N = 6$, $P < 0.01$). In the absence of Nif, $[Ca^{2+}]_i$ rose from 53 ± 9 to 104 ± 13 following the stimulation with ryanodine ($N = 6$, $P < 0.01$). Subsequent addition of Nif was without effect (102 ± 14, $N = 6$, $P > 0.7$ for comparison of the effect of ryanodine with or without Nif).

Before evaluating the possible role of ryanodine-stimulated depletion of SR Ca^{2+} in activating capacitative (store-operated) entry in preglomerular VSMCs, we performed control experiments to examine the effect (or lack thereof) of adding Ca^{2+} or Mn^{2+} to VSMCs previously maintained in nominally calcium-free buffer. Figure 3A is a composite of nine experiments in which Ca^{2+} (1 mmol/L) was added to VSMCs previously exposed to calcium-free medium for approximately 3.5 hours. The $[Ca^{2+}]_i$ of VSMCs in calcium-free buffer and that following the addition of Ca^{2+} were not different. Thus, extracellular calcium alone, in the absence of agonist, does not significantly change $[Ca^{2+}]_i$ in healthy preglomerular VSMCs. To ensure that these were indeed viable cells, they were then stimulated with a variety of agonists (Ang II, V1R, and KCl), which consistently increased $[Ca^{2+}]_i$. Manganese (1 mmol/L) likewise did not alter resting $[Ca^{2+}]_i$ in VSMCs (Fig. 3B). Baseline $[Ca^{2+}]_i$ was 97 ± 7 nmol/L and did not change after the addition of Mn^{2+} (90 ± 7 nmol/L, $N = 12$, $P > 0.7$).

As Figure 4A shows, stimulation of VSMCs with Ang II in calcium-free buffer containing 0.5 mmol/L EGTA and Nif (10^{-7} mol/L) immediately caused a rapid and sustained increase in $[Ca^{2+}]_i$ from 72 ± 9 to 101 ± 6 nmol/L ($P < 0.001$), indicating that Ang II mobilizes Ca^{2+} from intracellular stores as we have previously shown for V1R [6, 7]. The addition of Ca^{2+} (1 mmol/L) to the extracellular buffer caused a step increase in

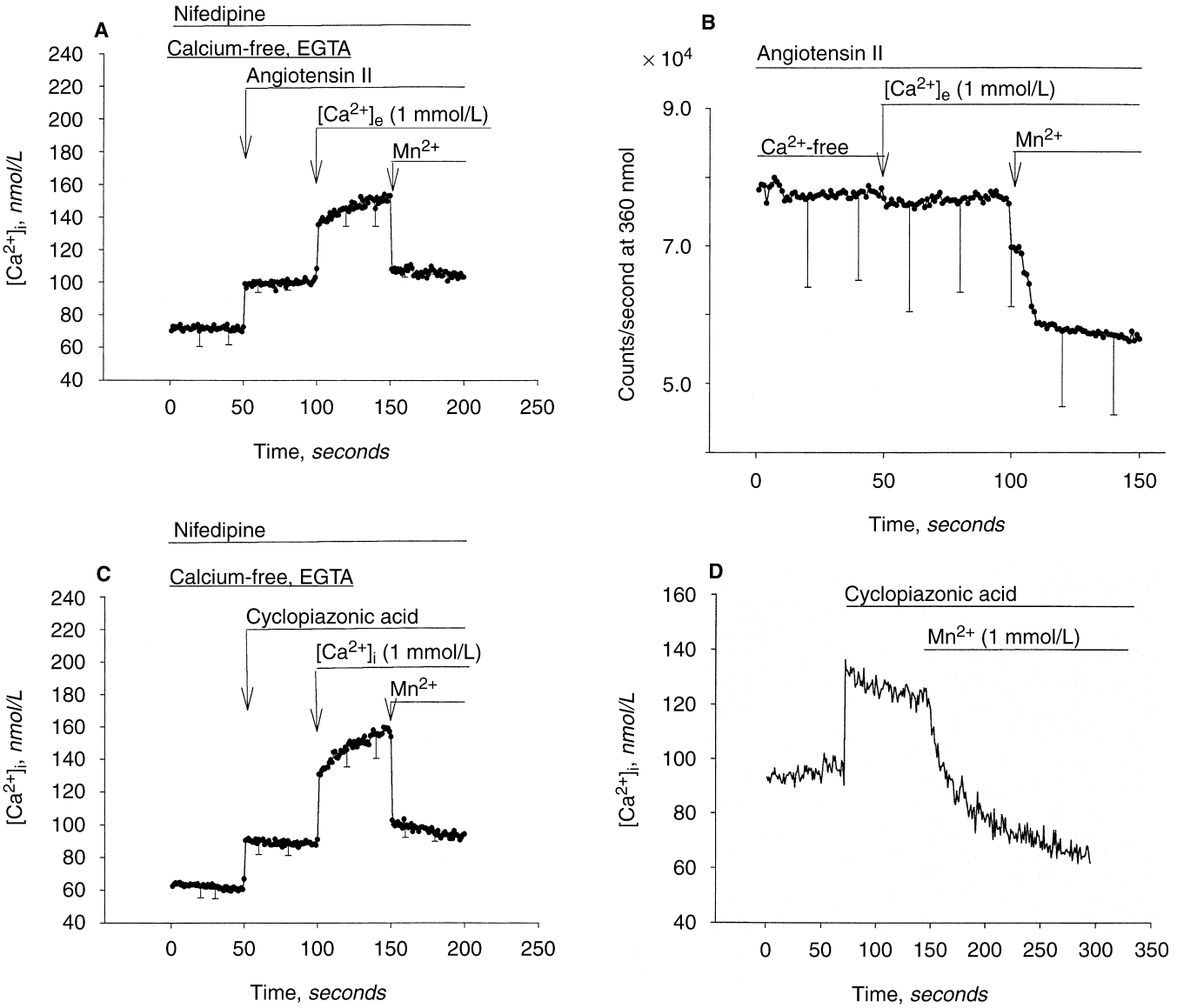

Fig. 4. Cytosolic calcium concentration ($[Ca^{2+}]_i$) in VSMCs freshly derived from preglomerular arterioles in calcium-free buffer containing EGTA (0.5 mol/L) and Nif (10^{-7} mol/L). (*A*) Effect of treatment with Ang II (10^{-7} mol/L) followed by the addition of Ca^{2+} (1 mmol/L) and then Mn^{2+} (approximately 1 mmol/L). The results are means ± SEM for seven experiments ($P < 0.001$ for each comparison). (*B*) VSMCs treated with angiotensin II (Ang II, 10^{-7} mol/L). Fluorescence in counts per second at 360 nm. The sequential addition of Ca^{2+} (1 mmol/L) and Mn^{2+} (approximately 1 mmol/L, $N = 7$). (*C*) Group results for the sequential addition of cyclopiazonic acid (CPA, 10^{-6} mol/L) and Ca^{2+} (1 mmol/L). The subsequent addition of Mn^{2+} (approximately 1 mmol/L) quenched the Ca^{2+}/fura signal ($N = 7$, $P < 0.001$ for all comparisons). Results are means ± SEM. (*D*) Representative tracing continued for 300 seconds to illustrate the decline of the ratio of 340/380 nm with time following the addition of Mn^{2+}.

$[Ca^{2+}]_i$ to 152 ± 10 nmol/L ($P < 0.001$). The Ca^{2+}/fura signal was then quenched by the addition of Mn^{2+} (approximately 1 mmol/L, $N = 7$, $P < 0.001$). Evidence for store-operated calcium entry in response to Ang II has not previously been demonstrated in preglomerular renal VSMCs. In Mn^{2+} quenching experiments, fluorescence at 360 nm was quantitated [25] to show that at the isobestic point, the signal did not change after a rise in $[Ca^{2+}]_i$, but fell after the addition of Mn^{2+} to the bathing buffer corroborating divalent cation entry (Fig. 4B). These data demonstrate that Ang II-initiated Ca^{2+} mobilization activates capacitative (store-operated) Ca^{2+} influx mechanisms in preglomerular VSMCs. They extend our previous findings that AVP V1 receptor agonist stimulates capacitative entry in renal resistance vessels [6].

To substantiate further the operation of capacitative Ca^{2+} entry in VSMCs derived from renal-resistance arteries and arterioles, we employed the Ca^{2+}-ATPase inhibitor CPA (10^{-6} mol/L), which depletes intracellular Ca^{2+} stores by preventing reaccumulation of calcium into

the SR [38]. In these experiments, baseline $[Ca^{2+}]_i$ in EGTA-calcium free buffer rose from 61 ± 7 to 88 ± 9 nmol/L following the addition of CPA and to 159 ± 15 nmol/L when Ca^{2+} (1 mmol/L) was added to the buffer ($N = 7$, $P < 0.01$ for each intervention). Subsequent addition of extracellular Mn^{2+} quenched the Ca^{2+}/fura signal, further demonstrating that depletion of calcium from the SR stimulated opening of a divalent ion entry channel that persists for at least 50 seconds (Fig. 4C). In several experiments, which were carried out for longer periods of time, the ratio of counts per second at 340/380 nm following addition of Mn^{2+} continued to decline (Fig. 4D).

Studies were conducted to determine whether ryanodine stimulation of calcium release from the SR would trigger store-operated Ca^{2+} entry. In these experiments, we used Nif to block possible entry via voltage-gated L-type channels. When VSMCs in calcium-free buffer containing Nif (10^{-7} mol/L) were stimulated with ryanodine (3 μmol/L), $[Ca^{2+}]_i$ increased from 80 ± 10 to 94 ± 10 nmol/L ($P < 0.001$); a subsequent addition of Ca^{2+} (1 mmol/L) caused a step increase to 166 ± 11 nmol/L ($N = 9$, $P < 0.001$). These data show that ryanodine-induced depletion of SR Ca^{2+} promoted the opening of a channel distinct from an L-type channel. That Mn^{2+} quenched the Ca^{2+}/fura signal further substantiates the opening of a divalent cation channel insensitive to Nif (Fig. 5A). Figure 5B shows the data for studies done in the absence of Nif. Ryanodine (3 μmol/L) increased $[Ca^{2+}]_i$ from 61 ± 7 to 78 ± 5 nmol/L, which then rose to 141 ± 18 nmol/L when Ca^{2+} (1 mmol/L) was added to the buffer ($N = 9$, $P < 0.001$ for both). Mn^{2+} quenched the Ca^{2+}/fura signal as before (Fig. 5C). It is noteworthy that the increase in $[Ca^{2+}]_i$ was similar whether or not Nif was present, confirming our earlier results showing that store-operated Ca^{2+} occurs via channel distinct from one that is dyhydropyridine sensitive.

DISCUSSION

We have established, to our knowledge for the first time, the presence of a functional RyR in fresh VSMCs isolated from preglomerular arterioles. The RyR of the SR exists in a number of different subtypes in skeletal and cardiac muscle [35]. More recently, RyR subtypes have been identified in nonexcitable cells [39–41] and nonrenal VSMCs [12–22, 42, 43]. Furthermore, we are the first to show that depleting SR Ca^{2+} by stimulation of the RyR with ryanodine promotes calcium influx via a store-operated or capacitative mechanism [43–45]. These data along with those obtained with CPA and with Ang II in the present study extend our previous observations with vasopressin V-1 receptor agonist and thapsigargin that a capacitative pathway exists in preglomerular VSMCs [6]. To our knowledge, capacitative calcium influx following the depletion of SR calcium with ryanodine has not previously been demonstrated in smooth muscle cells in any vascular tissue.

Store-operated or capacitative calcium entry is that process by which depletion of calcium in the endoplasmic or SR stimulates the opening of a divalent cation channel(s) in the plasmalemmal membrane, which permits the entry of Ca^{2+} or Mn^{2+} [43–45]. This entry channel is inhibited by Gd^{3+} and is distinct from voltage-gated L-type channels and from arachidonic acid metabolite-stimulated calcium entry [46]. Our studies show that stimulation of VSMCs with Ang II or with V1R [6] mobilized Ca^{2+} from the SR in calcium-free buffer containing CCB to block voltage-gated L-type channels. The subsequent addition of calcium to the extracellular buffer increased $[Ca^{2+}]_i$ through influx of Ca^{2+} via the putative capacitative entry pathway. The addition of extracellular Mn^{2+} quenched the Ca^{2+}/fura signal, demonstrating that a divalent cation channel had been opened to permit Mn^{2+} entry. Control experiments established that Mn^{2+} does not enter the cytosol under basal conditions when the divalent cation channels are inactive. Likewise, treatment of VSMCs in calcium-free buffer containing Nif (to block voltage-gated L-type channels) and the Ca^{2+}-ATPase inhibitors CPA (Fig. 4C) or thapsigargin [6], prevented refilling of the SR with Ca^{2+}, activated the store-operated pathway that opened divalent cation channels, and permitted an influx of Ca^{2+} when calcium was added to the extracellular buffer. It is highly unlikely that sodium/calcium exchange, operating in the reverse direction [47], could be responsible for calcium entry under our experimental conditions. Sodium concentration in the extracellular compartment was not lowered nor were drugs that increase intracellular sodium given.

Ryanodine (2 to 3 μmol/L, a concentration known to stimulate RyR) [17] and caffeine (10 mmol/L) both increased $[Ca^{2+}]_i$ by stimulating release of Ca^{2+} from the SR. At these concentrations, ryanodine and caffeine are specific for the RyR and do not have an effect on IP_3 receptors of the SR in VSMCs [11]. Ryanodine, like CPA or thapsigargin, depleted intracellular calcium stores and thus promoted calcium entry via a capacitative pathway.

The RyRs of skeletal and cardiac muscle have been extensively studied [35]. However, the identification and functional characterization of the RyR Ca^{2+}-gated Ca^{2+} release channel in VSMCs have only more recently been achieved [11]. Like the skeletal and cardiac muscle subtypes, RyR in aortic VSMCs is activated by μmol/L concentrations of ryanodine, activated by caffeine (mmol/L range), inhibited by Mg^{2+} (>2 mmol/L), and inhibited by ruthenium red (μmol/L) [15]. A wide variety of both conduit and resistance arteries contain RyR [12–22, 42, 43]. In studies conducted on rings of large arteries, smaller resistance arteries and submucosal ileal arterioles, functional RyRs were found to be present at

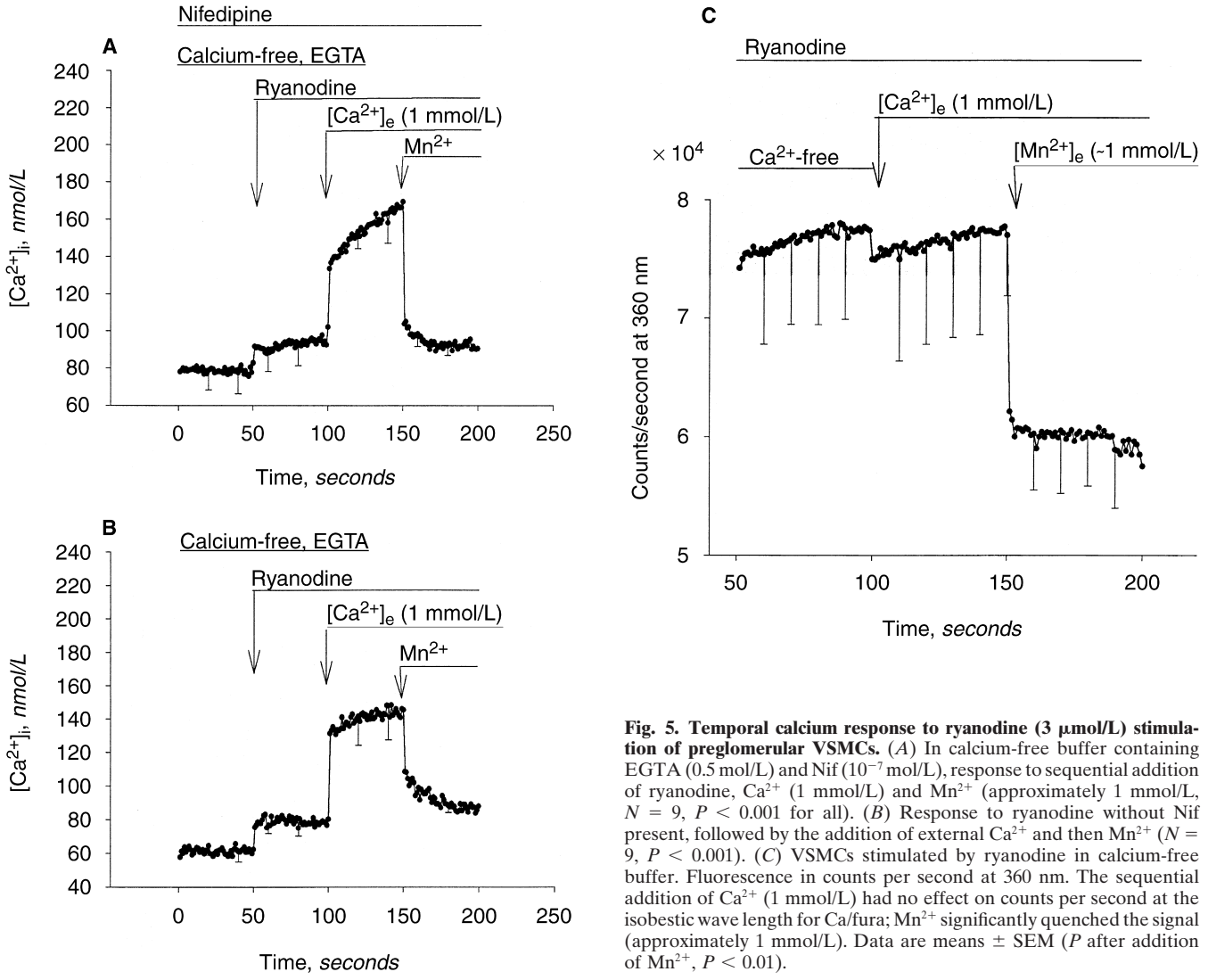

Fig. 5. Temporal calcium response to ryanodine (3 μmol/L) stimulation of preglomerular VSMCs. (*A*) In calcium-free buffer containing EGTA (0.5 mol/L) and Nif (10^{-7} mol/L), response to sequential addition of ryanodine, Ca^{2+} (1 mmol/L) and Mn^{2+} (approximately 1 mmol/L, $N = 9$, $P < 0.001$ for all). (*B*) Response to ryanodine without Nif present, followed by the addition of external Ca^{2+} and then Mn^{2+} ($N = 9$, $P < 0.001$). (*C*) VSMCs stimulated by ryanodine in calcium-free buffer. Fluorescence in counts per second at 360 nm. The sequential addition of Ca^{2+} (1 mmol/L) had no effect on counts per second at the isobestic wave length for Ca/fura; Mn^{2+} significantly quenched the signal (approximately 1 mmol/L). Data are means ± SEM (*P* after addition of Mn^{2+}, $P < 0.01$).

all levels of the vasculature; however, the contribution of the SR to the maintenance of vascular contractility appeared to diminish with decreasing caliber of the vessel [48].

Coupling of RyR to calcium entry via a store-operated mechanism has been suggested in studies with caffeine and thapsigargin in the excitable cell line of PC 12 cells [40]. Evidence for capacitative calcium influx was sought in the embryonic aortic A7r5 cells in culture, but was not found [43]. We have not identified any study in which capacitative entry linked to ryanodine-stimulated emptying of the SR has been proven to be operative in a blood vessel of any origin or caliber.

Ryanodine-sensitive receptors have been investigated in intact pressurized cerebral arteries and fresh VSMCs enzymatically derived from these arteries [36, 49, 50]. Ryanodine, by increasing $[Ca^{2+}]_i$, diminished the frequency of calcium sparks in these studies and thus decreased the activity of K_{Ca} channels. Also, the CCBs nimodipine and diltiazem (10^{-8} mol/L) decreased calcium sparks, demonstrating the voltage dependence of the sparks. Our studies were geared toward studying calcium entry mechanisms rather than the process of hyperpolarization generated by calcium sparks and K_{Ca} channels. We used both verapamil and Nif (10^{-7} mol/L) to inhibit effectively and selectively the voltage-gated L-type channels and found that the stimulatory effect of ryanodine on the SR was not influenced by these maneuvers.

Cyclic ADP ribose (cADPR), a recently identified nucleotide that is known to stimulate the RyR to release Ca^{2+} from the SR, is synthesized from β-NAD with the enzyme ADP-ribosyl cyclase [51]. It has been suggested that cADPR acts as a second messenger to release Ca^{2+} from the SR via the RyR pathway [51, 52]. Rat glomeruli, cultured mesangial cells, and cultured aortic VSMCs

have high levels of ADP-ribose cyclase activity, whereas proximal tubules do not [53]. Arteriolar VSMCs were not evaluated in these studies. In contrast to the glomerular localization of ADP-ribose cyclase in the kidney, RyR has been found in cultured MDCK cells [53], proximal tubular cells [54], and human embryonic kidney cells [55]. The functional role of the RyR in these cells awaits investigation.

Afferent arterioles are unique among all vascular beds by virtue of their ending in a complex glomerular network of capillaries, which, in turn, empties into the efferent arteriole rather than a venule. The critical role of these resistance vessels in controlling the glomerular filtration rate as well as sodium excretion may mandate a multiplicity of control mechanisms to regulate cytosolic calcium. Hence, it is not surprising that the characteristics of these pathways may differ from those of other vascular beds, particularly those of conduit arteries.

We have shown, to our knowledge for the first time, that: (*1*) RyRs exist in renal preglomerular arterioles of the rat kidney; (*2*) stimulation of the RyR channel to release Ca^{2+} from the SR, thereby depleting SR Ca^{2+} stores, promotes activation of a capacitative entry mechanism; and (*3*) inhibition of voltage-gated L-type channels with CCB does not block RyR pathway activity. The relative role of RyR in the regulation of renal vascular tone, glomerular filtration, and excretion of solute and water deserves further investigation.

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