Differential Mechanisms of Ca^{2+} Release from Vascular Smooth Muscle Cell Microsomes

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The release of Ca^{2+} from intracellular stores is a fundamental element of signaling pathways involved in regulation of vascular tone, proliferation, apoptosis, and gene expression. Studies of sea urchin eggs have led to the identification of three functionally distinct Ca^{2+} signaling pathways triggered by IP_3, cADPR, and NAADP. The coexistence and functional relevance of these distinct intracellular Ca^{2+} release systems has only been described in a few mammalian cell types. The purpose of this study was to determine whether the IP_3, cADPR, and NAADP Ca^{2+} release systems coexist in smooth muscle cells (SMC) and to determine the specificity of these intracellular Ca^{2+} release pathways. Microsomes were prepared from rat aortic SMC (VSMC) and were loaded with $^{45}Ca^{2+}$. cADPR, NAADP, and IP_3 induced Ca^{2+} release from VSMC microsomes in a dose-dependent fashion. Heparin blocked only IP_3-mediated Ca^{2+} release, whereas the ryanodine channel inhibitors 8-Br-cADPR and ruthenium red blocked only cADPR-induced Ca^{2+} release. Nifedipine, an L-type Ca^{2+} channel blocker, inhibited NAADP elicited Ca^{2+} release, but had no effect on IP_3- or cADPR-mediated Ca^{2+} release. An increase in pH from 7.2 to 8.2 inhibited cADPR-mediated Ca^{2+} release, but had no effect on IP_3- or NAADP-induced Ca^{2+} release. By RT-PCR, VSMC expressed ryanodine receptor types 1, 2, and 3. Ca^{2+}-dependent binding of [^{3}H]-ryanodine to VSMC microsomes was enhanced by the ryanodine receptor agonists 4-chloro-methyl-phenol (CMP) and caffeine, but was inhibited by ruthenium red and cADPR. We conclude that VSMC possess at least three functionally distinct pathways that promote intracellular Ca^{2+} release. IP_3-, cADPR-, and NAADP-induced intracellular Ca^{2+} release may play a critical role in the maladaptive responses of VSMC to environmental stimuli that are characteristically associated with hypertension and/or atherogenesis. [Exp Biol Med Vol. 227(1):36–44, 2002]

Key words: vascular smooth muscle; inositol-1,4,5-trisphosphate (IP_3); cyclic ADP-ribose (cADPR); nicotinic acid-adenine dinucleotide phosphate (NAADP); calcium (Ca^{++})

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The release of Ca^{2+} from intracellular stores and endoplasmic and sarcoplasmic reticulum (ER/SR) is one of the key signal transduction mechanisms in the regulation of numerous cellular functions (1–3), including contractility, protein synthesis and turnover, hormone secretion, proliferation, and activation. Currently, at least three distinct intracellular Ca^{2+}-mobilizing systems have been identified. The most comprehensively studied is the Ca^{2+} release triggered by inositol-1,4,5-trisphosphate (IP_3), an agonist that binds to a specific IP_3-receptor/Ca^{2+} channel (4–6). A second major Ca^{2+} signaling pathway is triggered by cyclic ADP-ribose (cADPR), an adenine nucleotide synthesized from β-NAD by the enzyme ADP-ribosyl cyclase (7–9). cADPR induces Ca^{2+} release via the ryanodine receptor/channel (RyR) by increasing the sensitivity of RyR for Ca^{2+}, thereby causing Ca^{2+} release by a Ca^{2+}-induced Ca^{2+} release mechanism (CICR) (9, 10). Ryanodine receptors have been described in vascular and cardiac muscle cells and in several nonexcitable cell types (11). We have recently demonstrated that rat mesangial cells, contractile cells with smooth muscle-like properties, possess elements of a cADPR→RyR→Ca^{2+} signaling pathway (12).

A third intracellular Ca^{2+} signaling pathway, activated by nicotinic acid-adenine dinucleotide phosphate (NAADP), has recently been identified through studies of Ca^{2+} release in sea urchin eggs (7, 9, 10, 13). NAADP, an analog of β-NADP, controls intracellular Ca^{2+} release by a mechanism fundamentally different from that of IP_3 or cADPR (13). We have recently shown that NAADP elicits specific Ca^{2+} release from microsomes prepared from a variety of cells and cell lines, suggesting that the capacity for NAADP-induced Ca^{2+} release is widespread in mammalian cells (14).

The physiologic relevance of intracellular Ca^{2+} signaling pathways triggered by IP_3, cADPR, and NAADP has not been adequately defined. The IP_3→IP_3R→Ca^{2+} release system is ubiquitously distributed (5, 15); this pathway mediates rapid intracellular Ca^{2+} release in response to vasoactive mediators such as endothelin or vasopressin (2, 6). In addition to promoting Ca^{2+}-induced Ca^{2+} release (9), the cADPR→RyR→Ca^{2+} release pathway is involved in insulin

release by pancreatic β cells (16) and gonadotrophin releasing hormone, signaling (17). Recent studies have shown that the activity of ADP-ribosyl cyclase, the enzyme responsible for cADPR synthesis, is upregulated in various tissues and cell types by steroid superfamily hormones such as retinoic acid (18), β-estradiol (19), or 3,5,3′-triiodothyronine (20). Although little is known about NAADP-elicited intracellular Ca^{2+} release in mammalian cells, we have recently demonstrated that retinoic acid increases capacity for NAADP biosynthesis in rat mesangial cells (21). The IP_3, cADPR, and NAADP signaling systems coexist in ascidian oocytes, and each system mediates distinct changes associated with fertilization and early development of the oocyte (22).

The IP_3, cADPR, and NAADP intracellular Ca^{2+} signaling pathways have not previously been directly compared in mammalian cells. Since alterations in intracellular Ca^{2+} have been shown to play a major role in vascular smooth muscle contractility, hypertrophy, and hyperplasia (23), these functionally distinct intracellular Ca^{2+} signaling pathways may direct adaptive cellular responses to pathobiologic stimuli. Here, we report that primary cultures of rat aortic smooth muscle cells (SMCs) possess functionally distinct Ca^{2+} signaling pathways triggered by IP_3, cADPR, and NAADP. IP_3 augments ryanodine binding to smooth muscle microsomes, indicating a potential site of crosstalk between the IP_3 and cADPR signaling pathways.

Materials and Methods

[^{3}H]-ryanodine and $^{45}Ca^{2+}$ were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). cADPR, 8-bromo-cyclic ADP ribose (8-Br-cADPR), ADPR, and ruthenium red (RR) were purchased from Calbiochem (La Jolla, CA). 4-Cl-methyl-phenol (CMP) was from Aldrich (Milwaukee, WI). Nicotinamide guanine dinucleotide (NGD), cyclic GDP-ribose (cGDPR), caffeine, and calmodulin were obtained from Sigma (St. Louis, MO). All other chemicals and biochemicals, all of highest purity grades, were from other standard suppliers.

Vascular SMC (VSMC) Isolation and Culture. VSMC were isolated and subcultured from rat aorta explant outgrowths as previously described (24). Briefly, the aorta was isolated from 200- to 250-g male Sprague-Dawley rats, cleaned in ice-cold sterile 0.9% NaCl, endothelium was removed by scraping, and adventitia was removed with surgical tweezers. The tissue was then minced and placed in 100-mm petri dishes with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B. VSMC outgrowths were characterized by positive immunocytochemical staining against α-smooth muscle actin and phase-contrast microscopy as previously described (24, 25). Cell cultures were maintained at 37°C in an atmosphere of 95% air and 5% CO_2. After reaching confluence on 100 mm-diameter dishes, cells were washed twice with phosphate-buffered saline (PBS) and they were incubated in serum-free DMEM. Cells were used between passages 4 and 12.

Isolation of Microsomes. Microsomal fractions were prepared from harvested cultured rat VSMC as previously described (21, 26). In brief, harvested cells were chilled to 4°C and were suspended in a buffer containing 300 mM sucrose, 10 mM HEPES, 0.1 mM EDTA, and 0.5 mM PMSF (pH 7.4) and homogenized by polytron (3 × 30 sec at setting 10). The homogenate was centrifuged for 10 min at 1600*g*, and the pellet was discarded. The supernatant was further centrifuged at 11,000*g* for 20 min, and the supernatant thus obtained was ultracentrifuged at 100,000*g* for 1 hr. The pellet was resuspended in a small volume of homogenizing buffer using a Dounce homogenizer. The microsomes were then either used fresh for measurement of $^{45}Ca^{2+}$ release, or were divided into aliquots, quickly frozen, and stored at −80°C for measurement of [^{3}H]-ryanodine binding (12). Storage at −80°C preserved the [^{3}H]-ryanodine binding capacity of microsomes. The protein content of fractions was determined by the method of Lowry *et al.* (27).

PCR Analysis. Total RNA was isolated from VSMC using the RNeasy Total RNA Isolation kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Reverse transcription and PCR amplification were performed using the GeneAmp System (Perkin Elmer, Branchburg, NJ). PCR analysis of RyR-1, RyR-2, and RyR-3 was performed essentially as previously described (28). Design of PCR primers was based upon GenBank accession numbers X83932 (RyR-1), X83933 (RyR-2), and X83934 (RyR-3) (28). The nucleotide sequence and length of expected PCR products for each primer pair are: RyR-1 (s), GAAGGTTCTGGACAAACACGGG; RyR-1 (as), TCGCTCTTGTTGTAGAATTTGCGG (435 bp); RyR-2 (s), GAATCAGTGAGTTACTGGGCATGG; RyR-2 (as), CTGGTCTCTGAGTTCTCCAAAAGC (635 bp); RyR-3 (s), AGAAGAGGCCAAAGCAGAGG; RyR-3 (as), GGAGGCCAACGGTCAGA (269 bp). All PCR products were was sequenced in both orientations. To exclude the possibility that genomic DNA rather than cDNA was amplified, control reactions were performed in which the reverse transcription step was omitted prior to PCR amplification. Additional negative control reactions were performed in which the template was omitted.

[^{3}H]-Ryanodine Binding. This was carried out as previously described (12, 29, 30). Microsomes (100–200 μg of protein) were incubated for 2 hr at 37°C in a medium containing (final concentration) 600 mM KCl, 100 μM EGTA, 150 μM Ca^{2+}, 0.2 mM PMSF, 25 mM HEPES (pH 7.2), and 30 nM [^{3}H]-ryanodine (54.7 Ci/mm). Free [^{3}H]-ryanodine was separated from [^{3}H]-ryanodine bound to microsomes by a rapid filtration technique using Whatman GF/B filters, followed by three subsequent washes with ice-cold water. The [^{3}H]-ryanodine radioactivity that remained

on the filters was measured by liquid scintillation counting. The high-affinity Ca^{2+}-dependent specific [^{3}H]-ryanodine binding was calculated as the difference of total binding and nonspecific binding, determined in the presence of RR (10 μM).

$^{45}Ca^{2+}$ Release from Microsomes. Freshly prepared microsomes (approximately 100 μg of protein) were passively loaded by incubating for 3 hr at room temperature (21°C) in a medium containing 100 mM NaCl, 25 mM HEPES (pH 7.2), 1 mM $CaCl_2$, and 1 μCi of $^{45}Ca^{2+}$. Release of $^{45}Ca^{2+}$ from loaded microsomes was initiated by 10-fold dilution of microsomal suspension with a buffer containing 100 mM NaCl, 1 mM EGTA, 1 mM $MgCl_2$, and 25 mM HEPES (pH 7.2), as previously described (26). After 10 sec, the suspension was further diluted in a medium of identical composition that contained agonists or antagonists to be tested. $^{45}Ca^{2+}$ efflux was stopped at 90 sec after the second dilution with added test agents. $^{45}Ca^{2+}$ retained in microsomes was separated from free $^{45}Ca^{2+}$ by rapid filtration using Whatman GF/B filters. The filters were rinsed three times with a solution containing 100 mM NaCl, 1 mM EGTA, 4 mM $MgCl_2$, 10 μM ruthenium red, and 25 mM HEPES (pH 7.2). The $^{45}Ca^{2+}$ retained in microsomes was determined by liquid scintillation counting.

Synthesis of NAADP. The NAADP synthetic capacity was assessed as previously described (21). In brief, membrane fraction of homogenized cells (0.3 mg/ml) was incubated with 0.2 mM β-NADP and 7 mM nicotinic acid at 37°C in a medium containing 0.25 M sucrose and 20 mM Tris HCl (pH 6.5) for 60 min. The content of NAADP was determined using a sea urchin egg homogenate Ca^{2+} release bioassay (21, 31).

Sea Urchin Egg Homogenate Bioassay. Homogenates from sea urchin eggs (*Lytechinus pictus*) were prepared as described previously (21, 31). Frozen homogenates were thawed in a 17°C water bath and diluted to 1.25% in a medium containing 2 U/ml creatine kinase, 4 mM phosphocreatine, 1 mM ATP, and 3 μM fluo-3. Fluo-3 fluorescence was monitored at 490 nm excitation and 535 nm emission in a 250-μl cuvette at 17°C with a circulating water bath and continuously mixed with a magnetic stirring bar using a Hitachi F-2000 spectrofluorimeter (Hitachi, Tokyo, Japan).

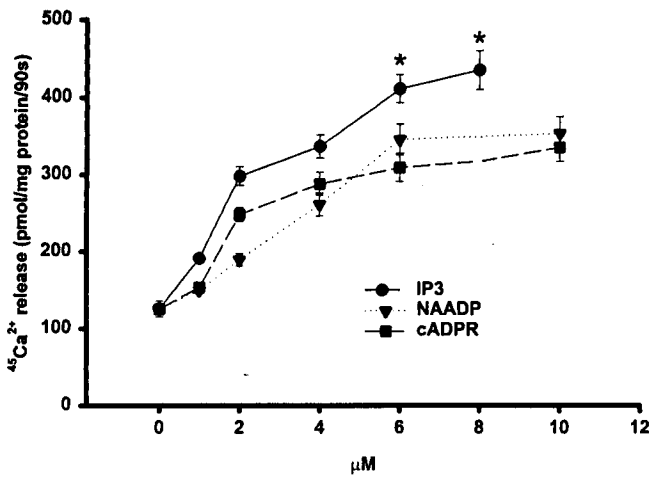

Figure 1. Dose-dependent effects of IP_3, ●; NAADP, ▼; and cADPR, ■ on Ca^{2+} release from VSMC microsomes. Data are representative of three independent experiments.

Statistical Analysis. Data presented are representative of at least three independent experiments performed in triplicate, as indicated in figure legends. Statistical analysis was performed using InStat (GraphPad, San Diego, CA). Group or pairwise comparisons were evaluated by the Students *t* test; *P* values of <0.05 were considered statistically significant.

Results

Dose-Dependent Ca^{2+} Release. IP_3, cADPR, and NAADP induced Ca^{2+} release from VSMC microsomes, indicating that VSMC possess elements of all three intracellular Ca^{2+} signaling pathways. Microsomal Ca^{2+} release by the agonists is dose-dependent. Maximal Ca^{2+} release by 10 μM cADPR and 10 μM NAADP was similar to that elicited by 8 μM IP_3 (Fig. 1).

Effect of Specific Ca^{2+} Release Antagonists. We further characterized specificity of microsomal Ca^{2+} release by IP_3, cADPR, and NAADP. Heparin (1 mg/ml), a specific IP_3 receptor blocker, abolished IP_3-elicited microsomal Ca^{2+} release. However, RR, an inhibitor of the ryanodine channel/receptor (RyR), failed to block IP_3-mediated microsomal Ca^{2+} release (Fig. 2A). cADPR-elicited microsomal Ca^{2+} release was completely blocked by coadministration of cADPR either with 8-bromo-cADPR (an antagonist of cADPR) (32) or with RR (a ryanodine channel blocker; Fig. 2B). Heparin, an IP_3 receptor blocker, had no effect on cADPR-elicited Ca^{2+} release (data not shown). NAADP-triggered Ca^{2+} release was not inhibited by heparin or by RR (Fig. 2C). β-NADP, a biosynthetic precursor of NAADP, failed to induce Ca^{2+} release from VSMC microsomes (Fig. 2C). Pretreatment of NAADP with alkaline phosphatase (25 U/mL) at 35°C for 10 min abolished its Ca^{2+} releasing activity (data not shown). These studies provide evidence that Ca^{2+} release from VSMC is specific for NAADP and not its biosynthetic precursor (β-NADP) or its degradation product (NAAD). Nifedipine, an L-type Ca^{2+} channel blocker, completely abolished NAADP-elicited Ca^{2+} release (Fig. 3). Nifedipine had no effect on IP_3- or cADPR-mediated Ca^{2+} release (Fig. 3).

Effect of pH on Ca^{2+} Release by IP_3, cADPR, and NAADP. In agreement with our previous findings in sea urchin eggs and our observations in cultured rat mesangial cells (14, 33), IP_3- or NAADP-induced Ca^{2+} release was not affected by changing the pH of the incubation buffer from 7.2 to 8.2 (Fig. 4). In contrast, the cADPR-induced Ca^{2+} release was inhibited by alkalinization of media (Fig. 4).

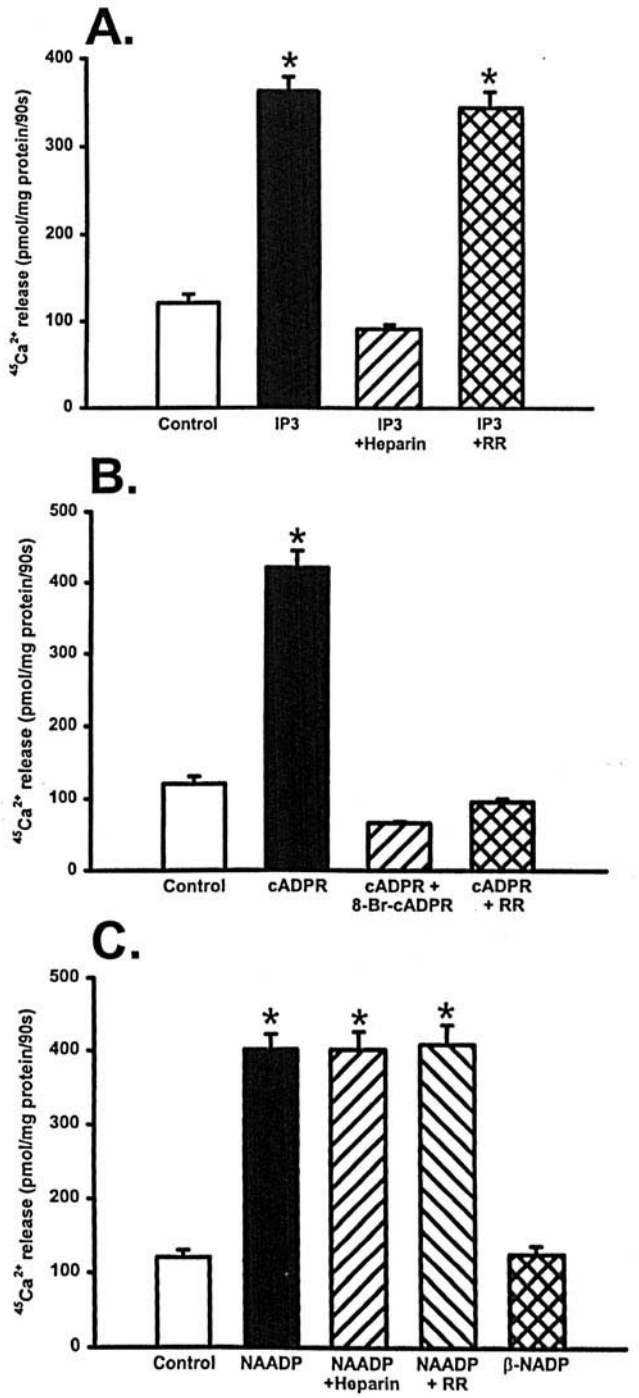

Figure 2. Effects of various calcium mobilizing agents and their antagonists on Ca^{2+} release from VSMC microsomes. (A) Specific Ca^{2+} release from VSMC microsomes by IP_3. Values are mean ± SEM ($n = 3$). *, Significantly different from control values (t test, $P < 0.05$). Open bar, control (no addition); closed bar, 8 μM IP_3; hatched bar, 8 μM IP_3 + 1 mg/ml heparin; double-hatched bar, 8 μM IP_3 + 10 μM ruthenium red. (B) Specific Ca^{2+} release from VSMC microsomes by cADPR. Values are mean ± SEM ($n = 3$). * = significantly different from control values (t test, $P < 0.05$); open bar, control (no addition); closed bar, 10 μM cADPR; hatched bar, 10 μM cADPR + 40 μM 8-Br-cADPR; double-hatched bar, 10 μM cADPR + 10 μM ruthenium red. (C) Specific Ca^{2+} release from VSMC microsomes by NAADP. Values are mean ± SEM ($n = 3$). * = significantly different from control values (t test, $P < 0.05$). Open bar, control (no addition); closed bar, 10 μM NAADP; first hatched bar, 10 μM NAADP + 1 mg/ml heparin; second hatched bar, 10 μM NAADP + 10 μM ruthenium red; double-hatched bar, 10 μM β-NADP.

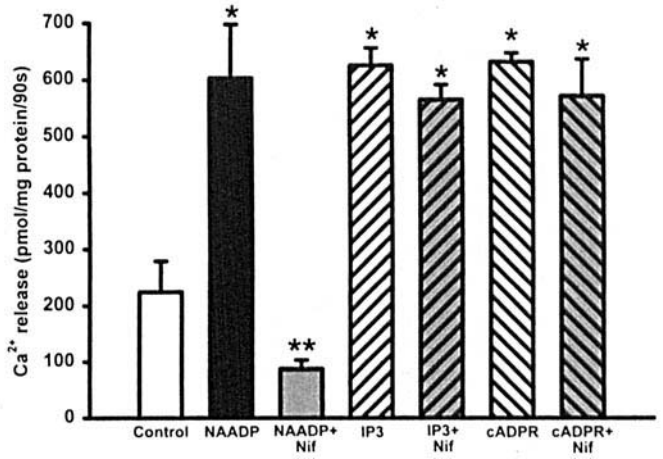

Figure 3. The effect of nifedipine, an L-type Ca^{2+} channel blocker, on $^{45}Ca^{2+}$ release from microsomes treated with NAADP, IP_3, or cADPR. Open bar, control; closed bar, 10 μM NAADP; gray bar, 10 μM NAADP + 100 μM nifedipine; first white hatched bar, 8 μM IP_3; first hatched gray bar, 8 μM IP_3 + 100 μM nifedipine; second white hatched bar, 10 μM cADPR; second hatched gray bar, 10 μM cADPR + 100 μM nifedipine. Values are mean ± SEM ($n = 3$). *, Significantly different from control values (t test, $P < 0.05$). **, Significantly different from both control and NAADP-treated values ($P < 0.01$).

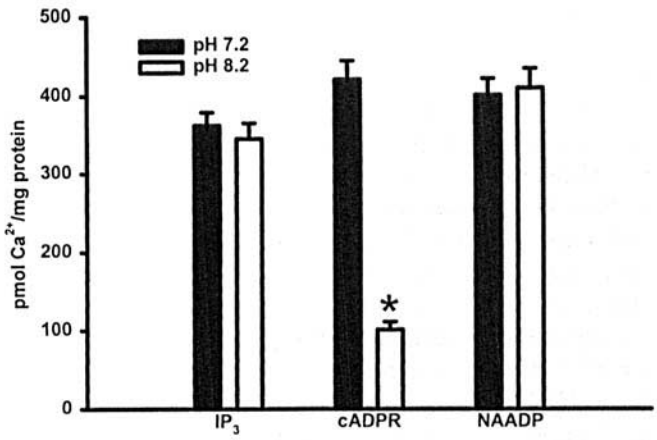

Figure 4. Effect of pH on Ca^{2+} release elicited by IP3, cADPR, and NAADP from VSMC microsomes. Ca^{2+} release from microsomes treated with 8 μM IP_3, 10 μM cADPR, or 10 μM NAADP was assessed as described in the "Materials and Methods." Data represent specific Ca^{2+} release by agonist minus Ca^{2+} release in controls, which was 121 ± 10 pmol/90 sec per mg of protein. Values are mean ± SEM. *, Values significantly different from values observed at pH 7.2 for three separate experiments.

Presence of Functional RyR in VSMC Microsomes. Previous studies have shown that intracellular Ca^{2+} release by cADPR is mediated by the RyR. We next sought to determine which isoforms of RyR are present in primary VSMC cultures and their role in microsomal Ca^{2+} release. RT-PCR studies revealed the presence of type 1 (RyR-1), type 2 (RyR-2), and type 3 (RyR-3) ryanodine receptors (Fig. 5). Sequence analysis of the PCR products revealed essentially 100% homology to the previously reported rat RyR-1, RyR-2, and RyR-3 sequences (see "Materials and Methods"). Negative control reactions in which the template was omitted or the reverse transcriptase step

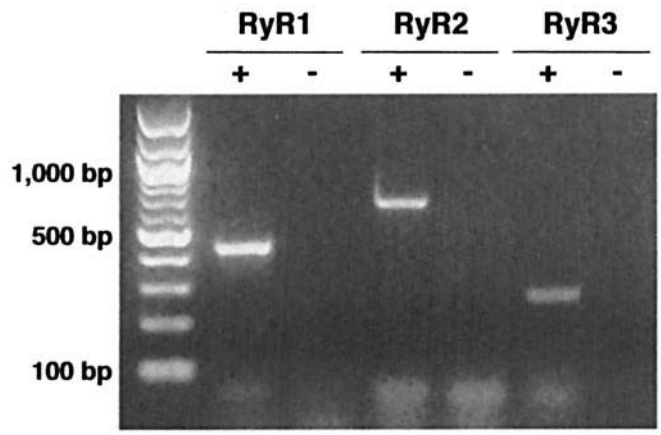

Figure 5. NRyR expression by VSMC. cDNA fragments of RyR-1, RyR-2, and RyR-3 were amplified from VSMC cDNA, as described in "Materials and Methods." VSMC express RyR-1 (435 bp), RyR-2 (635 bp), and RyR-3 (269 bp). – refers to negative control reaction in which the reverse transcriptase step was omitted prior to PCR amplification.

was not performed prior to PCR amplification failed to produce a product (Fig. 5). Assessment of Ca^{2+}-dependent, high-affinity [^{3}H]-ryanodine binding is a well-established method for detection and quantification of RyRs (15, 29, 30). We observed that [^{3}H]-ryanodine (30 nM) binds to VSMC microsomes with high affinity. [^{3}H]-ryanodine binding was Ca^{2+}-dependent and was blocked by 10 μM RR (Fig. 6A). Ca^{2+} (50 μM) enhanced [^{3}H]-ryanodine binding to VSMC microsomes by 76% compared with nonspecific binding in the absence of Ca^{2+} (Fig. 6A). This Ca^{2+}-dependent [^{3}H]-ryanodine binding was completely blocked by 10 μM RR, an RyR blocker (Fig. 6A). Further studies were undertaken to define the specificity of Ca^{2+}-dependent [^{3}H]-ryanodine binding (as defined by [^{3}H]-ryanodine binding to VSMC microsomes in the presence of 50 μM Ca^{2+} that is inhibited by 10 μM RR). We measured the binding in the presence of caffeine and CMP, established agonists of RyR (26, 30, 34). Both caffeine and CMP significantly enhanced Ca^{2+}-dependent specific [^{3}H]-ryanodine binding (Fig. 6B). Moreover, Ca^{2+}-dependent binding was inhibited by calmodulin (CaM), a protein that interacts with the RyR and appears to be required for cADPR-elicited Ca^{2+} release (35).

Effect of IP_3, cADPR, and NAADP on Ca^{2+}-dependent [^{3}H]-Ryanodine Binding. cADPR significantly inhibited Ca^{2+}-dependent [^{3}H]-ryanodine binding to VSMC microsomes (Fig. 7), whereas NAADP had no effect on [^{3}H]-ryanodine binding. IP_3 significantly augmented Ca^{2+}-dependent [^{3}H]-ryanodine binding to VSMC microsomes (Fig. 7).

The functional relevance of RyR in VSMC was determined by examining the release of Ca^{2+} from passively preloaded microsomes with $^{45}Ca^{2+}$. Both caffeine and CMP significantly enhanced Ca^{2+} release from VSMC microsomes (Fig. 8). The increment of Ca^{2+} release as well as enhanced [^{3}H]-ryanodine binding in response to both caffeine and CMP were all blocked by RR (Fig. 8).

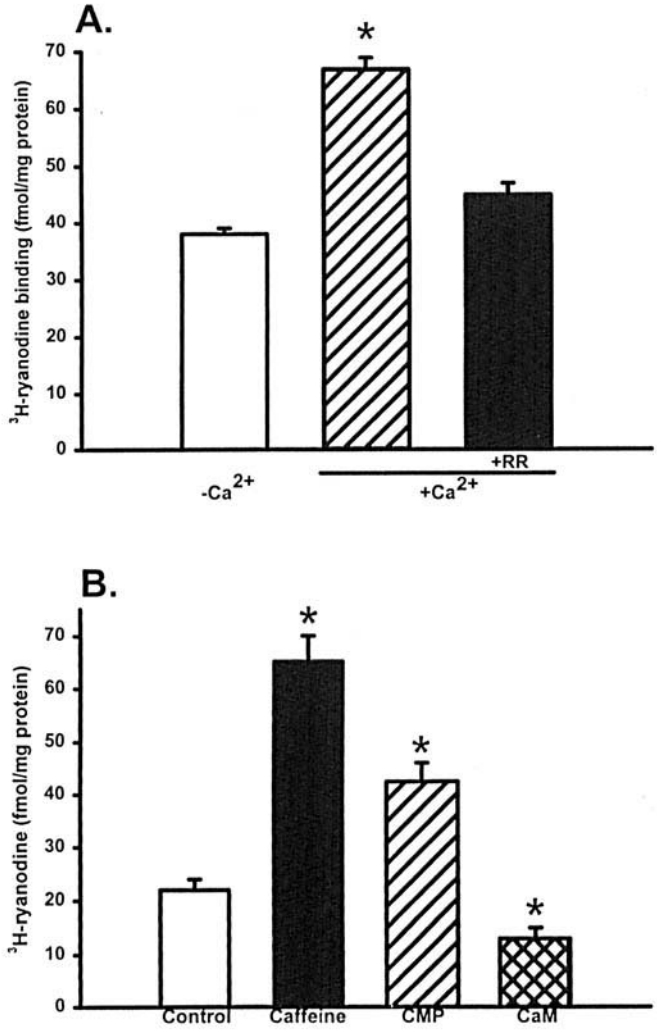

Figure 6. [^{3}H]-ryanodine binding to VSMC microsomes. (A) Specific [^{3}H]-ryanodine binding. The microsomes were incubated with 30 nM [^{3}H]-ryanodine (see "Materials and Methods" for details) without adding Ca^{2+} (control, open bar); or in the presence of 50 μM Ca^{2+} without (hatched bar) or with (closed bar) 10 μM ruthenium red. Each bar denotes mean ± SEM, n = 3 experiments. * denotes value significantly different from control (t test, $P < 0.05$). (B) The effect of RyR agonists or antagonists upon specific Ca^{2+}-dependent [^{3}H]-ryanodine binding to VSMC microsomes. All binding studies were performed in the presence of 50 μM Ca^{2+}. Open bar, control, no additions; closed bar, 20 mM caffeine; hatched bar, 0.5 mM 4-Cl-methyl-phenol (CMP); double-hatched bar, 5 μM calmodulin (CaM). * denotes values significantly different from control (t test, $P < 0.05$).

Synthesis of cADPR and NAADP by VSMC. Recently, we reported the ability of VSMC to synthesize cADPR by ADPR cyclase (20). However, the capacity for NAADP synthesis in primary VSMC cultures has not yet been documented. Membrane fractions of VSMC were incubated with β-NADP and nicotinic acid as described in "Materials and Methods" (21), and NAADP content was assessed by the sea urchin egg homogenate Ca^{2+} release bioassay (36). VSMC have a high capacity for NAADP synthesis, similar to that of rat mesangial cells, which are contractile cells with smooth muscle-like properties (37, 38) (Fig. 9).

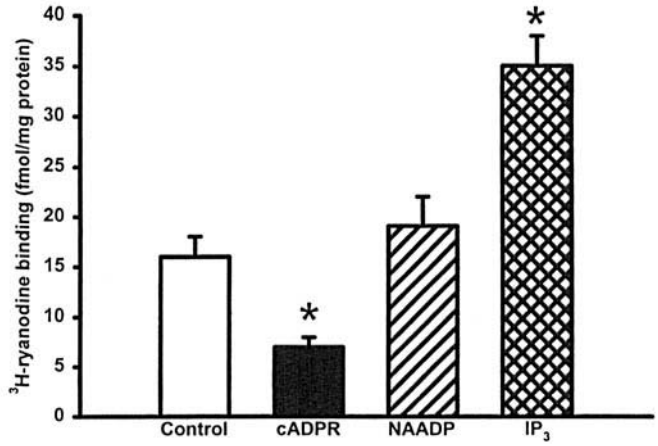

Figure 7. Effects of 10 μM cADPR, 10 μM NAADP, and 8 μM IP_3 on calcium-dependent [^{3}H]-ryanodine binding to VSMC microsomes. All binding studies were performed in the presence of 50 μM Ca^{2+}. Each bar denotes mean ± SEM, $n = 3$ experiments. * values are significantly different (t test, $P < 0.05$) from controls.

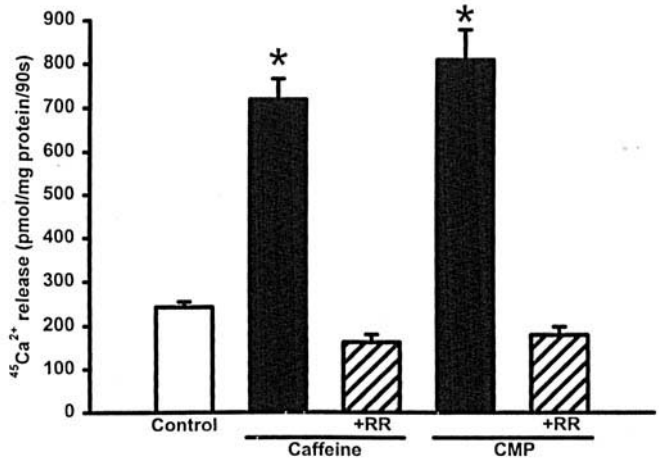

Figure 8. Microsomal Ca^{2+} release from VSMC by RyR agonists. Open bar, control; 20 mM caffeine without (first closed bar) or with (first hatched bar) 10 μM RR and 0.5 M 4-Cl-methyl-phenol (CMP) without (second closed bar) or with (second hacted bar) 10 μM ruthenium red. Each bar denotes mean ± SEM, $n = 3$ experiments. * values are significantly different (t test, $P < 0.05$) from controls.

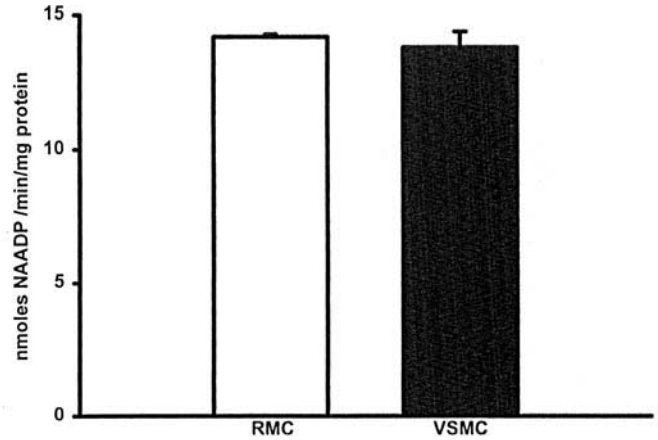

Figure 9. Biosynthesis of nicotinic acid adenine dinucleotide phosphate (NAADP) by rat mesangial cells (open bar) and VSMC (closed bar) using sea urchin egg homogenate bioassay as described in "Materials and Methods."

Discussion

Intracellular Ca^{2+} release is fundamental to many responses to environmental stimuli, including contractility, proliferation, apoptosis, and gene expression (39, 40). Studies of sea urchin eggs (7, 9, 10) and ascidian eggs (22) have led to the identification of three functionally distinct intracellular Ca^{2+} signaling pathways. Of the three, the IP_3-dependent mechanism has a ubiquitous distribution (41). In general, the IP_3 signaling system is involved in transduction of signals elicited by fast-onset, short-acting agents such as vasoactive peptides (2, 41). Since the initial description of the cADPR signaling pathway in sea urchin eggs (7, 42), more recent studies have shown that a variety of both contractile and noncontractile cells possess this signaling pathway (9, 11, 43). cADPR sensitizes RyR to Ca^{2+} and activates CICR (9, 10). cADPR production is increased by a variety of pro-inflammatory cytokines and hormones, including retinoic acid and triiodothyronine (12, 20). Unlike the IP_3 and cADPR Ca^{2+} release systems, much less is known about regulation of NAADP-elicited Ca^{2+} release in mammalian cells. It has been shown that NAADP-sensitive Ca^{2+} stores can be physically separated from intracellular Ca^{2+} stores sensitive to IP_3 and cADPR (9, 44). We have recently demonstrated that the capacity for NAADP-induced Ca^{2+} release may be widespread in mammalian cells (14). Cells possess many discrete intracellular Ca^{2+} stores (45, 46), which may be differentially regulated by these functionally distinct intracellular Ca^{2+} signaling pathways. However, the coexistence of all three signaling pathways has only been described in a few cell types, including sea urchin eggs, mouse pancreatic acinar cells, and human Jurkat T lymphocytes (46). The primary aim of this study was to directly compare these Ca^{2+} signaling pathways in primary cultures of VSMC.

We found that IP_3, cADPR, and NAADP elicited Ca^{2+} release in a dose-dependent fashion from VSMC microsomes, providing evidence that all three Ca^{2+} signaling pathways are present in cultured VSMC (Fig. 1). Specificity of Ca^{2+} release triggered by the IP_3, cADPR, and NAADP pathways was verified using specific agonists and antagonists (Figs. 2 and 3). It has been previously reported that Ca^{2+} release through intracellular Ca^{2+} channels can be regulated by pH (47). Ca^{2+} release from VSMC microsomes was differentially affected by IP_3, cADPR and NAADP at pH 7.2 and 8.2 (Fig. 4), providing further evidence that these Ca^{2+} agonists signal through functionally distinct pathways. Alkalinization of the media may alter the binding of cADPR to its receptor or may affect activation of RyRs by pharmacological agonists (48).

Previous studies in other cell systems have provided evidence that IP_3 and cADPR promote intracellular Ca^{2+} release through activation of different receptor complexes; the receptor involved in NAADP-elicited intracellular Ca^{2+} release has not yet been characterized. In VSMC, IP_3R-1 is

the predominant intracellular Ca^{2+} release channel activated through binding of IP_3 (49, 50). Several lines of evidence support the presence of functionally competent ryanodine receptors in VSMC (51). RyR-3 and possibly RyR-1 receptors have been identified in VSMC (52). By RT-PCR we have identified the presence of functional RyR-1, RyR-2, and RyR-3 in VSMC (Fig. 5). We found that [^{3}H]-ryanodine specifically binds to VSMC microsomes in a Ca^{2+}-dependent manner. Specificity of Ca^{2+}-dependent [^{3}H]-ryanodine binding was verified using established RyR agonists and antagonists.

We report here for the first time that [^{3}H]-ryanodine binding to VSMC microsomes is significantly enhanced by IP_3 (Fig. 7). This observation provides evidence that the IP_3 receptor complex can activate the ryanodine receptor/channel to facilitate Ca^{2+}-induced Ca^{2+} release (CICR) by cADPR or other agonists of RyR. IP_3 and cADPR may thereby complement each other in inducing Ca^{2+} release by CICR in VSMC microsomes.

We found that cADPR inhibits [^{3}H]-ryanodine binding to VSMC microsomes (Fig. 7). This observation would suggest that cADPR and ryanodine compete for binding to a similar site on the ryanodine receptor (10). However, in other cell systems, both stimulatory (53–55) and inhibitory (56) effects of cADPR on [^{3}H]-ryanodine binding have been described. Ca^{2+} release from VSMC microsomes was induced by RyR agonists (caffeine or CMP) and inhibited by RyR antagonists (RR), providing further evidence for the functional relevance of RyRs in VSMC. Unlike IP_3 and cADPR, NAADP had no effect on [^{3}H]-ryanodine binding (Fig. 7), again confirming that Ca^{2+} signaling by NAADP is independent of IP_3 and cADPR controlled Ca^{2+} signaling pathways. Previous studies have demonstrated that the enzyme ADP-ribosyl cyclase, which synthesizes cADPR from β-NAD, also catalyzes the production of NAADP from β-NADP (57, 58). Recent studies have shown that a variety of mammalian cells possess a capacity for NAADP biosynthesis, utilizing a reaction that requires high concentrations of β-NADP and nicotinic acid (14). The physiologic relevance of this pathway, or the presence of other pathways responsible for NAADP biosynthesis *in vivo*, remain to be established. In addition, the intracellular receptor for NAADP has not yet been characterized. However, widespread distribution of binding sites for NAADP has recently been reported in brain tissues (59).

In summary, we have established the presence of functionally distinct intracellular Ca^{2+} signaling pathways triggered by IP_3, cADPR, and NAADP in VSMC. Although the presence of all three intracellular Ca^{2+} signaling pathways has only been described in a few cell types (46), it is likely that future studies will document the coexistence of these pathways in a wide variety of both contractile and noncontractile cells. The functional relevance of intracellular Ca^{2+} release directed by IP_3, cADPR, and NAADP remains to be established, and may depend in large part upon cell type. For example, the IP_3 and cADPR pathways are functionally redundant during fertilization of sea urchin eggs (60, 61). In pancreatic acinar cells, the IP_3, cADPR, and NAADP pathways interact to coordinate apical release of secretory granules (62). IP_3R-1-deficient T cells are resistant to apoptosis in response to a variety of stimuli (39) and have defects at antigen-specific signaling (63), indicating that an intact IP_3 system is essential for these processes.

Abnormalities in intracellular Ca^{2+} signaling have recently been described in a variety of cardiovascular diseases. For example, end-stage heart disease is associated with downregulation of RyR-2 and an upregulation of IP_3R-1 (64). Angiotensin II-mediated hypertrophy of VSMC is dependent upon an increase in intracellular Ca^{2+} levels (23). The specific intracellular Ca^{2+} signaling pathway(s) responsible for this effect have yet to be elucidated. Future studies will define the role of IP_3, cADPR, and NAADP in normal VSMC function and response to pathobiologic stimuli.

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