Crucial Role of Type 2 Inositol 1,4,5-Trisphosphate Receptors for Acetylcholine-Induced Ca\(^{2+}\) Oscillations in Vascular Myocytes

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Objective—The aim of this study was to correlate the expression of InsP\(_3\)R subtypes in native vascular and visceral myocytes with specific Ca\(^{2+}\)-signaling patterns.

Methods and Results—By Western blot and immunostaining, we showed that rat portal vein expressed InsP\(_3\)R1 and InsP\(_3\)R2 but not InsP\(_3\)R3, whereas rat ureter expressed InsP\(_3\)R1 and InsP\(_3\)R3 but not InsP\(_3\)R2. Acetylcholine induced single Ca\(^{2+}\) responses in all ureteric myocytes but only in 50% of vascular myocytes. In the remaining vascular myocytes, the first transient peak was followed by Ca\(^{2+}\) oscillations. By correlating Ca\(^{2+}\) signals and immunostaining, we revealed that oscillating vascular cells expressed both InsP\(_3\)R1 and InsP\(_3\)R2 whereas nonoscillating vascular cells expressed only InsP\(_3\)R1. Acetylcholine-induced oscillations were not affected by inhibitors of ryanodine receptors, Ca\(^{2+}\)-ATPases, Ca\(^{2+}\) influx, and mitochondrial Ca\(^{2+}\) uniporter but were inhibited by intracellular infusion of heparin. Using specific antibodies against InsP\(_3\)R subtypes, we showed that acetylcholine-induced Ca\(^{2+}\) oscillations were specifically blocked by the anti-InsP\(_3\)R antibody. These data were supported by antisense oligonucleotides targeting InsP\(_3\)R2, which selectively inhibited Ca\(^{2+}\) oscillations.

Conclusions—Our results suggest that in native smooth muscle cells, a differential expression of InsP\(_3\)R subtypes encodes specific InsP\(_3\)-mediated Ca\(^{2+}\) responses and that the presence of the InsP\(_3\)R2 subtype is required for acetylcholine-induced Ca\(^{2+}\) oscillations in vascular myocytes. (Arterioscler Thromb Vasc Biol. 2003;23:1567-1575.)

Key Words: calcium ■ InsP\(_3\)-gated channel ■ Ca\(^{2+}\) oscillations ■ smooth muscle ■ acetylcholine

Stimulation of plasma membrane receptors results generally in the generation of inositol 1,4,5-trisphosphate (InsP\(_3\)) via the activation of phospholipase C. InsP\(_3\) releases Ca\(^{2+}\) from intracellular stores by binding to InsP\(_3\) receptors (InsP\(_3\)Rs), which form tetrameric InsP\(_3\)-gated Ca\(^{2+}\) release channels. Molecular cloning studies have revealed that there are at least 3 subtypes of InsP\(_3\)Rs derived from distinct genes, designated as subtype 1 (InsP\(_3\)R1), subtype 2 (InsP\(_3\)R2), and subtype 3 (InsP\(_3\)R3). Multiple subtypes of InsP\(_3\)Rs may be expressed in different cell types and form both homotetramers and heterotetramers. Therefore, expression of different InsP\(_3\)Rs may support the generation of specific Ca\(^{2+}\) responses. Recent studies using incorporation of InsP\(_3\)Rs into lipid bilayers and overexpression of recombinant InsP\(_3\)Rs have suggested that the 3 InsP\(_3\)R subtypes may be differentially regulated by InsP\(_3\) and Ca\(^{2+}\), calmodulin, ATP, and phosphorylation. In genetically engineered DT40 B cells that express either a single or a combination of InsP\(_3\)R subtypes, distinct patterns of Ca\(^{2+}\) signals have been reported. However, expression of InsP\(_3\)R subtypes have not been analyzed in different native cells under equivalent experimental conditions, and it has not been demonstrated that the differential expression of InsP\(_3\)R subtypes may be responsible for the different Ca\(^{2+}\) signal patterns recorded in situ.

The aim of the present study was to correlate the functional expression of InsP\(_3\)R subtypes with specific Ca\(^{2+}\)-signaling patterns in native vascular and visceral smooth muscle cells. Our results show that the functional InsP\(_3\)R subtypes expressed in rat portal vein and ureteric myocytes differ and may support different acetylcholine-induced Ca\(^{2+}\) responses, ie, Ca\(^{2+}\) oscillations and single Ca\(^{2+}\) responses.

Methods

Cell Preparation

The investigation conforms with the European Community and French guiding principles in the care and use of animals. Authorization to perform animal experiments was obtained from the French Ministère de l’Agriculture et de la Pêche.

Wistar rats (150 to 170 g) were killed by cervical dislocation. Isolated myocytes were obtained from portal vein and ureter by enzymatic dispersion, as previously described. Cells were seeded on glass slides in physiological solution and maintained in short-term primary culture in medium M199 containing FCS (2% for portal vein myocytes and 5% for ureteric myocytes), 2 mmol/L glutamine,
1 mmol/L pyruvate, 20 U/mL penicillin, and 20 μg/mL streptomycin; they were kept in an incubator gassed with 95% air and 5% CO₂ at 37°C and used either within 20 to 30 hours or within 2 to 4 days for cells injected with the antisense oligonucleotides.

Microinjection of Oligonucleotides

Phosphorothioate antisense oligonucleotides (denoted with the prefix “as”) used in the present study were designed on the known cloned InsP₃R sequences deposited in the GenBank sequence database with Lasergene software (DNASTAR). Sequences of all 3 InsP₃R CDAs were aligned with each other, and specific antisense oligonucleotide sequences were chosen in the region of the cDNA of interest, completely different from the sequences of the 2 other InsP₃R subtypes. Then antisense sequences displaying putative binding to any other mammalian sequences deposited in GenBank were discarded. Oligonucleotides were injected into the nuclei of myocytes by a manual injection system (Eppendorf). Intracellular oligonucleotide injection with Femtotips II (Eppendorf) was performed as previously described. The myocytes were then cultured for 2 to 4 days in culture medium, and the glass slides were transferred into the perfusion chamber for physiological experiments. The sequence of asInsP₃R1 is ATCTGTTGACTGTTGCC, corresponding to nucleotides 566 to 584 of cDNA; that of asInsP₃R2 is TATTTCA-CAATTTCTCC, corresponding to nucleotides 580 to 596 of InsP₃R₂ cDNA; and that of asInsP₃R3 is TGTGCAGAAGCTGAATCA, corresponding to nucleotides 494 to 513 of InsP₃R₃ cDNA.

Western Blot Analysis

Western blot analyses of rat tissue extracts and COS-1 cells (50 μg protein) were performed as previously described.¹⁶

Fluorescence Measurements

Measurements of [Ca²⁺], were performed with an indo-1 setup, as described elsewhere. Cells were either preloaded with the membrane-permeant indo-1 AM (1 μmol/L) for 30 minutes or 50 μmol/L indo-1 AM for 1 hour and was added to the pipette solution and entered the cells after establishment of the whole-cell recording mode. [Ca²⁺] was estimated from the 405/480-nm fluorescence ratio using a calibration determined within cells. All measurements were made at 25±1°C. Voltage clamp was made with a standard patch-clamp technique using a List EPC-7 patch-clamp amplifier (Darmstadt-Ebersbardt). Patch pipettes had resistances of 3 to 4 MΩ. Anti-InsP₃R₁, anti-InsP₃R₂, and anti-InsP₃R₃ antibodies were added to the pipette solution to allow dialysis of the cell after a breakthrough in whole-cell recording mode for at least 5 minutes, a time longer than that expected for diffusion of substances in solution.¹⁷

InsP₃R Immunostaining

Myocytes were washed with PBS, fixed with 4% (vol/vol) formaldehyde and 0.5% glutaraldehyde for 10 minutes at room temperature, and permeabilized in PBS containing 3% FCS and 1 mg/mL of saponin for 20 minutes. Cells were incubated with PBS, saponin (1 mg/mL), and anti-InsP₃R antibody (1 μg/mL) overnight at 4°C. Then cells were washed (4×5 minutes) and incubated with the secondary antibody conjugated to fluorescein-isothiocyanate during 45 minutes at room temperature. After washing in PBS, cells were mounted in Vectashield (Valbiotech). Images of the stained cells were obtained with a confocal microscope (Bio-Rad MRC 1024, Bio-Rad), and fluorescence was estimated by gray level analysis using IDL software (RSI) in 0.5-μm confocal sections. On each cell, fluorescence was acquired from a z-series analysis (20±5 sections) using Lasersharp software (Bio-Rad) and expressed by volume unit. Cells were compared by keeping acquisition parameters (such as gray scale, exposure time, iris aperture, gain, and laser power) constant. A similar protocol was applied to portal vein strips (longitudinal muscle) except that anti-InsP₃R₁ (Affinity BioReagents) and anti-InsP₃R₂ (Santa Cruz Biotechnology) antibodies were conjugated to Alexa 488 and Alexa 568 secondary antibodies, respectively.

Solutions

The physiological solution contained (in mmol/L) NaCl 130, KCl 5.6, MgCl₂, Cl₂, MgCl₂, glucose 11, and HEPES 10, pH 7.4, with NaOH. The basic pipette solution contained (in mmol/L) CsCl 130 and HEPES 10, pH 7.3, with CsOH. Acetylcholine and active compounds were applied to the recorded cell by pressure ejection for the period indicated on the records.

Chemicals and Drugs

Collagenase was obtained from Worthington. M199 medium, streptomycin, penicillin, and pyruvate were from Invitrogen. FCS was from BioMedia. Indo-1, indo-1 AM, and the mouse monoclonal anti-RYR antibody (559279) were from Calbiochem, Meudon, France. Anti-InsP₃R₁, anti-InsP₃R₂, and anti-InsP₃R₃ antibodies (SC6093, SC7278, and SC7277) were from Santa Cruz Biotechnology (Santa Cruz, Calif), from Affinity BioReagents (anti-InsP₃R₁ antibody, COGER, Paris, France), from AbCys (anti-InsP₃R₂ antibody, Valbiotech), and from Transduction Laboratories (anti-InsP₃R₃ antibody, BD Sciences, Le Pont de Claiix, France). Alexa 488 and Alexa 568 secondary antibodies were from Molecular Probes (Eugene, Ore). Anti-PCNA (proliferating cell nuclear antigen) antibody was from Chemicon (Temecula, Calif). All other products were from Sigma.

Data Analysis

Data are expressed as mean±SEM; n represents the number of cells or experiments. Significance was tested by means of paired Student’s t test when cells were their own control; otherwise, unpaired test was used. P<0.05 was considered significant.

Results

Ca²⁺ Responses Evoked by Acetylcholine

In portal vein myocytes, application of 1 μmol/L acetylcholine (ACH) for 30 seconds induced 2 types of Ca²⁺ responses. In 51% of cells (138 of 270 cells, 18 dissociations), ACH initiated a Ca²⁺ peak that was followed by regenerative oscillations (Figure 1A). The amplitude of the first response reached 218±9 nmol/L from a resting level of 66±11 nmol/L (n=138). The amplitude of oscillations varied strongly, but the frequency of oscillations was reproducible independently of the duration of ACH applications (4.0±0.4 oscillations/min, n=138). Although the percentage of oscillating cells was similar in the ACH concentration range from 1 nmol/L to 10 μmol/L, the highest and more reproducible frequency of oscillations was obtained at 1 μmol/L ACH (n=138), so that the following experiments were performed with this ACH concentration. The nonoscillating cells (49%) responded by single Ca²⁺ responses (Figure 1A). On application of ACH, the peak response reached 198±10 nmol/L from a resting level of 67±9 nmol/L (n=132) and was followed by a sustained plateau of 50±25 nmol/L (n=132). It is noteworthy that the duration at half-maximal amplitude of the first Ca²⁺ peak in oscillating cells (2.62±0.22 seconds, n=138) was smaller than that of single Ca²⁺ transients in nonoscillating cells (6.74±0.62 seconds, n=132). Similarly, application of noradrenaline (0.1 to 10 μmol/L) induced both Ca²⁺ oscillations and single Ca²⁺ transients in vascular myocytes (n=64). In contrast, in ureteric myocytes, Ca²⁺ oscillations were never observed after application of 0.1 to 10 μmol/L ACH (0 of 125 cells tested, 6 dissociations). As shown in Figure 1A, the amplitude of the transient Ca²⁺ response evoked by 1 μmol/L ACH was 368±17 nmol/L from a resting level of 72±9
nmol/L (n=92). This Ca²⁺ peak was followed by a sustained plateau of 41±15 nmol/L (n=92).

Effects of different pharmacological substances and external ion solutions were tested on both amplitude (first Ca²⁺ peak) and frequency of Ca²⁺ oscillations induced by 1 μmol/L ACh in vascular myocytes (Table). Ca²⁺ oscillation frequency was not affected after removal of external Ca²⁺ for 30 seconds (in Ca²⁺-free solution containing 0.5 mmol/L EGTA), but the responses decreased in amplitude within 3 to 4 minutes as a consequence of the rapid depletion of the intracellular Ca²⁺ store. Both amplitude of the first ACh-induced Ca²⁺ peak and frequency of oscillations were not significantly affected in the presence of 1 μmol/L oxodipine (a specific inhibitor of voltage-dependent Ca²⁺ channels) or 1 μmol/L RU-360 (a selective inhibitor of the mitochondrial Ca²⁺ uniporter). Although 10 μmol/L ryanodine or 10 μg/mL anti-ryanodine receptor antibody (inhibitors of ryanodine-sensitive Ca²⁺ release channels) had no effect on Ca²⁺ oscillation frequency, they decreased the amplitude of the Ca²⁺ peak, as previously reported. Depletion of the Ca²⁺ store can be modulated by thapsigargin (a Ca²⁺-ATPase inhibitor). Different concentrations of thapsigargin were applied for 1 minute before testing the effects of 1 μmol/L ACh. Thapsigargin inhibited in a concentration-dependent manner the ACh-induced Ca²⁺ responses (Figure 1B). Complete inhibition was obtained with 10 μmol/L thapsigargin, indicating that the store was empty. At 50 nmol/L, thapsigargin partially depleted the Ca²⁺ store, because the first ACh-induced Ca²⁺ peak was decreased by approximately 50% (Figure 1B). Under these conditions, the number of cells producing Ca²⁺ oscillations in response to ACh (9 of 17 cells tested) and the frequency of Ca²⁺ oscillations were not significantly different from those obtained in control conditions (Table, Figure 1C). However, a second application of ACh 5 minutes later was ineffective, as expected when the agonist-sensitive Ca²⁺ store was completely depleted. Finally, intracellular application of 1 mg/mL heparin (an inhibitor of InsP₃ Rs) for 5 minutes suppressed the ACh-induced Ca²⁺ responses in both vascular (n=14) and ureteric (n=12) myocytes. These results indicate that the ACh-mediated Ca²⁺ release occurs from the sarcoplasmic reticulum and that Ca²⁺ oscillations can be obtained from a partially depleted Ca²⁺ store.

To check the possibility that the capacitative Ca²⁺ entry could be different in the 2 types of vascular myocytes, Ca²⁺ entry was stimulated either by a pathway involving a Gq coupled receptor (ACh) or by thapsigargin in cells identified...
as oscillating or nonoscillating during previous applications of ACh. After complete depletion of the Ca\(^{2+}\) store by applications of 10 \(\mu\)mol/L ACh or 10 \(\mu\)mol/L thapsigargin in Ca\(^{2+}\)-free 0.5 mmol/L EGTA-containing solution, readmission of 2 mmol/L external Ca\(^{2+}\) caused a transient and significant increase in \([\text{Ca}^{2+}]_i\).\(^{20}\) In oscillating cells, peak Ca\(^{2+}\) responses activated by ACh and by store depletion were 98±17 nmol/L (n=17) and 106±11 nmol/L (n=18), respectively, whereas in nonoscillating cells, they were 100±14 nmol/L (n=19) and 101±14 nmol/L (n=43), respectively. In addition, the Ca\(^{2+}\) loading of the store was assessed by applications of 10 mmol/L caffeine after complete refilling of the Ca\(^{2+}\) store in 2 mmol/L external Ca\(^{2+}\) for 15 minutes. Peak amplitudes of caffeine-induced Ca\(^{2+}\) responses were not significantly different in oscillating (183±19 nmol/L, n=18) and nonoscillating (210±21 nmol/L, n=19) cells. Taken together, these results indicate that ACh-induced Ca\(^{2+}\) oscillations in vascular myocytes depend only on Ca\(^{2+}\) release from the sarcoplasmic reticulum through activation of InsP\(_3\) Rs and that both Ca\(^{2+}\) content of the intracellular store and capacitative Ca\(^{2+}\) entry were not different in oscillating and nonoscillating vascular myocytes.

Expression of InsP\(_3\)R Subtypes

Expression of InsP\(_3\)R subtypes was examined by using specific antibodies directed against InsP\(_3\)R1, InsP\(_3\)R2, and InsP\(_3\)R3 in rat portal vein and ureteric myocytes and in COS-1 cells. Western blot detection of InsP\(_3\)R subtypes (Figure 2A) indicated that InsP\(_3\)R1 and InsP\(_3\)R2 were expressed in portal vein myocytes and InsP\(_3\)R1 and InsP\(_3\)R3 in ureteric myocytes. As previously reported,\(^{21}\) COS-1 cells expressed InsP\(_3\)R2 and InsP\(_3\)R3 but not InsP\(_3\)R1. The molecular weight of each subtype was determined in 3 different experiments and corresponded to that expected from primary sequences.\(^4\)

Immunodetection of InsP\(_3\)R subtypes in confocal sections from ureteric and portal vein myocytes was performed with a second set of antibodies from Santa Cruz Biotechnology. With these antibodies, the binding sites were revealed with the same fluorescein-isothiocyanate–conjugated secondary antibody and the specificity was attested by the use of available antigenic peptides. Nonspecific fluorescence (NSF) was determined when specific anti-InsP\(_3\)R antibody was preincubated with its antigenic peptide 1 hour before application of the immunostaining protocol. When the cell fluorescence obtained with the anti-InsP\(_3\)R antibody was higher than NSF, the cell was considered as positive and specific fluorescence (F-NSF) could be estimated. The antigenic peptide for InsP\(_3\)R1 blocked the ability of the anti-InsP\(_3\)R1 antibody to bind to its target (n=41) but did not interfere with the activity of both anti-InsP\(_3\)R2 and anti-InsP\(_3\)R3 antibodies (n=41). Similarly, the antigenic peptides for InsP\(_3\)R2 and InsP\(_3\)R3 blocked the activity of the anti-InsP\(_3\)R2 and anti-InsP\(_3\)R3 antibodies, respectively, but did not interfere with the ability of the anti-InsP\(_3\)R1 antibody to bind to its target (n=32). Figure 2B illustrates typical immunostainings obtained in portal vein myocytes from the same dissociation with each of the anti-InsP\(_3\)R subtype antibodies. All of the cells were stained with the anti-InsP\(_3\)R1 antibody (Figure 2B; please see Figure IA, available in the online data supplement at http://atvb.ahajournals.org). In contrast, no specific staining was obtained with the anti-InsP\(_3\)R3 antibody. Staining with the anti-InsP\(_3\)R2 antibody revealed that only a fraction of myocytes expressed InsP\(_3\)R2 (7 of 15 cells; Figure 2B; please see online Figure IA). A similar distribution of cell fluorescence was obtained from 6 different dissociations (please see online Figure IB), supporting the idea that all of the cells expressed InsP\(_3\)R1 whereas approximately 50% of the cells expressed InsP\(_3\)R2. Quantitatively, specific fluorescence with the anti-InsP\(_3\)R1 antibody was observed in 271 of 288 vascular myocytes tested (6 dissociations), whereas specific InsP\(_3\)R2 immunostaining was detected in 231 of 452 cells tested (6 dissociations) and InsP\(_3\)R3 labeling was never observed (217 cells tested, 6 dissociations) (please see online Figure IC). In ureteric myocytes, specific InsP\(_3\)R1 and InsP\(_3\)R3 immunostainings were detected (Figure 2B), respectively, whereas in nonoscillating myocytes, InsP\(_3\)R2 labeling was never observed (96 cells tested, 5 dissociations). In COS-1 cells, specific immunostaining was obtained for InsP\(_3\)R2 and InsP\(_3\)R3 (in 58 and 55 of 60 cells tested, respectively) whereas InsP\(_3\)R1 staining was never observed (63 cells tested, 4 dissociations; please see online Figure IC). Immunostainings in confocal sections of intact portal vein muscle revealed that InsP\(_3\)R2 was not expressed in all of the myocytes compared with InsP\(_3\)R1 (Figure 2C). These results confirm that immunostainings with the anti-InsP\(_3\)R antibodies are specific for the corresponding InsP\(_3\)R subtypes and reveal a differential expression of InsP\(_3\)R subtypes in vascular and ureteric myocytes.

The fact that the InsP\(_3\)R2 subtype was detected by immunostaining in approximately 50% of all of the vascular cells tested prompted us to verify the expression of InsP\(_3\)R1 and InsP\(_3\)R2 in myocytes showing either Ca\(^{2+}\) oscillations or single Ca\(^{2+}\) responses. We found that all of the oscillating vascular myocytes expressed both InsP\(_3\)R1 and InsP\(_3\)R2 (n=116; 5 dissociations), whereas the nonoscillating myocytes expressed only InsP\(_3\)R1 (n=121; 5 dissociations).

Specific InsP\(_3\)R Isoforms Required for Ca\(^{2+}\) Oscillations

To assess the role of InsP\(_3\)R subtypes in Ca\(^{2+}\) oscillations, we used both subtype-specific antibodies and inhibition of InsP\(_3\)R subtypes by an antisense strategy. For the antibody experiments, the following protocol was performed. On each myocyte, ACh (1 \(\mu\)mol/L) was first ejected in non–voltage-clamped conditions to detect the oscillating cells, and the parameters of the Ca\(^{2+}\) responses were measured. Then, voltage-clamp conditions were established and a second application of ACh was applied 5 minutes later on the same cells maintained at a holding potential of −50 mV. In portal vein myocytes, the peak Ca\(^{2+}\) response corresponding to the first ACh application (in non–voltage-clamped cells) was 270±39 nmol/L from a resting [Ca\(^{2+}\)] level of 66±8 nmol/L (n=19). The amplitude of the second ACh-induced Ca\(^{2+}\) response (in voltage-clamped cells) was 225±21 nmol/L from a resting level of 69±9 nmol/L (n=19). Similarly, the mean frequency of Ca\(^{2+}\) oscillations during the second application of ACh was 4.2±0.8 oscillations/min, not differ-
ent from that obtained during the first application of ACh (4.0±0.8 oscillations/min, n=19). Therefore, this protocol was used to examine the role of the InsP₃ R subtypes on the ACh-induced Ca²⁺ responses by infusing the cells through the patch pipette with the specific InsP₃ R subtype antibodies for 5 minutes.

We tested the anti-InsP₃ R antibodies in nonoscillating vascular cells expressing only InsP₃ R1 to determine the effective concentrations of these antibodies. In nonoscillating vascular cells, the single Ca²⁺ responses were inhibited in a concentration-dependent manner by intracellular applications of the anti-InsP₃ R1 antibody with maximal inhibition obtained at 1 μg/mL (please see online Figures IIA and IIB), whereas both anti-InsP₃ R2 and anti-InsP₃ R3 antibodies had no significant effects at concentrations up to 10 μg/mL (please see online Figures IIC and IID). The inhibitory effect of the anti-InsP₃ R1 antibody is specific, as shown by the absence of effect of boiled (90°C for 30 minutes) anti-InsP₃ R1 antibody (see online Figure IIB) or antibody preincubated with its antigenic peptide (not shown). In oscillating vascular myocytes, intracellular application of the anti-InsP₃ R1 antibody inhibited the amplitude of Ca²⁺ peaks, but oscillations were detected in all the cells tested (Figure 3A and 3B). Interestingly, in the presence of anti-InsP₃ R1 antibody, the frequency of Ca²⁺ oscillations was not significantly affected (control, 4.1±0.5 oscillations/min; in the presence of the antibody, 3.9±0.4 oscillations/min; n=6). Intracellular applications of anti-InsP₃ R2 antibody inhibited both ampli-

Figure 2. Expression of InsP₃ R subtypes in portal vein, ureteric, and COS-1 cells. A, Microsomal proteins (50μg) were separated by SDS/PAGE and analyzed by Western blot with anti-InsP₃ R1 (1:1000, Affinity BioReagents), anti-InsP₃ R2 (1:100, AbCys), and anti-InsP₃ R3 (1:1000, Transduction Laboratories) antibodies. Molecular weights are in kilodaltons. Similar results were obtained from 3 different experiments. B, Typical confocal cell sections showing a differential expression of InsP₃ R subtypes in portal vein (PV1 and PV2) and ureteric (Ur) myocytes. Anti-InsP₃ R subtype antibodies were from Santa Cruz Biotechnology. C, Confocal micrographs of rat portal vein sections (longitudinal muscle) costained with the anti-InsP₃ R1 and anti-InsP₃ R2 antibodies (Santa Cruz Biotechnology).
ACh was able to induce a single Ca\(^{2+}\) InsP\(_3\) R3 in vascular myocytes. Similar results were obtained (not illustrated), in agreement with the absence of detection of asInsP3 R to inhibit the expression of InsP3 R isoform and the cleotides efficiency was determined by checking the ability of

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\frac{\Delta [Ca^{2+}]_{\text{m}}}{\text{with/without Ab}}
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bars are obtained with boiled antibody.

Figure 3. Effects of anti-InsP3R1 and anti-InsP3R2 antibodies on Ca\(^{2+}\) oscillations evoked by ACh (1 \(\mu\)mol/L) in portal vein myocytes. A, Typical recordings showing the effect of 1 \(\mu\)g/mL anti-InsP3R1 or anti-InsP3R2 antibody on Ca\(^{2+}\) oscillations. B, Compiled data showing the effect of increasing concentrations of the anti-InsP3R1 antibody (Affinity BioReagents) on the amplitude of the first Ca\(^{2+}\) peak and the percentage of oscillating cells. Filled bars are obtained with boiled antibody. \(\Delta [Ca^{2+}]\) is expressed as the ratio of measurements obtained in the presence and absence of anti-InsP3R1 antibody. Data are mean±SEM, with the number of cells indicated in parentheses (*, \(P<0.05\)).

Reverse transcriptase–polymerase chain reaction performed on RNA extracts prepared from rat portal vein and ureteric myocytes confirmed the expression of the different InsP,R subtypes, as identified by Western blotting. Therefore, we designed antisense oligonucleotides specifically targeting each InsP,R subtype. The time course of antisense oligonucleotides efficiency was determined by checking the ability of asInsP-R to inhibit the expression of InsP-R isoform and the amplitude of ACh-induced Ca\(^{2+}\) responses in vascular myocytes. Immunostaining with the anti-InsP,R1 antibody was

maximally decreased 2 days after nuclear injection of 10 \(\mu\)mol/L asInsP,R1 in vascular myocytes located in a marked area of glass slides, whereas noninjected cells outside this marked area (used as controls) were normally immunostained (please see online Figure IIIA). Recovery began the next day, and staining of injected cells returned to control 4 days after injection of asInsP,R1. Increasing asInsP,R1 concentration to 20 \(\mu\)mol/L had no additional inhibitory effect on InsP,R1 staining (n=17). Based on this time scale, we verified that the Ca\(^{2+}\) responses were maximally decreased 2 days after injection of asInsP,R1 plus asInsP,R2 (10 \(\mu\)mol/L, each) and recovery began the next day (please see online Figure IIIB). Non-specific effects of antisense oligonucleotides were detected only at concentrations higher than 50 \(\mu\)mol/L (n=10). We also verified that each InsP,R subtype was specifically decreased by the corresponding asInsP,R 2 days after injection. Vascular myocytes injected with asInsP,R2 or asInsP,R3 were normally stained with the anti-InsP,R1 antibody (please see online Figure IIIC). In cells injected with asInsP,R, staining with the anti-InsP,R2 antibody was strongly reduced in immunopositive cells, which represented approximately 50% of the cells examined (please see online Figure IIDD). In contrast, in asInsP,R1- and asInsP,R3-injected cells, stainings with the anti-InsP,R2 antibody were similar to those obtained in noninjected cells (please see online Figure IIDD). These results indicate that the antisense oligonucleotides were efficient and specific in inhibiting expression of InsP,R subtypes within 2 days after nuclear injection and that they could be used to study the role of each InsP,R subtype on ACh-induced Ca\(^{2+}\) responses. In vascular myocytes injected with 10 \(\mu\)mol/L asInsP,R1, the amplitude of the first Ca\(^{2+}\) peak evoked by 1 \(\mu\)mol/L ACh was reduced by approximately 50% compared with noninjected cells, but oscillations were observed in most of the cells tested (41 of 50 cells, Figure 4). In cells injected with asInsP,R2, the amplitude of the first Ca\(^{2+}\) peak was slightly reduced, but the percentage of oscillating cells was strongly inhibited (7 of 72 cells). No effect on the Ca\(^{2+}\) peak and the percentage of oscillating cells (26 of 50 cells) was observed in cells injected with asInsP,R3 (Figures 4B and 4C). The frequency of oscillations in asInsP,R1-injected cells (4.0±0.3 oscillations/min, n=41) was not different of that in noninjected cells (4.3±0.2 oscillations/min, n=82). All of the cells used induced large Ca\(^{2+}\) responses when 10 mmol/L caffeine was added at the end of this protocol. Cell proliferation was not detected with the anti-PCNA antibody in both freshly dissociated and cultured vascular myocytes (n=250).

Taken together, these results suggest that Ca\(^{2+}\) oscillations in vascular myocytes require the presence of InsP,R2 and that InsP,R1 alone or both InsP,R1 and InsP,R3 are unable to produce Ca\(^{2+}\) oscillations in vascular and ureteric myocytes, respectively.

Discussion

The results of the present study establish the functional role of InsP,R subtypes in native smooth muscle cells in response to acetylcholine. We obtained evidence that endogenous expression of InsP,R2 was critical for Ca\(^{2+}\) oscillations in rat portal vein myocytes, whereas endogenous expression of
InsP,R1 alone or both InsP,R1 and InsP,R3 was responsible for single Ca\\(^{2+}\) responses in vascular and ureteric myocytes, respectively.

For studying the role of each InsP,R subtype in various smooth muscle cells, we used 2 complementary methods, ie, specific anti-InsP,R antibodies and antisense oligonucleotides, which specifically inhibited the expression of each InsP,R subtype. Specificity of the anti-InsP,R antibodies is based on the following observations: (1) in different cell types, Western blotting reveals a differential expression of InsP,R subtypes; (2) similar differential immunostainings were obtained in the same cells with these anti-InsP,R antibodies; (3) immunostaining for 1 anti-InsP,R antibody is blocked by the COOH-terminal antigenic peptide of this antibody but not by any other antigenic peptides; (4) in functional experiments, these antibodies neutralized by their antigenic peptides or by heating (95°C for 30 minutes) have no effect on ACh-induced Ca\\(^{2+}\) responses in 2 different smooth muscle cell types; and (5) the inhibitory effects of each anti-InsP,R antibody on the Ca\\(^{2+}\) responses are concentration-dependent, with a maximal effect of approximately 1 µg/mL. For the antisense strategy, appropriate controls have been carried out to demonstrate the efficiency of antisense oligonucleotides. We evaluated the specificity of antisense oligonucleotides to reduce the expression of the InsP,R subtypes labeled with the specific antibodies by showing (1) the specific inhibition of the targeted InsP,R subtype by 1 given antisense oligonucleotide and the lack of effect of this antisense oligonucleotide on the other InsP,R subtypes; (2) the lack of effect of antisense oligonucleotides against InsP,R3 in myocytes that did not express this subtype; and (3) the reduction of the InsP,R-mediated Ca\\(^{2+}\) response produced by each effective oligonucleotide compared with the suppression of Ca\\(^{2+}\) response after injection of a mixture of oligonucleotides targeting the expressed InsP,R subtypes.

Ca\\(^{2+}\) oscillations can be supported by different Ca\\(^{2+}\) channels and Ca\\(^{2+}\) stores.\(^{14,22-24}\) We showed that the pharmacological inhibition of the mitochondrial Ca\\(^{2+}\) uniporter, RYR, voltage-gated Ca\\(^{2+}\) channels, and Ca\\(^{2+}\) influx had no effect on ACh-induced Ca\\(^{2+}\) oscillations in rat portal vein myocytes. Because inhibition of InsP,R2 by specific antibodies and antisense oligonucleotides suppressed these responses, InsP,R2 seems necessary to support the ACh-induced Ca\\(^{2+}\) oscillations. In vascular myocytes, distribution of InsP,R2 has been found at the plasma membrane and associated with the nucleus in proliferating cells.\(^{25,26}\) We show that freshly dissociated and short-term cultured rat portal vein myocytes express InsP,R1 whereas only 50% of the cells express InsP,R2 and generate Ca\\(^{2+}\) oscillations. It is unlikely that these 2 populations of myocytes correspond to cells being in different states of development, because staining with the anti-PCNA antibody was not detected in freshly dissociated and short-term cultured myocytes. Immunostaining of intact vascular muscle revealed that both InsP,R1 and InsP,R2 were expressed and that some cells expressed only InsP,R1, confirming the results obtained in dissociated vascular myocytes.

Targeted gene knockouts of InsP,R subtypes in DT40 B cells have revealed that cells expressing different InsP,R subtypes may show distinct patterns of Ca\\(^{2+}\) signaling.\(^{13}\) When expressed singly, activation of InsP,R1 or InsP,R3 triggers 1 or 2 Ca\\(^{2+}\) peaks, whereas activation of InsP,R3 supports long-lasting, regular Ca\\(^{2+}\) oscillations.\(^{15}\) In native cells, such as hepatocytes, cardiac myocytes, and pancreatic islets that show Ca\\(^{2+}\) oscillations,\(^{27-29}\) the presence of InsP,R has been demonstrated in separate experiments.\(^{30}\) With specific antibodies and antisense oligonucleotides, we demonstrate that native vascular myocytes expressing only InsP,R2 may generate Ca\\(^{2+}\) oscillations of small amplitude, confirming the results obtained in DT40 B cells. In agreement with these data, in native cells that do not express InsP,R2, like A7r5 cells\(^{31}\) or ureteric myocytes (the present study), InsP,R-mediated Ca\\(^{2+}\) oscillations have not been observed. Conversely, Ca\\(^{2+}\) oscillations were observed in cells expressing a high proportion of InsP,R2 (RBL-2H3 mast cells\(^{32}\) or ex-
pressing both InsP,R2 and InsP,R3 (NIH-3T3 cells). In HeLa cells that show Ca\(^{2+}\) oscillations with a low expression of InsP,R2, it has been shown that these oscillations are dependent on the extracellular Ca\(^{2+}\) concentration, suggesting that Ca\(^{2+}\) influx may be involved in triggering Ca\(^{2+}\) oscillations in these cells.

Our results support a model in which Ca\(^{2+}\) oscillations depend on Ca\(^{2+}\) release from the intracellular store through both InsP,R1 and InsP,R2. Because InsP,R1 alone is unable to trigger Ca\(^{2+}\) oscillations, it is likely that the properties of InsP,R2 are critical for these oscillations. InsP,R2 has a higher InsP\(_i\) sensitivity than InsP,R1. In native cells expressing predominantly InsP,R2, a sustained inhibition is obtained by increasing [Ca\(^{2+}\)]\(_i\), a result that is not found in studies using planar lipid bilayers. In contrast, InsP,R1 inhibition by high [Ca\(^{2+}\)]\(_i\), has been reported in both bilayers and native cells. Because our preliminary experiments using GFP-PH PLC \(_\text{G12}\), expression indicate that the InsP\(_i\) production in vascular myocytes does not oscillate in response to 1 \(\mu\)mol/L ACh, we speculate that the Ca\(^{2+}\)-dependent inhibition of InsP,R2 may be important in the rapid fall of [Ca\(^{2+}\)]\(_i\), during the first peak, as revealed by the duration at half-maximal amplitude of the first Ca\(^{2+}\) peak, which is shorter in oscillating cells than in nonoscillating cells. Because [Ca\(^{2+}\)]\(_i\), decreases near basal value, InsP,R2 can reopen, and a second Ca\(^{2+}\) peak is initiated with the concomitant participation of InsP,R1. The InsP\(_i\)- and Ca\(^{2+}\)-dependences of InsP,R1 and InsP,R2 are under investigation in native vascular myocytes, because intracellular accessory proteins can strongly modulate InsP,R activity.

The physiological role of InsP,R2 has not been previously examined in freshly dissociated and short-term cultured vascular myocytes. However, in agonist-stimulated adult cardiomyocytes, it has been reported that activation of InsP,R may trigger additional action potentials leading to arrhythmias. Moreover, intracellular Ca\(^{2+}\) oscillations have been shown to support spontaneous contractions during early stages of cardiomyogenesis, before complete expression of ion channels. It is tempting to speculate that vascular myocytes expressing both InsP,R1 and InsP,R2 may function as pacemaker cells, initiating and modulating rhythmic electrical and mechanical activities in portal vein. We have previously shown that in these cells, InsP\(_i\) is generated under basal conditions and that the InsP\(_i\) production is strongly increased by neurotransmitters. Because vascular myocytes are electrically coupled by gap junctions, a small fraction of pacemaker cells activated by the basal InsP\(_i\) concentration may trigger the spontaneous contractile activity of the vessel. When neurotransmitters are delivered, the frequency of rhythmic contractions is strongly increased, as expected from an increase in the InsP\(_i\) production, which can stimulate Ca\(^{2+}\) oscillations in all the cells expressing both InsP,R1 and InsP,R2.

In conclusion, the differential expression of InsP,R subtypes seems a determinant factor in the generation of specific Ca\(^{2+}\)-signaling patterns in native smooth muscle cells.

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Crucial Role of Type 2 Inositol 1,4,5-Trisphosphate Receptors for Acetylcholine-Induced Ca\(^{2+}\) Oscillations in Vascular Myocytes

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Figure I on-line

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Figure I. Immunostaining of InsP₃R subtypes in portal vein, ureteric and COS-1 cells. Fluorescence was estimated by grey level measurements from z-series of confocal cell sections and expressed by volume unit in the presence of the specific anti-InsP₃R subtype antibody alone (F) or after a pre-incubation with its antigenic peptide for 1 h (NSF). (A) Fluorescence of vascular myocytes obtained with the three anti-InsP₃R subtype antibodies in a typical microscope field and expressed as F (filled symbols) and NSF (open symbols). (B) Distribution of fluorescence expressed as F (filled bars) and NSF (open bars) in vascular myocytes from 6 different dissociations. (C) Specific fluorescence (F-NSF) obtained with the three anti-InsP₃R subtype antibodies in portal vein (PV), ureteric (Ur) and COS-1 cells. Data are means ± SEM with the number of positive cells/total number of cells tested indicated in parentheses. Cells were stained with anti-InsP₃R antibodies from Santa Cruz Biotechnology.
Figure II on-line
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Figure II. Effects of anti-InsP₃R antibodies on the single Ca²⁺ responses evoked by ACh (1 µM) in non-oscillating portal vein myocytes. (A) Typical recordings showing the effect of the anti-InsP₃R1 antibody (1 µg/ml) on single Ca²⁺ response. (B-D) Compiled data showing the effect of increasing concentrations of anti-InsP₃R1 antibody (B, Affinity BioReagents), anti-InsP₃R2 antibody (C, AbCys) and anti-InsP₃R3 antibody (D, Transduction Laboratories) on Ca²⁺ peaks. Results are expressed as the ratio of Ca²⁺ measurements obtained in presence and absence of antibodies. Filled bar is obtained with boiled antibody. Data are means ± SEM with the number of cells indicated in parentheses (★, p < 0.05).
Figure III on-line

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Figure III. Efficiency of antisense oligonucleotides directed against mRNAs of the three InsP₃R subtypes in portal vein myocytes. (A) Time course of inhibition of specific staining measured with the anti-InsP₃R1 antibody in cells injected with 10 µM asInsP₃R1 (filled bars) and in non-injected cells (open bars). (B) Time course of inhibition of ACh-induced Ca²⁺ responses in cells injected with a mixture of asInsP₃R1 + asInsP₃R2 (10 µM each, hatched bars) and in non-injected cells (open bars). (C) Staining with anti-InsP₃R1 antibody in non-injected cells (control) and in cells injected with 10 µM asInsP₃R1, asInsP₃R2 or asInsP₃R3. (D) Staining with anti-InsP₃R2 antibody in non-injected cells (control) and in cells injected with 10 µM of asInsP₃R2, asInsP₃R1 or asInsP₃R3. Anti-InsP₃R antibodies were from Santa Cruz Biotechnology. Data are means ± SEM with the number of positive cells/total number of cells tested indicated in parentheses (★, p < 0.05).
On line materials

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