Ca^{2+} signals mediated by Ins(1,4,5)P_3-gated channels in rat ureteric myocytes

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Localized Ca^{2+}-release signals (puffs) and propagated Ca^{2+} waves were characterized in rat ureteric myocytes by confocal microscopy. Ca^{2+} puffs were evoked by photorelease of low concentrations of Ins(1,4,5)P_3 from a caged precursor and by low concentrations of acetylcholine; they were also observed spontaneously in Ca^{2+}-overloaded myocytes. Ca^{2+} puffs showed some variability in amplitude, time course and spatial spread, suggesting that Ins(1,4,5)P_3-gated channels exist in clusters containing variable numbers of channels and that within these clusters a variable number of channels can be recruited. Immunodetection of Ins(1,4,5)P_3 receptors revealed the existence of several spots of fluorescence in the confocal cell sections, supporting the existence of clusters of Ins(1,4,5)P_3 receptors. Strong Ins(1,4,5)P_3 photorelease and high concentrations of acetylcholine induced Ca^{2+} waves that originated from an initiation site and propagated in the whole cell by spatial recruitment of neighbouring Ca^{2+}-release sites. Both Ca^{2+} puffs and Ca^{2+} waves were blocked selectively by intracellular applications of heparin and an anti-Ins(1,4,5)P_3-receptor antibody, but were unaffected by ryanodine and intracellular application of an anti-ryanodine receptor antibody. mRNAs encoding for the three subtypes of Ins(1,4,5)P_3 receptor and subtype 3 of ryanodine receptor were detected in these myocytes, and the maximal binding capacity of [^{3}H]Ins(1,4,5)P_3 was 10- to 12-fold higher than that of [^{3}H]-ryanodine. These results suggest that Ins(1,4,5)P_3-gated channels mediate a continuum of Ca^{2+} signalling in smooth-muscle cells expressing a high level of Ins(1,4,5)P_3 receptors and no subtypes 1 and 2 of ryanodine receptors.

Key words: calcium puff, calcium-release channel, confocal microscopy, sarcoplasmic reticulum, smooth muscle.

INTRODUCTION

The release of Ca^{2+} ions from the intracellular stores into the cytosol plays a central role in the control of many cellular activities in excitable and non-excitable cells. Ins(1,4,5)P_3 receptors and ryanodine receptors represent the two major types of intracellular Ca^{2+}-release channels, and share some functional similarities. Global Ca^{2+} signals in intact cells have been reported to be due to the co-ordinated recruitment of 'elementary' Ca^{2+}-release units [1,2], inducing either Ca^{2+} sparks when the units contain mainly ryanodine receptors, i.e. in cardiac and vascular myocytes [3–5], or Ca^{2+}-release signals, or 'puffs', when the units contain mainly Ins(1,4,5)P_3 receptors, i.e. in *Xenopus* oocytes and HeLa cells [6,7]. Elementary Ca^{2+} events are spatially localized, short transient signals that dissipate rapidly due to diffusion of Ca^{2+} into the cytoplasm and sequestration into the intracellular stores.

In smooth muscles, it is believed generally that Ca^{2+} release from the intracellular store can be generated by Ins(1,4,5)P_3 and ryanodine receptors [8]. In rat portal-vein myocytes, there are indications that these two Ca^{2+}-release channels are co-localized on the same intracellular store [9]. In these myocytes, ryanodine receptors are highly expressed and organized in clustered units, so that they can generate Ca^{2+} sparks. In contrast, Ins(1,4,5)P_3 receptors are not organized in clustered units and Ins(1,4,5)P_3-evoked Ca^{2+} puffs have not been observed in portal-vein myocytes [9]. Up to now, Ca^{2+} puffs activated by Ins(1,4,5)P_3 have never been reported in any smooth muscle. On the basis of contraction and cytosolic Ca^{2+} measurements, it has been suggested recently that rat ureter myocytes may possess only an Ins(1,4,5)P_3-sensitive Ca^{2+} store [10,11] and may therefore represent a good model with which to study the hierarchy of Ca^{2+} signals evoked by Ins(1,4,5)P_3-gated channels.

The present study identifies for the first time in smooth-muscle cells Ca^{2+} puffs evoked by activation of clusters of Ins(1,4,5)P_3-gated channels in response to photorelease of Ins(1,4,5)P_3 and application of low doses of acetylcholine. Ca^{2+} puffs can be observed spontaneously in Ca^{2+}-overloaded myocytes. The Ca^{2+} puffs display some variability in size, time course and spatial spread. Increasing concentrations of Ins(1,4,5)P_3 and acetylcholine induce a spatial recruitment of localized Ca^{2+} signals leading to propagated Ca^{2+} waves. The functional experiments are supported by immunodetection of Ins(1,4,5)P_3 receptors, showing the existence of several spots of fluorescence in confocal cell sections, and by binding data indicating that the density of Ins(1,4,5)P_3 receptors is 10–12-fold higher than that of ryanodine receptors in these myocytes. These results suggest that in visceral smooth-muscle cells expressing a high level of Ins(1,4,5)P_3 receptors and no subtypes 1 and 2 of ryanodine receptors, a continuum of Ins(1,4,5)P_3-gated channel-mediating Ca^{2+} signalling can be detected.

EXPERIMENTAL PROCEDURES

Cell preparation, solutions and membrane-current recordings

Wistar rats (150–160 g) were stunned and killed by cervical dislocation. Ureters were removed quickly, cut into several pieces and incubated for 10 min in low-calcium (40 μM) physiological

Abbreviations used: AM, acetoxymethyl ester; $[Ca^{2+}]_i$, cytoplasmic calcium concentration; $[Ca^{2+}]_o$, external calcium concentration; FWHM, full width at half-maximal amplitude; Fluo-3, 1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxo-3*H*-xanthen-9-yl)]-2-(2′-amino-5′-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid penta-ammonium; Indo-1, 1-[2-amino-5-(6-carboxyindol-2-yl)phenoxy]-2-(2′-amino-5′-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid pentasodium.

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solution; then 0.8 mg/ml collagenase, 0.25 mg/ml Pronase E and 1 mg/ml BSA were added at 37 °C for 15 min. After this time, the solution was removed and the ureter pieces were incubated again in a fresh enzyme solution at 37 °C for 15 min. Tissues were then placed in enzyme-free solution and triturated using a fire-polished Pasteur pipette to release cells. Cells were stored on glass coverslips and used within 10 h. Portal-vein myocytes were obtained as described previously [5,9].

The normal physiological solution used contained 130 mM NaCl, 5.6 mM KCl, 1 mM $MgCl_2$, 1.7 mM $CaCl_2$, 11 mM glucose and 10 mM Hepes, pH 7.4. The basic pipette solution contained 130 mM CsCl, 10 mM Hepes and 60 μM Fluo-3 {1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxo-3*H*-xanthen-9-yl)]-2-(2′-amino-5′-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid penta-ammonium}, pH 7.3, with CsOH. Substances were applied externally by pressure ejection from a glass pipette for the periods indicated in the relevant Figure legends. All the experiments were performed at 28 ± 1 °C.

Voltage-clamp and membrane-current recordings were made with a standard patch-clamp technique using a List EPC-7 patch-clamp amplifier (List, Darmstadt-Eberstadt, Germany). Patch pipettes had resistances of 4–6 MΩ. Membrane potential and whole-cell membrane current were stored and analysed using an IBM-PC computer (P-clamp System, Axon Instruments, Foster City, CA, U.S.A.).

Confocal microscopy and fluorescence measurements

A Bio-Rad MRC 1000 (Bio-Rad, Paris, France) confocal scanning head was coupled to a Nikon Diaphot (Nikon, Tokyo, Japan) inverted microscope. In all experiments a Nikon Plan Apo ×60, 1.4 numerical aperture objective lens was used. The iris aperture was set to 40–50 % of maximum, providing an axial (z) resolution of about 1.5 μm and an x–y resolution of 0.4 μm. Illumination was provided by a 25-mW argon ion laser (Ion Laser Technology, Salt Lake City, UT, U.S.A.). The excitation wavelength (488 nm) was selected using interference filters and the emitted fluorescence was collected at wavelengths greater than 515 nm. For some experiments, Fluo-3 (60 μM) was dialysed into the cells through the patch-pipette, as reported previously [9]. The fluorescence beam was filtered and detected using a photomultiplier tube. For other experiments, cells were loaded by incubation in physiological solution containing 1 μM Fluo-3 acetoxymethyl ester (AM) for 1 h at room temperature. These cells were washed and allowed to cleave the dye to the active Fluo-3 compound for at least 1 h. To compensate for uneven distribution of the Fluo-3, self ratios were calculated ($R = F/F_{rest}$, where F is fluorescence and F_{rest} is resting-level fluorescence). Note that no change in fluorescence corresponds to $F/F_{rest} = 1$ and $\Delta F/F_{rest} = 0$. Image acquisition and data analysis were performed by using COMOS, TCSM and MPL-1000 software (Bio-Rad). Images were acquired in the line-scan mode of the confocal microscope; this mode repeatedly scanned a single line through the cell every 6 ms. Spatial average fluorescence can be measured (after filtering with a 3×3 rank filter) in the entire line-scan image or in a 2-μm region on the x-axis, illustrating temporal changes of $[Ca^{2+}]_i$, the cytoplasmic calcium concentration.

In some experiments, cells were loaded by incubation in physiological solution containing 1 μM Indo-1 {1-[2-amino-5-(6-carboxyindol-2-yl)phenoxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid pentasodium}/AM for 30 min. Measurement of fluorescence to monitor $[Ca^{2+}]_i$ using Indo-1 has been reported previously [12].

Flash photolysis

Caged Ins(1,4,5)P_3 [Ins(1,4,5)P_3, $P^{4(5)}$-1-(2-nitrophenyl) ethyl ester] was introduced into the cell via the patch pipette, allowing 3–4 min for equilibration. Photolysis was produced by a 1-ms pulse from a xenon flash lamp (Hi-Tech Scientific, Salisbury, Wilts., U.K.) focused to a spot of about 2 mm in diameter around the cell. Light was band-pass filtered with a UG11 glass between 300 and 350 nm. Flash intensity could be adjusted by varying the capacitor charging voltage between 0 and 385 V, which corresponded to a change in the energy input into the flash lamp from 0 to 240 J. Upon flash photolysis, Ins(1,4,5)P_3 was released within 2 ms. A small percentage of conversion of caged compounds (about 10 %) was useful if repetitive pulses were applied in order to obtain similar responses. Flash intensities up to 60 J could be applied repetitively without altering the reserve of caged Ins(1,4,5)P_3 (20 μM) and, consequently, the amount of photoreleased compounds.

Immunocytochemistry

Myocytes were immunostained as described previously [13], except that donkey serum was used instead of fetal calf serum. Myocytes were incubated in the presence of anti-Ins(1,4,5)P_3 receptor antibodies (at 1:200 dilution) for 20 h at 4 °C, and the secondary antibodies (donkey anti-rabbit IgG conjugated to FITC, diluted 1:200) were incubated for 3 h at 20 °C. Thereafter, cells were mounted in Vectashield.

Microsomal-membrane preparation

Microsomal membranes of ureter and cerebellum from Wistar rats were prepared by homogenization with a Kontes potter in a solution containing 20 mM Tris/HCl, 1 mM EGTA and 1 mM PMSF, pH 7.4. The homogenate was centrifuged at 170 ***g*** for 10 min at 4 °C. Microsomal membranes were obtained as a pellet by centrifugation of the supernatant at 185000 ***g*** for 90 min at 4 °C. Microsomal membranes were then resuspended in the buffer and stored at −80 °C. Protein concentration was determined according to Bradford [14].

Immunoblotting

For Western-blotting analysis, microsomal proteins were separated on SDS/PAGE (7.5 %) minigels and transferred to PVDF membranes for 16 h at 30 V in a transfer buffer containing 192 mM glycine and 25 mM Tris/HCl (pH 8.3). Membranes were blocked for 1 h in blocking buffer containing 20 mM Tris/HCl and 3 % BSA (pH 7.4) and then incubated for 5 h at room temperature with the primary anti-Ins(1,4,5)P_3 receptor antibody at 1:200 dilution. After extensive washing, membranes were incubated for 2 h with the secondary antibody coupled to horseradish peroxidase (anti-rabbit, 1:5000 dilution). Specific antigen detection was performed using H_2O_2 and diaminobenzidine to detect horseradish peroxidase activity on PVDF membranes and Kodak EDAS 120 (Kodak, Rochester, NY, U.S.A.).

[^{3}H]Ryanodine- and [^{3}H]Ins(1,4,5)P_3-binding assays

[^{3}H]Ryanodine binding to microsomal membranes of rat ureters was measured, as reported previously on rat portal vein [15], in a medium containing 1 M KCl, 25 mM Hepes (pH 7.8 at 37 °C), 1 mM dithiothreitol, 0.1 mM $CaCl_2$, 1 mg/ml BSA and 0.1 mM PMSF. [^{3}H]Ryanodine was used in the concentration range of

2–30 nM. After a 3-h incubation at 37 °C, aliquots were filtered through Whatmann GF/C fibre-glass filters and washed three times with 5 ml of ice-cold binding buffer. The filters were placed in scintillation vials filled with 4 ml of liquid-scintillation cocktail, shaken for 1 h, and counted in a Packard 1500 Tri-Carb. Non-specific binding was measured in the presence of 10 μM ryanodine and was subtracted before calculation. At 20 nM [^{3}H]ryanodine, non-specific binding was $< 60\,\%$ of total binding.

[^{3}H]Ins(1,4,5)P_3 binding was measured in a medium containing 0.1 M KCl, 50 mM Tris/HCl (pH 8.3 at 2–4 °C) and 1 mM EGTA. [^{3}H]Ins(1,4,5)P_3 was used in the concentration range of 2–200 nM. After a 10-min incubation on ice, binding reactions were terminated by centrifugation (15000 ***g***, 15 min, 4 °C). The supernatant was aspirated and the pellet was rinsed quickly with 0.2 ml of ice-cold binding buffer. Pellets were solubilized with 0.1 ml of Soluene-100 (55 °C, 30 min). After transfer into scintillation vials with 4 ml of liquid-scintillation cocktail, radioactivity was counted in a Packard 1500 Tri-Carb. Non-specific binding was measured in the presence of a 1000-fold excess of Ins(1,4,5)P_3 over [^{3}H]Ins(1,4,5)P_3 concentration. At 100 nM [^{3}H]Ins(1,4,5)P_3, non-specific binding was $< 55\,\%$ of total binding.

Maximal binding capacity (B_{max}) and dissociation constant (K_d) were obtained from saturation binding experiments.

Reverse transcriptase PCR

Total RNA was extracted from about 500 cells using the RNeasy mini kit (Qiagen, Hilden, Germany). The reverse-transcription reaction was performed using the Sensiscript RT kit (Qiagen). Total RNA was incubated first with random primers (Promega, Lyon, France) at 65 °C for 5 min, then, after a cooling time of 15 min (at 25 °C), reverse transcriptase mix was added and the total mixture was incubated 60 min at 37 °C. The resulting cDNA was stored at −20 °C. PCR was performed with 2 μl of cDNA (in reverse transcriptase PCR mix), 1.25 units of HotStart *Taq* DNA polymerase (Qiagen), 1 μM of each primer and 200 μM of each deoxynucleotide triphosphate, in a final volume of 50 μl. The solution was supplemented with 2.5 mM $MgCl_2$, except for subtype 1 of Ins(1,4,5)P_3 receptors. The PCR conditions were 95 °C for 15 min, then 35 cycles at 94 °C for 1 min, 60 °C (subtypes 1 and 2 of ryanodine receptors and all subtypes of Ins(1,4,5)P_3 receptors) or 56 °C (subtype 3 of ryanodine receptor) for 1 min and 72 °C for 1.5 min and at the end of PCR, samples were kept at 72 °C for 10 min for final extension and then stored at 4 °C. Reverse transcription and PCR were performed with a thermal cycler (Techne, Cambridge, U.K.). Amplification products were separated by electrophoresis (2 % agarose gel) and vizualized by ethidium bromide staining. Gels were photographed with EDAS 120 and analysed with KDS 1D 2.0 software (Kodak Digital Science, Paris, France). Sense and antisense primer pairs used to amplify fragments of subtypes 1, 2 and 3 of Ins(1,4,5)P_3 and ryanodine receptors were designed on the basis of the known cloned receptor sequences deposited in GenBank (accession numbers J05510, X61677, L06096, X83932, X83933 and X83934, respectively) with DNASTAR software (Lasergene, DNAstar, Madison, WI, U.S.A.). The nucleotide sequences and the lengths of the expected PCR products (in parentheses) for each primer pair were as follows. Subtype 1 of Ins(1,4,5)P_3 receptor, sense, 5′-ACTGGAAGACCATAAAAGGGGTGA-3′, and antisense, 5′-TCCGTCAGCCGGCAGAAAAATGAG-3′ (368 bp); subtype 2 of Ins(1,4,5)P_3 receptor, sense, 5′-GGCACAGCCTCTCCATGTGGCAGG-3′, and antisense, 5′-TTCCTGTCTCGCTGACCCCAGTGC-3′ (197 bp); subtype 3 of Ins(1,4,5)P_3 receptor, sense, 5′-CACGGAGCTGCCACATTTATGGGC-3′, and antisense, 5′-TCCTCAGTCCGTGGTTCATGACGG-3′ (168 bp) [16]; subtype 1 of ryanodine receptor, sense, 5′-GAAGGTTCTGGACAAACACGGG-3′, and antisense, 5′-TCGCTCTTGTTGTAGAATTTGCGG-3′ (435 bp); subtype 2 of ryanodine receptor, sense, 5′-GAATCAGTGAGTTACTGGGCATGG-3′, and antisense, 5′-CTGGTCTCTGAGTTCTCCAAAAGC-3′ (635 bp); subtype 3 of ryanodine receptor, sense, 5′-CCTTCGCTATCAACTTCATCCTGC-3′, and antisense, 5′-TCTTCTACTGGGCTAAAGTCAAGG-3′ (505 bp) [17].

Chemicals and drugs

Collagenase was obtained from Worthington (Freehold, NJ, U.S.A.); Pronase E, BSA, acetylcholine, heparin and Ins(1,4,5)P_3 were from Sigma (St Louis, MO, U.S.A.). Ryanodine, Indo-1/AM and caged Ins(1,4,5)P_3 [Ins(1,4,5)P_3, $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester] were from Calbiochem (Meudon, France). Caffeine was from Merck (Nogent sur Marne, France). Fluo-3 and Fluo-3/AM were from Molecular Probes Europe (Leiden, The Netherlands). Oxodipine was a gift from Dr Galiano (Instituto de Investigacion y Desarrollo Quimico Biologico, Madrid, Spain). [^{3}H]Ryanodine (68 Ci/mmol) and [^{3}H]Ins(1,4,5)P_3 (20 Ci/mmol) were from DuPont NEN (Boston, MA, U.S.A.). Primers specific for Ins(1,4,5)P_3 and ryanodine receptors were synthesized by Eurogentec (Seraing, Belgium).

Antibodies directed against Ins(1,4,5)P_3 and ryanodine receptors were added to the pipette solution to allow dialysis of the cell after a breakthrough in whole-cell recording mode for at least 5–8 min, a time longer than that expected theoretically for diffusion of substances in solutions [18]. The rabbit anti-Ins(1,4,5)P_3-receptor antibody was raised to the C-terminal amino acids (GGVGDVLRKPS) of the Ins(1,4,5)P_3 receptors (407143-S, Calbiochem). The mouse anti-(ryanodine receptor) antibody was raised to the C-terminal amino acids (DQQEQVKEDMETK) of the ryanodine receptor (559279-S, Calbiochem). For immunological detection, FITC-conjugated affinity-pure donkey anti-rabbit IgG and donkey serum were from Jackson Immunoresearch Laboratories (West Grove, PA, U.S.A.) and Vectashield was from Biosys (Compiègne, France).

Analysis of data

The results are expressed as means ± S.E.M. Significance was tested by means of Student's t test. P values of < 0.05 were considered significant.

RESULTS

Expression of Ins(1,4,5)P_3 and ryanodine receptors in rat ureteric myocytes

The expression of Ins(1,4,5)P_3- and ryanodine-receptor subtypes was examined by using subtype-specific primers designed to amplify cDNA fragments of subtypes 1, 2 and 3 of these receptors in rat ureter and cerebellum. Amplified fragments of all three Ins(1,4,5)P_3-receptor subtypes were observed (Figure 1A), indicating that the three Ins(1,4,5)P_3-receptor mRNAs were expressed in ureter and cerebellum. In contrast, amplification of fragments of mRNAs encoding the ryanodine-receptor subtypes showed that only the subtype 3 was expressed in rat ureteric myocytes, whereas all three subtypes were revealed in cerebellum (Figure 1B). Amplified products obtained with the primers designed to amplify the Ins(1,4,5)P_3- or ryanodine-receptor subtypes displayed sizes and sequences (results not shown) corresponding to those of the cloned Ins(1,4,5)P_3- or ryanodine-receptor subtypes. Scatchard analysis of [^{3}H]Ins(1,4,5)P_3 and

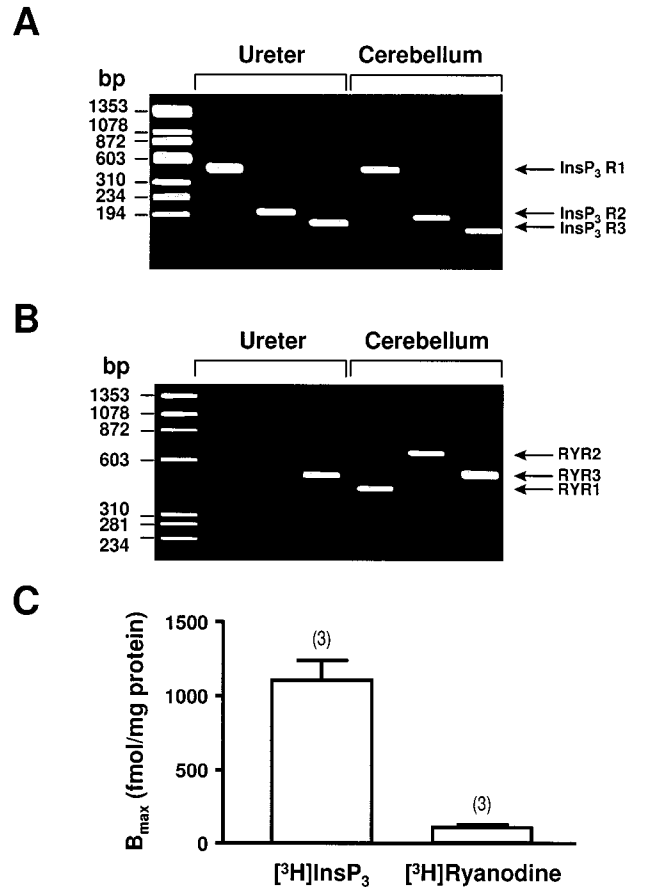

Figure 1 Expression of Ins(1,4,5)P_3 and ryanodine receptors in rat ureteric myocytes

(**A**) Amplified DNA fragments of subtypes 1, 2 and 3 (R1–3) of Ins(1,4,5)P_3 receptors were separated on a 2% agarose gel and visualized by staining with ethidium bromide using rat ureter and cerebellum RNA. Numbers on the left indicate molecular-size standards. (**B**) Amplified DNA fragments of isoforms 1, 2 and 3 of ryanodine receptors (RYR1–3) using rat ureter and cerebellum RNA. For RNA purification and PCR conditions, see the Experimental procedures section. (**C**) Specific binding of [^{3}H]Ins(1,4,5)P_3 and [^{3}H]ryanodine to ureteric smooth-muscle membranes. Maximal binding capacity (B_{max}) corresponding to the total number of binding sites (high-affinity sites for [^{3}H]ryanodine; high-affinity plus low-affinity sites for [^{3}H]Ins(1,4,5)P_3) is the mean $\pm$ S.E.M. of three experiments, each carried out in duplicate. The mean K_d values for [^{3}H]ryanodine and [^{3}H]Ins(1,4,5)P_3 (high-affinity and low-affinity) were 2.5, 5.3 and 207.5 nM, respectively.

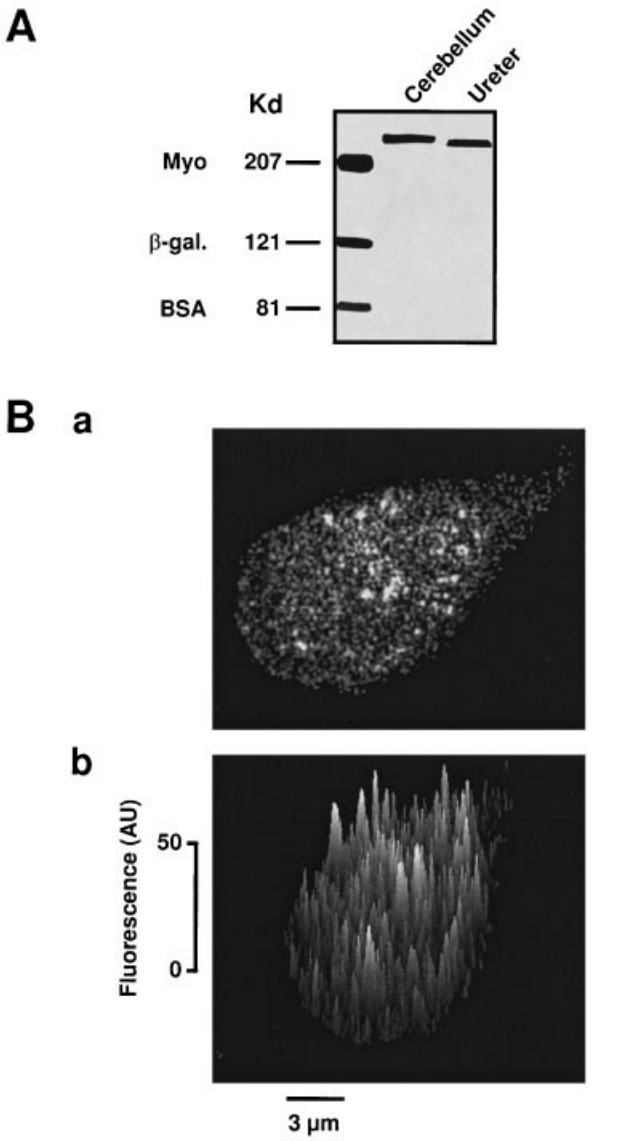

Figure 2 Immunological detection of Ins(1,4,5)P_3 receptors in rat ureteric myocytes

(**A**) Microsomes of ureter smooth muscle (200 μg) and cerebellum (100 μg) were separated by SDS/PAGE (7.5% gel) and analysed by Western blot with an anti-Ins(1,4,5)P_3-receptor antibody. Numbers on the left indicate molecular-mass standards (in kDa). Myo, myosin; β-gal, β-galactosidase. (**B**) Immunolocalization of Ins(1,4,5)P_3 receptors. (**a**) A typical confocal cell section of a freshly dissociated ureteric myocyte performed above the nucleus, showing several spots of fluorescence near the plasma membrane and in the cytoplasm. (**b**) Three-dimensional representation of the fluorescence (in arbitrary units, AU) from the same confocal cell section. In absence of primary antibody or after inactivation of the antibody with its antigen, only a faint background fluorescence was observed.

[^{3}H]ryanodine binding on rat ureter membranes showed that the B_{max} value corresponding to the total number of Ins(1,4,5)P_3-binding sites (high-affinity plus low-affinity sites) was 1113 $\pm$125 fmol/mg of protein ($n = 3$), whereas the B_{max} value corresponding to the total number of ryanodine-binding sites was 109$\pm$21 fmol/mg of protein ($n = 3$; Figure 1C). Finally, Western-blot detection on samples of rat ureter and cerebellum (Figure 2A) revealed a similar molecular-mass band ($\approx$ 240 kDa) corresponding to the value reported previously for Ins(1,4,5)P_3 receptors [19]. These results indicate that rat ureteric myocytes possess mainly Ins(1,4,5)P_3 receptors.

Immunodetection of Ins(1,4,5)P_3 receptors in 0.5-μm confocal sections from freshly dissociated ureteric myocytes was performed with the anti-[Ins(1,4,5)P_3 receptor] antibody (used for protein detection) and the binding sites were revealed with FITC-conjugated secondary antibody. As illustrated in Figure 2(B), Ins(1,4,5)P_3 receptors appeared to be distributed in the whole confocal section, with several marked spots of fluorescence localized near the plasma membrane and in the cytoplasm. Taken together, these results suggest that ureter smooth muscle can be a suitable model for studying Ca^{2+} signalling induced by Ins(1,4,5)P_3-gated channels.

Acetylcholine- and Ins(1,4,5)P_3-induced localized Ca^{2+} signals

Line-scan images of ureteric myocytes were obtained by scanning of a single line in a confocal section. Most of the scanned lines

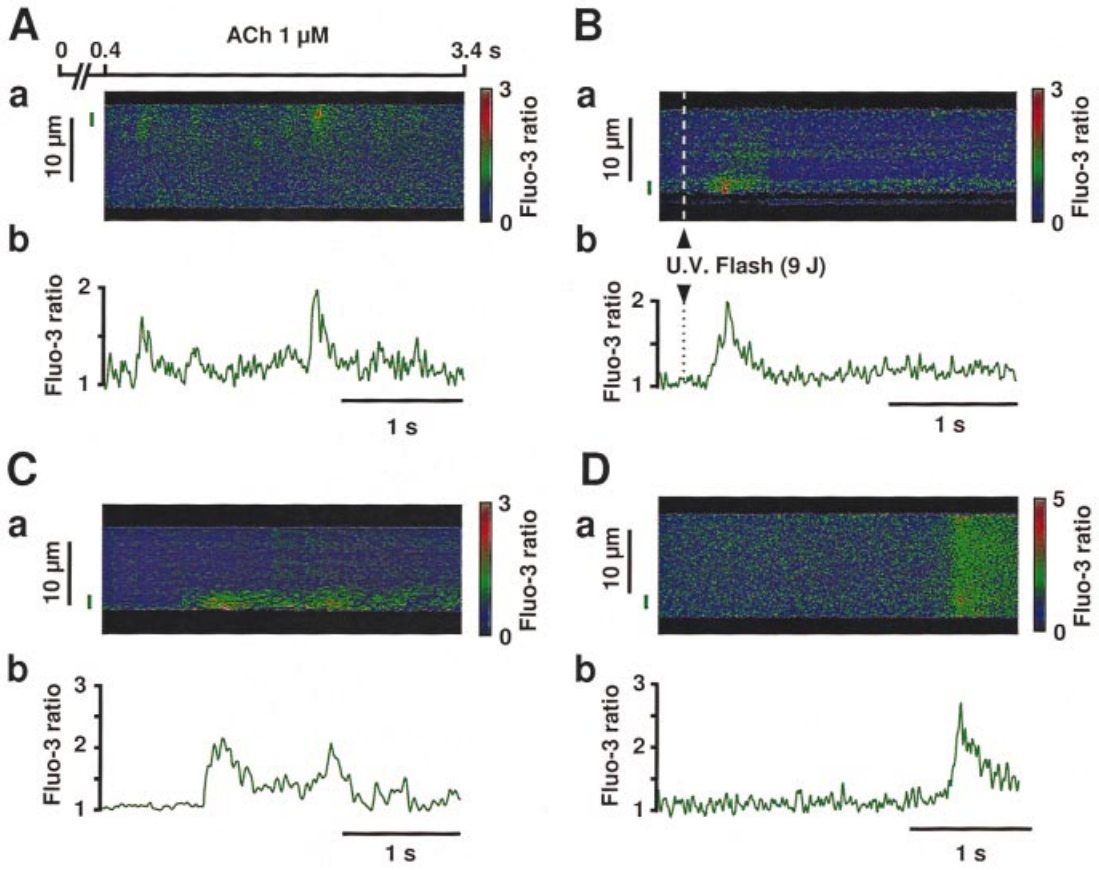

Figure 3 Triggered and spontaneous Ca^{2+}-release signals in rat ureteric myocytes

(**A**) Typical Ca^{2+} response evoked by 1 μM acetylcholine (ACh; horizontal bar) in physiological (1.7 mM Ca^{2+}) solution, shown as a line-scan image (**a**) and averaged fluorescence from a 2-μm region (**b**), indicated by the vertical bar on the line-scan image. Myocytes were loaded with Fluo-3/AM and not patch-clamped. (**B**) Ca^{2+} responses evoked by a 9-J flash pulse in physiological solution, displayed as in (**A**), indicated by the vertical bar on the line-scan image. Myocytes were loaded with Fluo-3 and caged Ins(1,4,5)P_3 through the patch-pipette and held at -50 mV. (**C**, **D**) Spontaneous Ca^{2+} puff (**C**) and Ca^{2+} spike (**D**), displayed as in (**A**), indicated by the vertical bar on the line-scan image in myocytes superfused with 10 mM Ca^{2+} from 30 min. Myocytes were loaded with Fluo-3/AM and not patch-clamped. External solution contained 10 μM ryanodine and 1 μM oxodipine. No change in fluorescence corresponds to a Fluo-3 ratio of 1.

performed above the nucleus detected Ca^{2+} signals, in agreement with immunostaining of Ins(1,4,5)P_3 receptors (Figure 2B). Experiments were performed in either voltage-clamped (holding potential, -50 mV) or non-voltage-clamped myocytes, in the continuous presence of 1 μM oxodipine (a light-stable dihydropyridine) to inhibit voltage-dependent Ca^{2+} channels. Fluorescence signals are expressed as a pseudo-ratio (F/F_{rest}) and are depicted by a pseudocolour scale as indicated by the colour bars (see Figures 3 and 5, below). The basal $[Ca^{2+}]_i$ level measured with the Indo-1 dye was 91 ± 7 nM ($n = 25$).

In non-voltage-clamped ureteric myocytes, applications of 1 μM acetylcholine induced localized and transient Ca^{2+} signals that originated from 1 or 2 initiation sites in the line-scan images and appeared after a delay of 0.55 ± 0.01 s ($n = 29$; Figure 3A). However, these signals showed some heterogeneity in amplitude, time course and spatial spread, even when they originated from the same site. The largest Ca^{2+}-release events are characterized by a relatively rapid upstroke (with time-to-peak of 93.6 ± 9.7 ms, $n = 10$) followed by a decline, with a time to decay to half-maximal amplitude of 107.4 ± 16.4 ms, and were restricted to a small area with full width at half-maximal amplitude (FWHM) of 1.89 ± 0.23 μm. The peak amplitude of such events was 1.13 ± 0.16 ($\Delta F/F_{rest}$, $n = 10$) and they appear generally similar to the Ca^{2+} puffs reported in non-excitable cells [6,7]. The smallest Ca^{2+}-release events had a lower peak amplitude ($\Delta F/F_{rest} = 0.39 \pm 0.02$, $n = 19$) and a more restricted spatial spread (1.03 ± 0.07 μm). The kinetics of these events were more rapid than those of Ca^{2+} puffs, with time-to-peak and time at half-decay of 49.2 ± 3.9 ms and 60.9 ± 8.5 ms, respectively ($n = 19$). Such small events are unlikely to be due to out-of-focus Ca^{2+} puffs, since such out-of-focus Ca^{2+} signals would exhibit a reduced amplitude and a slower time course [20], whereas the detected small events are generally much faster than the other localized Ca^{2+} events.

Distributions of peak fluorescence, time-to-peak, time at half-decay and FWHM of 29 elementary Ca^{2+} signals obtained from 11 different cells are illustrated in Figure 4. The broad range of amplitude, time-to-peak, time at half-decay and FWHM is consistent with the notion of a continuum of Ca^{2+} puffs rather than a hierarchy of discrete amplitudes. This conclusion is also supported by the localization of Ca^{2+} events of different amplitude originating from the same site (Figure 3A). In addition, a noticeable variation was also observed in spatial spread (Figure 4D). Such a variation in FWHM would be expected for Ca^{2+} events that differed in amplitude, since a high-amplitude signal should have a larger spatial spread than a signal of lower amplitude, providing the Ca^{2+} buffering and sequestration mechanisms were equal for both events. As shown in Figure 4(D)

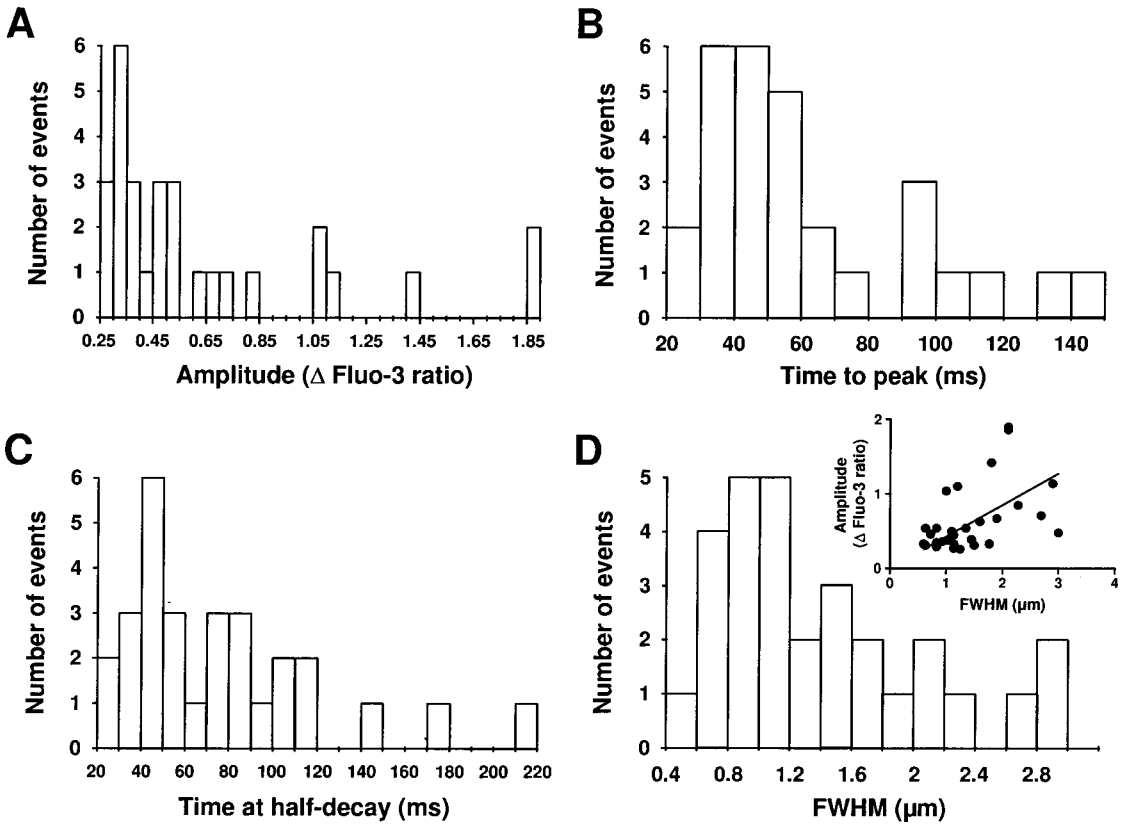

Figure 4 Characteristics of localized Ca^{2+}-release signals evoked by low concentrations of acetylcholine

(**A**) Amplitude histogram of 29 elementary Ca^{2+} signals (from 11 different cells) stimulated by 1 μM acetylcholine. Distribution of the time to reach peak Ca^{2+} signals (**B**), the time to reach half-decay (**C**) and the FWHM (**D**) for the same 29 elementary Ca^{2+} signals. Inset shows the correlation between amplitude of localized Ca^{2+} signals and FWHM. The linear regression coefficient (r^2) was 0.72. Myocytes were loaded with Fluo-3/AM and not patch-clamped. External solution contained 1 μM oxodipine.

(inset), such a relationship between amplitude and spatial spread could be observed, although the correlation was not high ($r^2 = 0.72$).

In voltage-clamped myocytes, photorelease from a caged Ins(1,4,5)P_3 precursor by UV-light flashes of low-intensity (9 J) evoked localized and transient Ca^{2+} events (Figure 3B) with variable amplitudes and time courses ($n = 15$), similar to those obtained with 1 μM acetylcholine.

Spontaneous Ca^{2+} puffs in Ca^{2+}-overloaded myocytes

In accordance with previous data [21], the potency of muscle cells to generate spontaneous localized Ca^{2+} signals may depend on the Ca^{2+}-loading status. Superfusion of ureteric myocytes with 10 mM $[Ca^{2+}]_o$ (the external calcium concentration) for at least 30 min resulted in a significant increase in the mean amplitude ($\Delta F/F_{rest}$) of the Ca^{2+} responses evoked by 10 μM acetylcholine from 2.48 ± 0.15 ($n = 32$) in normal (1.7 mM Ca^{2+}) physiological solution to 2.89 ± 0.17 ($n = 32$) in 10 mM $[Ca^{2+}]_o$, suggesting a Ca^{2+} loading of the store. With a $[Ca^{2+}]_o$ of 1.7 mM, most ureteric myocytes had a steady-state $[Ca^{2+}]_i$ level and spontaneous Ca^{2+} signals were very scarce. With a $[Ca^{2+}]_o$ of 10 mM for at least 30 min, the basal $[Ca^{2+}]_i$ level was not significantly affected (97 ± 12 nM, $n = 20$) but discrete, spontaneous events occurred more frequently (Figure 3C). The majority of these localized Ca^{2+} events were not associated with Ca^{2+} waves. Occasionally, localized Ca^{2+} events appeared to spread and generate transient Ca^{2+} spikes (Figure 3D). The peak amplitude of spontaneous Ca^{2+} events was 1.24 ± 0.20 ($\Delta F/F_{rest}$, $n = 31$). The time-to-peak, the half-time of decay and the FWHM were 110 ± 25, 190 ± 35 and 2.51 ± 0.25 μm, respectively ($n = 31$). Although Ca^{2+} overload appeared to slow the time course of the Ca^{2+}-signal decline and to increase slightly the FWHM, the kinetic parameters of the spontaneous Ca^{2+} events resembled those of Ca^{2+} puffs. The frequency of spontaneous Ca^{2+} puffs increased significantly from 0.03 ± 0.01 puff$\cdot s^{-1}$ ($n = 54$) in control conditions to 0.31 ± 0.3 puff$\cdot s^{-1}$ ($n = 31$) in Ca^{2+}-loading conditions. These results show that in muscle cells overloaded with Ca^{2+}, clustered Ins(1,4,5)P_3 receptors may underlie spontaneous Ca^{2+} releases, as in non-excitable cells [22]. Spontaneous Ca^{2+} puffs were obtained in the continuous presence of 10 μM ryanodine and 1 μM oxodipine to inhibit ryanodine receptors and L-type Ca^{2+} channels, respectively. In addition, in patch-clamped myocytes intracellular applications of 1 mg/ml heparin or 10 μg/ml anti-[Ins(1,4,5)P_3 receptor] antibody for 7–8 min suppressed any spontaneous Ca^{2+}-release signals ($n = 12$).

Acetylcholine- and Ins(1,4,5)P_3-induced Ca^{2+} waves

It is proposed generally that the spatio-temporal summation of localized Ca^{2+} signals due to Ins(1,4,5)P_3-receptor activation gives rise to the whole-cell increase in $[Ca^{2+}]_i$. Increases in the frequency or amplitude of localized Ca^{2+} signals, or spatial recruitment of additional initiation sites, have been reported to

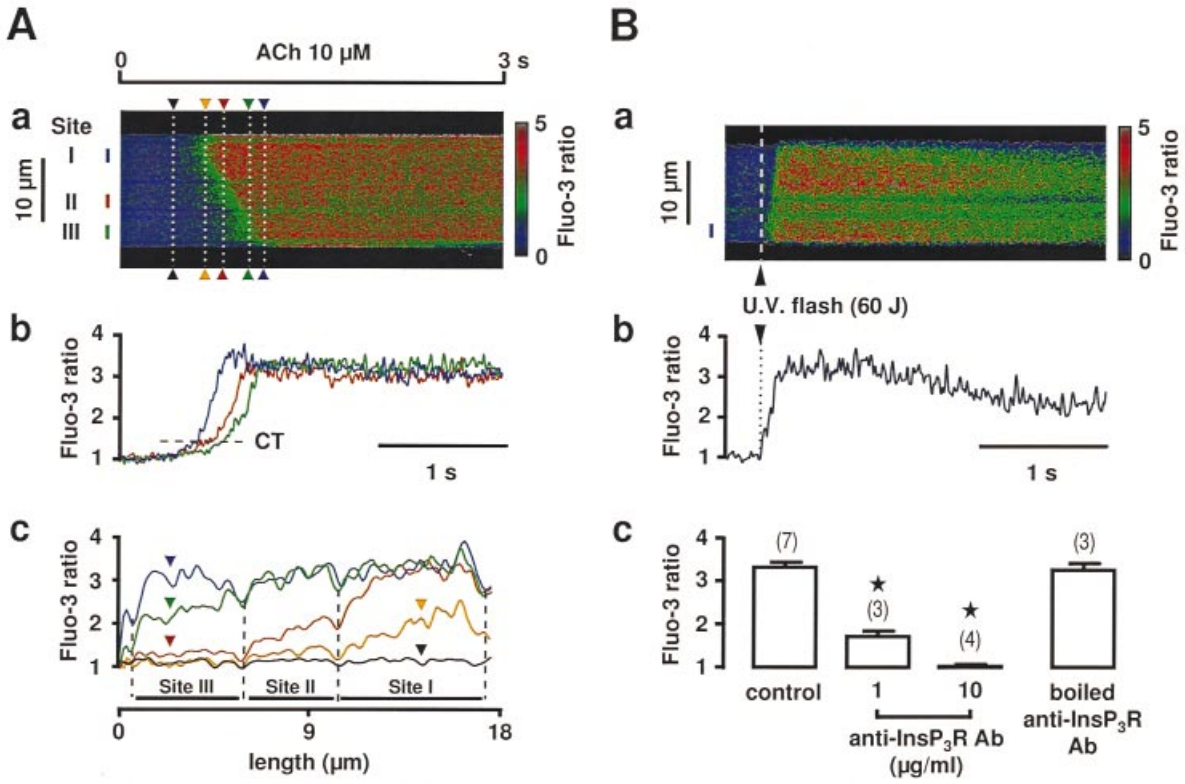

Figure 5 Activation of propagated Ca^{2+} waves by high doses of acetylcholine (ACh) and high-intensity flash photolysis of caged Ins(1,4,5)P_3

(**A**) Line-scan image (**a**) in response to the continuous application of 10 μM acetylcholine, as indicated by the horizontal bar. (**b**) Averaged fluorescence from three 2-μm regions (indicated by the vertical bars on the line-scan image). CT, control $[Ca^{2+}]_i$ threshold. (**c**) Spatial profiles (measured in 2-μm-wide areas indicated by coloured arrow heads) of the line-scan image shown in (**A**, **a**). In this myocyte, the Ca^{2+} wave started at one site (site I, yellow trace) and then propagated through the entire line-scan image by recruiting neighbouring Ca^{2+}-release sites (sites II and III). Myocytes were loaded with Fluo-3/AM and not patch-clamped. (**B**) Ca^{2+} waves evoked by flash photolysis of caged Ins(1,4,5)P_3 in voltage-clamped rat ureteric myocytes. (**a**) Line-scan image evoked by a high-intensity (60 J) flash pulse. (**b**) Averaged fluorescence from a 2-μm region indicated by the vertical bar on the line-scan image. (**c**) Compiled data showing the effects of the anti-Ins(1,4,5)P_3-receptor [anti-Ins(1,4,5)P_3R] antibody (Ab), applied intracellularly for 7–8 min. Data are means $\pm$ S.E.M. with the number of experiments indicated in parentheses. ★, Values significantly different from those obtained under control conditions ($P < 0.05$). Myocytes were loaded with Fluo-3 and caged Ins(1,4,5)P_3 through the patch-pipette and held at -50 mV. External solution contained 1 μM oxodipine. No change in fluorescence corresponds to a Fluo-3 ratio of 1.

be necessary for triggering propagated Ca^{2+} waves [23]. In all the cells tested ($n = 16$), we never observed increases in frequency or amplitude of Ca^{2+} puffs in response to 10 μM acetylcholine, which were able to initiate propagated Ca^{2+} waves. In contrast, propagated Ca^{2+} waves appeared from a first initiation site that progressively recruited neighbouring initiation sites by Ca^{2+} diffusion (Figure 5A). The delay between application of acetylcholine and the onset of the Ca^{2+} wave was estimated to be 0.50 ± 0.01 s ($n = 16$). The mean amplitude ($\Delta F/F_{rest}$) and the mean overall wave velocity of the acetylcholine-evoked Ca^{2+} waves were 2.56 ± 0.25 and 38 ± 3 μm/s ($n = 16$), respectively. The propagation velocity of Ca^{2+} waves depends on the distance between Ca^{2+}-release sites as well as their Ca^{2+} sensitivity and the amount of Ca^{2+} released. As shown in Figure 5(Ab), recordings of localized fluorescence signals at release sites activated during Ca^{2+} wave propagation showed an initial slow Ca^{2+} elevation preceding an abrupt rise. This is consistent with the triggering of regenerative Ca^{2+} release by an early rise in $[Ca^{2+}]_i$ resulting from summation of localized Ca^{2+} signals or from Ca^{2+} ions diffusing from neighbouring active sites. Fluorescence analysis in the spatial domain at different times during the onset of the Ca^{2+} wave (Figure 5Ac) revealed clearly the recruitment of neighbouring Ca^{2+}-release sites from the early initiation site (site I in Figure 5Ac). The mean number of initiation sites per line-scan image was 2.4 ± 0.4 ($n = 16$).

Photorelease of caged Ins(1,4,5)P_3 by high-intensity (30–60 J) flash pulses evoked large Ca^{2+} responses that started either from a simultaneous increase in $[Ca^{2+}]_i$ in the scanned line or from an initiation site leading to activation of a propagating wave (Figure 5B). The mean amplitudes ($\Delta F/F_{rest}$) of the Ins(1,4,5)P_3- and acetylcholine-evoked Ca^{2+} waves in voltage-clamped myocytes were 2.32 ± 0.11 and 2.48 ± 0.31 ($n = 7$), respectively.

To identify the molecular nature of the Ca^{2+}-release channels involved in both Ins(1,4,5)P_3- and acetylcholine-induced Ca^{2+} signals, we tested the effects of Ins(1,4,5)P_3- and ryanodine-receptor inhibitors. Intracellular infusion of 1 mg/ml heparin for 7–8 min through the patch-pipette suppressed both Ins(1,4,5)P_3- and acetylcholine-induced Ca^{2+} waves ($n = 5$; results not shown). Similarly, intracellular application of the anti-Ins(1,4,5)P_3-receptor antibody for 7–8 min inhibited in a concentration-dependent manner the Ca^{2+} responses evoked by flash photolysis of caged Ins(1,4,5)P_3 (Figure 5Bc) or application of 10 μM acetylcholine (Figure 6A). This antibody-induced inhibition was specific, in as much as boiled (95 °C for 30 min) anti-Ins(1,4,5)P_3-receptor antibody had no significant effect on both Ins(1,4,5)P_3-and acetylcholine-activated Ca^{2+} responses (Figures

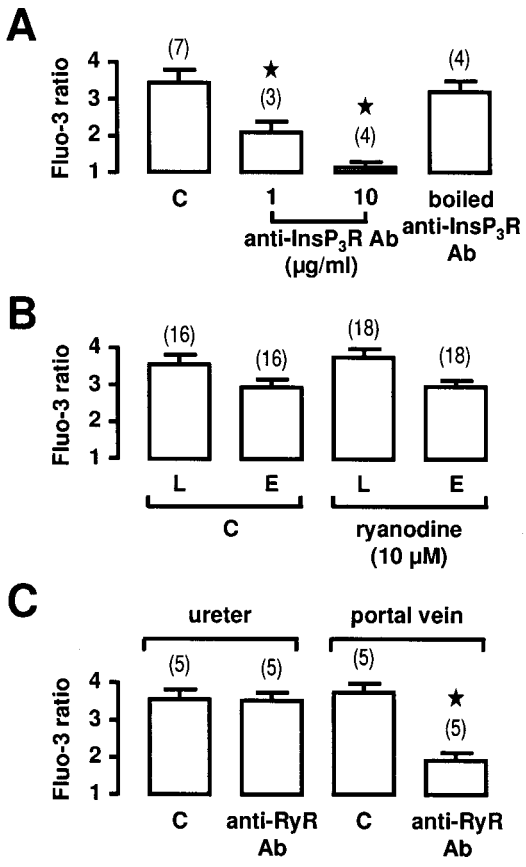

Figure 6 Effects of inhibitors of Ins(1,4,5)P_3 and ryanodine receptors on the acetylcholine-induced Ca^{2+} waves in voltage-clamped myocytes

(**A**) Compiled data showing the effects of increasing concentrations of anti-Ins(1,4,5)P_3 receptor [anti-Ins(1,4,5)P_3R] antibody (Ab) and of 10 μg/ml boiled antibody, applied intracellularly for 7–8 min on 10 μM acetylcholine-induced Ca^{2+} waves in ureteric myocytes. (**B**) Compiled data showing the effect of 10 μM ryanodine (applied externally for 15–17 min) on 10 μM acetylcholine-induced Ca^{2+} waves in ureteric myocytes. The fluorescence was measured either in the entire line-scan image (E) or in a localized 2-μm region of the line-scan image (L). (**C**) Compiled data showing the effect of 10 μg/ml anti-ryanodine receptor (anti-RyR) antibody, applied intracellularly for 7–8 min on 10 μM acetylcholine-induced Ca^{2+} waves in ureteric and portal-vein myocytes. Data are means $\pm$ S.E.M. with the number of cells tested indicated in parentheses. ★, Values significantly different from those obtained under control conditions ($P < 0.05$). Holding potential was -50 mV. External solution contained 1 μM oxodipine. No change in fluorescence corresponds to a Fluo-3 ratio of 1. C, control.

5Bc and 6A). In vascular myocytes, extracellular applications of 10 μM ryanodine have been shown to inhibit ryanodine receptors without depleting the internal Ca^{2+} store in a manner similar to that induced by an anti-ryanodine-receptor antibody [24]. Application of 10 μM ryanodine for 15–17 min in non-voltage-clamped ureteric myocytes had no significant effect on the amplitude of acetylcholine-induced global Ca^{2+} waves, measured in a 2-μm region or in the entire line-scan image (Figure 6B). Similarly, intracellular applications of 10 μg/ml anti-ryanodine-receptor antibody for 7–8 min did not affect the acetylcholine-induced Ca^{2+} wave (Figure 6C) as well as the Ins(1,4,5)P_3-induced Ca^{2+} response in ureteric myocytes. In contrast, in portal-vein myocytes, the acetylcholine-induced Ca^{2+} wave was decreased by about 60 % in the presence of 10 μg/ml of the same anti-ryanodine-receptor antibody (Figure 6C), in good agreement with previous data obtained from noradrenaline-induced Ca^{2+} response [24]. It is noteworthy that 10 μM anti-Ins(1,4,5)P_3-receptor antibody suppressed the Ca^{2+} puffs evoked by low concentrations of acetylcholine or Ins(1,4,5)P_3 using low-intensity flashes, in all the cells tested ($n = 12$). In contrast, 10 μM anti-ryanodine-receptor antibody was ineffective on Ca^{2+} puffs ($n = 9$).

DISCUSSION

This study describes for the first time in smooth-muscle cells the localized and transient Ca^{2+} events (Ca^{2+} puffs) evoked by Ins(1,4,5)P_3-gated channels in response to low concentrations of Ins(1,4,5)P_3 either released from caged Ins(1,4,5)P_3 or produced by low doses of acetylcholine. In addition, spontaneous Ca^{2+} puffs were detected frequently in myocytes overloaded with Ca^{2+}. At high concentrations of Ins(1,4,5)P_3 or acetylcholine, recruitment of several localized Ca^{2+} signals generates Ca^{2+} waves that appear to propagate between several Ca^{2+}-release sites in the scanned line. The functional results are supported by both binding and immunochemical data showing that Ins(1,4,5)P_3 receptors are highly expressed when compared with ryanodine receptors and that spots of Ins(1,4,5)P_3 receptors are detected in several areas of the cell confocal sections.

In rat ureteric myocytes, immunodetection of Ins(1,4,5)P_3 receptors in cell confocal sections showed that spots of Ins(1,4,5)P_3 receptors were detected in several areas in addition to an homogeneous distribution in the whole confocal sections. This observation is in good agreement with the fact that Ins(1,4,5)P_3 receptors are highly expressed in these smooth-muscle cells, as supported by binding experiments. The maximal binding capacity of [^{3}H]Ins(1,4,5)P_3 to ureteric microsomal preparations is 10–12 times higher than that of [^{3}H]ryanodine. The reverse-transcriptase PCR analysis showed that the RNAs encoding the three subtypes of the Ins(1,4,5)P_3 receptor and subtype 3 of the ryanodine receptor were detected in rat ureteric myocytes. However, binding experiments revealed that the level of expression of ryanodine-receptor subtype 3 was very low in these cells. This low expression level together with the fact that ryanodine-receptor subtype 3 is less sensitive to Ca^{2+}-induced channel activation than the other ryanodine-receptor subtypes [25] may explain why ryanodine and the anti-ryanodine-receptor antibody do not affect both localized and global Ca^{2+} signals evoked by flash photolysis of caged Ins(1,4,5)P_3 and acetylcholine. This is in contrast with results obtained in vascular myocytes, where both ryanodine and the anti-ryanodine-receptor antibody inhibit the Ca^{2+} responses evoked by noradrenaline [24] or acetylcholine (the present study). However, in vascular myocytes the density of ryanodine receptors is 3–4 times higher than that of Ins(1,4,5)P_3 receptors [24]. Taken together, these results indicate that in rat ureteric myocytes the Ca^{2+} responses to photorelease of Ins(1,4,5)P_3 and acetylcholine depend only on activation of Ins(1,4,5)P_3 receptors, in agreement with previous contraction experiments and $[Ca^{2+}]_i$ measurements in intact and permeabilized rat ureteric strips [10,11]. Accordingly, both Ins(1,4,5)P_3- and acetylcholine-evoked Ca^{2+} signals were blocked by heparin and by an anti-Ins(1,4,5)P_3-receptor antibody, which recognized in Western-blot analysis a molecular-mass band of about 240 kDa in rat ureter and cerebellum, but were unaffected by high concentrations of ryanodine and by an anti-ryanodine-receptor antibody.

The spatio-temporal characteristics of Ca^{2+} puffs evoked by photorelease of Ins(1,4,5)P_3 or low concentrations of acetylcholine were relatively variable, suggesting that they are not stereotypic. Therefore, the variable amplitude of Ca^{2+} puffs may depend on the number and organization of the Ins(1,4,5)P_3

receptors in the local volume as well as the local state of Ca^{2+} loading in the sarcoplasmic reticulum. Our results are compatible with a model where Ins(1,4,5)P_3-gated Ca^{2+} channels would exist in clusters containing variable numbers of channels and that within these clusters a variable number of channels could be recruited. A similar explanation has been proposed recently in non-excitable cells to account for hormone-evoked elementary Ca^{2+} signals [20,26]. In addition, successive activation of a given cluster of Ins(1,4,5)P_3 receptors may decrease the amount of Ca^{2+} locally available in this area of the sarcoplasmic reticulum and modify the amplitude of localized Ca^{2+} signals. It has been also proposed that the gating properties of Ins(1,4,5)P_3-gated channels may influence the amplitude and time course of Ca^{2+} events. An interesting possibility is that the individual clusters may be composed with different subtypes of Ins(1,4,5)P_3 receptors, which may have different sensitivity for Ca^{2+}-dependent modulation [27]. For example, subtype 3 of the Ins(1,4,5)P_3 receptor does not exhibit a Ca^{2+}-dependent inhibition, whereas subtype 1 shows a bell-shaped dependence on $[Ca^{2+}]_i$ [27,28]. Another possibility is that the gating of Ins(1,4,5)P_3 receptors may depend on the luminal Ca^{2+} concentration which may, in turn, regulate their sensitivity to Ins(1,4,5)P_3 [22,29]. In agreement with this proposal is the observation of spontaneous Ca^{2+} puffs and spikes in ureteric myocytes overloaded with 10 mM Ca^{2+} for 30 min. Spontaneous Ca^{2+} release from Ins(1,4,5)P_3-sensitive Ca^{2+} stores has been reported previously from permeabilized hepatocytes overloaded with Ca^{2+} [22]. To our knowledge, spontaneous Ca^{2+} puffs have never been described in intact cells at a basal Ins(1,4,5)P_3 level. The possibility that spontaneous Ca^{2+} puffs may activate various membrane conductances, as shown for Ca^{2+} sparks [4,5], is under investigation. It has been shown recently that co-expression of Ins(1,4,5)P_3-receptor subtypes 1 and 2 facilitates Ca^{2+} oscillations, whereas expression of Ins(1,4,5)P_3-receptor subtype 3 alone generates monophasic Ca^{2+} transients [30]. Since Ins(1,4,5)P_3-receptor subtypes 1 and 2 are more sensitive to Ins(1,4,5)P_3 and Ca^{2+} [30], it can be postulated that they are required for spontaneous Ca^{2+} puffs. In contrast, the Ins(1,4,5)P_3-receptor subtype 3 is activated by higher $[Ca^{2+}]_i$ and may participate in the acetylcholine-induced Ca^{2+} wave. Further experiments are needed to identify the expression pattern of Ins(1,4,5)P_3-receptor subtypes in ureteric myocytes and their specific roles in Ca^{2+} signalling.

As illustrated in Figure 5, Ca^{2+} waves propagate throughout ureteric myocytes by alternating between regeneration at several Ca^{2+}-release sites along the scanned line and Ca^{2+} diffusion between these release sites. In HeLa cells and *Xenopus* oocytes, Ca^{2+} wave activation has been associated with an increased frequency of elementary Ca^{2+} events at a single site, with an increased amplitude of Ca^{2+} events or with the recruitment of Ca^{2+}-release sites in the spatial domain [23,31]. In ureteric myocytes, Ca^{2+} waves were triggered by activation of an initiation site, frequently located at the edge of the cell, without any detectable increase in frequency or amplitude of preceding Ca^{2+} puffs. Diffusion of Ca^{2+} from this early initiation site activated neighbouring Ca^{2+}-release sites in the scanned lines, and this may account for wave-propagation velocity in ureteric myocytes. The notion that a sub-threshold pacemaker elevation of $[Ca^{2+}]_i$ precedes regenerative Ca^{2+} waves is not new [32], but our results emphasize further the importance of the pacemaker Ca^{2+} phase in cells expressing mainly Ins(1,4,5)P_3 receptors.

Visceral and vascular smooth muscles display a large variability in the type of intracellular Ca^{2+} store and in the role of these stores in initiating increase in $[Ca^{2+}]_i$ in response to mediators and hormones. Rat portal-vein myocytes possess a single functional intracellular Ca^{2+} store on which both ryanodine and Ins(1,4,5)P_3 receptors have been identified [9]. However, in these cells, only clusters of ryanodine receptors are present whereas Ins(1,4,5)P_3 receptors are distributed homogeneously on the sarcoplasmic reticulum, and the B_{max} value of [^{3}H]ryanodine binding is 3–4 times higher than that of [^{3}H]Ins(1,4,5)P_3 binding [24]. A physiological consequence is that a co-operativity between Ins(1,4,5)P_3- and ryanodine-sensitive Ca^{2+} channels is detected in these vascular myocytes to amplify Ca^{2+} release responsible for fast propagated Ca^{2+} waves in response to neuromediators [24]. In rat mesenteric artery myocytes, it has been proposed that Ca^{2+} stores are organized into small, spatially distinct compartments [33]. When these compartments possess only ryanodine or Ins(1,4,5)P_3 receptors, they function as separate Ca^{2+} stores and neuromediators may only activate the Ins(1,4,5)P_3-sensitive Ca^{2+} store. In contrast, when the compartments possess both ryanodine and Ins(1,4,5)P_3 receptors, they function as mixed Ca^{2+} stores and a co-operativity between Ins(1,4,5)P_3 and ryanodine receptors may be effective. In rat ureteric smooth muscle, as a consequence of the high expression of Ins(1,4,5)P_3 receptors and the absence of subtypes 1 and 2 of ryanodine receptors, Ca^{2+} release from the intracellular store is only activated by Ins(1,4,5)P_3 receptors and the amplifying mechanism is a pure Ins(1,4,5)P_3-induced Ca^{2+} release. In contrast, in guinea-pig ureter, the amplifying mechanism is described as dependent only on Ca^{2+}-induced Ca^{2+} release [11]. It can be proposed that in phasic smooth muscles showing electrical pacemaker activity, the Ca^{2+}-induced Ca^{2+}-release mechanism may play a major role in modulating contraction and cell excitability, whereas in tonic smooth muscles the Ins(1,4,5)P_3-induced Ca^{2+}-release mechanism predominantly controls Ca^{2+} release and contraction. Taken together, these data illustrate the variability of intracellular Ca^{2+} stores and Ca^{2+}-amplifying mechanisms in different cell types expressing different amounts of Ins(1,4,5)P_3 and ryanodine receptors. The results emphasize the importance of combining molecular experiments, immunostaining and functional approaches to identify the specific characteristics of Ca^{2+} signalling in each cell type studied.

In conclusion, these findings show that in rat ureteric smooth-muscle cells localized and transient Ca^{2+} events induced by the opening of clusters of Ins(1,4,5)P_3-sensitive Ca^{2+}-release channels can be triggered in response to neuromediator activation or recorded spontaneously in Ca^{2+}-overloaded myocytes. They represent a continuum of Ca^{2+} signals with variable amplitude, duration and diffusion rather than a strict hierarchy of Ca^{2+} events. Propagated Ca^{2+} waves appear to result from the spatial recruitment of several Ca^{2+}-release sites by Ca^{2+} diffusion. Both Ca^{2+} signals provide an integrated mechanism to regulate contractility in smooth-muscle cells where ryanodine receptors are not or are poorly expressed.

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