Acta Pharmacologica Sinica 2006 Jul; 27 (7): 939–944

Blackwell Publishing

Full-length article

Localized Ca^{2+} uncaging induces Ca^{2+} release through IP_3R in smooth muscle

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Key words

two photon flash photolysis; DMNP-EGTA; calcium release; smooth muscle; IP_3R; mice

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Received 2006-04-17
Accepted 2006-05-19

doi: 10.1111/j.1745-7254.2006.00389.x

Abstract

Aim: Our previous study indicated that there are two types of Ca^{2+} release events seen in intact mouse bladder tissue. In this study our aim is to investigate the mechanism that underlies the phenomena of Ca^{2+} release in smooth muscle. **Methods:** Single cells were isolated and tissue segments were prepared by cutting the detrusor into 0.1 cm×0.5 cm strips running along the axis from the neck to the fundus. Single cells and intact tissue strips were co-loaded with the Ca^{2+} indicator and caged Ca^{2+} by incubation with 10 μmol/L Fluo-4 AM and DMNP-EDTA-AM. Fluo-4 AM fluorescence was detected by laser scanning confocal microscopy, and local uncaging of DMNP-EGTA was achieved by brief exposure to the output of a diode-pumped, Ti:sapphire laser tuned to 730 nm. **Results:** Local uncaging of caged Ca^{2+} was able to trigger Ca^{2+} release events in both single cells and tissue strips from mouse bladder. The Ca^{2+} release events could not be blocked by ryanodine alone, but the property of the Ca^{2+} release was markedly altered. Surprisingly, in the presence of ryanodine, Xestospongin C completely inhibited the Ca^{2+} release events both in single cell and tissue experiments. **Conclusion:** (1) Two photon flash photolysis (TPFP) triggers Ca^{2+} induced Ca^{2+} release. This process involves release through type 2 ryanodine receptor channels; (2) TPFP results in the release of Ca^{2+} through inositol 1,4,5-trisphosphate receptors in the absence of phospholipase C activation.

Introduction

As in other muscle types, the major Ca^{2+}-store in smooth muscle is the sarcoplasmic reticulum (SR). Evidence indicates that there are two types of Ca^{2+} release receptors, ryanodine receptor (RYR) and inositol 1,4,5-trisphosphate receptor (IP_3R), in SR in the smooth myomytes[1–4]. Three receptor subtypes, or isoforms, have been identified for each type of receptor[5–8]. The release of SR-Ca^{2+} results from activation of either RYR or IP_3R. However, the role of IP_3R in regulation of Ca^{2+} release in smooth muscle, especially in mouse bladder smooth muscle, has not been identified, although it has been well established for type 2 ryanodine receptor (RYR2). In urinary bladder myocytes, activation of the voltage-dependent Ca^{2+} current (I_{Ca}) evokes Ca^{2+} induced Ca^{2+} release (CICR) in the form of Ca^{2+} sparks or global Ca^{2+} waves in a graded fashion[9]. Genetic evidence indicates that RYR2 channel proteins play a predominate role in SR Ca^{2+} release in bladder myocytes[10], similar to CICR in heart cells.

We previously reported that there are two types of Ca^{2+} release events in smooth muscle[11]. One is rapid, whole-cell Ca^{2+} transients, or "Ca^{2+} flashes" and the other is slowly propagating Ca^{2+} waves. The Ca^{2+} flashes occur through RYR-mediated Ca^{2+} release, whereas Ca^{2+} waves arise from IP_3-mediated Ca^{2+} release. However, ours and other studies suggest that IP_3R is not involved in Ca^{2+} release-induced either by stretching or by membrane voltage depolarization. Evidence of this has been found using 2-APB, an IP_3R inhibitor that does not block Ca^{2+} release in mouse bladder single cell studies[12]. There is no evidence to date that IP_3R is functional in bladder smooth myocytes.

Two photon flash photolysis (TPFP) provides the capability to photorelease molecules in a subcellular volume on

the order of 1 femtoliter and this method has been used to release and examine CICR in cardiac myocytes[13,14]. In the present study, we used TPFP to formally test the hypothesis that localized increases in Ca^{2+} in a small subcellular domain evokes Ca^{2+} release from the SR in smooth muscle independent of the gating of sarcolemmal Ca^{2+} channels. We also aimed to examine the mechanism underlying this process. We reported that TPFP is capable of triggering CICR and results in the release of Ca^{2+} through IP_3R in the absence of PLC in smooth muscle.

Materials and methods

Cell, tissue strip preparation and solutions Animals were euthanized with CO_2 and bladders were rapidly removed and dissected in cold water. Single cells were prepared as described previously[12]. After removing of mucosal and fibrosal layers, bladders were cut into small pieces and digested in enzymatic solution. The enzymatic solution containing (mmol/L) 80 Na-glutamate, 55 NaCl, 6 KCl, 2 $MgCl_2$, 10 HEPES, and 10 glucose. Two-step digestions were used for single cell dissociation. First, detrusor muscle was incubated for 20 min at 37 °C in dissociation solution containing 1 mg/mL dithioerythreitol, 1 mg/mL papain, and 1 mg/mL bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA), and the partially digested tissue was then transferred to a solution containing 1 mg/mL collagenase type II (Worthington Biochemical, Lakewood, New Jersey,USA), 1 mg/mL bovine serum albumin, and 100 μmol/L Ca^{2+}. After incubation for 10 min, the digested tissue was washed and gently triturated in dissociation solution to yield single smooth muscle cells. Tissue strip was prepared as reported previously[11]. Detrusor muscle running along the axis from the neck to the fundus was cut into strips about 0.1 cm×0.5 cm. The strips were transferred into an optical recording chamber and fixed with a Kevlar fiber retaining clip (Warner Instruments, Hamden, CT, USA). The extracellular solution used for single cell and tissue strip perfusion was (mmol/L) 137 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 10 glucose, 10 HEPES, and pH 7.4 adjusted with NaOH.

Imaging and local uncaging of caged Ca^{2+} Single cells and intact tissue strips were co-incubated with Fluo-4 AM 10 μmol/L (Molecular Probes, Eugene, Oregon, USA) and 1-(4,5-dimethoxy-2-nitrophenyl)-1,2-diaminoethane-*N*′,*N*,*N*′, *N*′,-tetraacetic acid, tetra (acotoxymethyl ester) (DMNP-EDTA, AM) 10 μmol/L (Molecular Probes, Eugene, Oregon, USA) in a bath solution containing 0.02% pluronic acid for 10 min (single cell) or 60 min (tissue) at room temperature (20–24 ºC). Fluo-4 AM was excited by a 488 nm laser light and emission was collected through a long-pass filter of 500 nm. Confocal images were captured using a Bio-Rad Radiance 2000 confocal head attached to a Nikon TMIII inverted microscope with 60×water immersion objective. Confocal X-y images are 128×20 pixels in size and with the line frequency of 1200 Hz. Line-scan images were acquired at sampling rates of 0.83 ms/pixel and 0.73 μm/pixel.

Local uncaging of caged Ca^{2+}, DMNP-EGTA, was achieved by exposure to an ultraviolet (UV) laser light beam (Mai Tai, Diode-pumped, mode-locked Ti: sapphire laser, Spectra-Physics, Mountain View, CA, USA) at wavelength of 730 nm and a power of 4.5 mW. Image processing, data analysis, and presentation were performed using software of MATLAB 6.5. Data were reported as mean±SEM. Student's *t*-test was applied to determine statistical significance of measured differences. A *P*-value of less than 0.05 was considered statistically significant.

Results

Ryanodine did not inhibit Ca^{2+} releases induced by two photon flash photolysis in single cells Our previous studies indicated that RYR2, but neither type 3 nor IP_3R, plays an important functional role in smooth muscle[5]. In the present study, we directly tested the function of RYR and IP_3R Ca^{2+} release channels using localized uncaging of caged Ca^{2+}, DMNP-EGTA, in mouse bladder smooth muscles. First we examined the uncaging of caged Ca^{2+} effect in single cells. As shown in Figure 1A, cells co-loaded with DMNP-EGTA and Fluo-4 AM repeatedly exposed to UV (30 ms, 4.3 mW) was able to trigger Ca^{2+} release (sparks), which is consistent with our previous study[12]. Next we tested the effects of Ca^{2+} release receptor inhibitors on the Ca^{2+} release events triggered by exposure to the bleaching laser light. Application of Xestospongin C (final concentration 10 μmol/L), a selective IP_3R inhibitor, there was almost no visible effect on Ca^{2+} sparks (data not shown). Surprisingly, application of ryanodine (10 μmol/L) did not completely block Ca^{2+} release induced by uncaging of caged Ca^{2+}, but rather markedly altered the characteristics of CICR (Figure 1B). This encouraged us to test what would happen if ryanodine and Xestospongin C were co-applied. The results indicated that in the presence of both ryanodine (10 μmol/L) and Xestospongin C (10 μmol/L) Ca^{2+} sparks and/or waves induced by local uncaging of DMNP-EGTA by exposure to UV laser light were completely ablated (Figure 1C). This suggests that in the presence of RYR inhibitor, increase in the cytosolic Ca^{2+} concentration level by local uncaging of caged Ca^{2+} could directly trigger IP_3R Ca^{2+} release channel function and hence, induce Ca^{2+} sparks and/or waves in bladder smooth muscle.

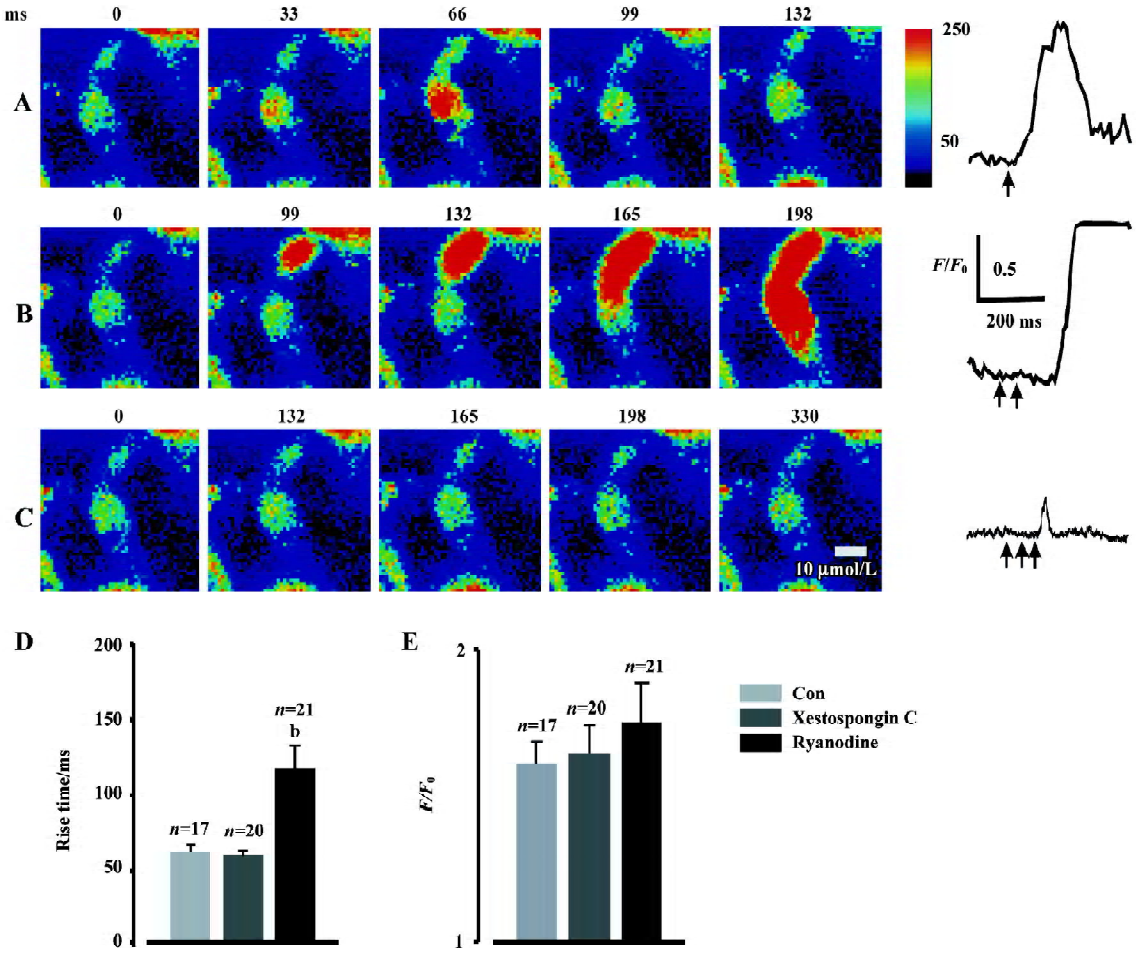

Figure 1. Ryanodine and IP_3 receptors mediate local uncaging of caged Ca^{2+}-induced CICR in single cells. (A) Single cell x-y images indicating a Ca^{2+} spark triggered by TPFP in a control cell. (B) Incubation of cells with ryanodine (10 μmol/L) did not prevent cells from releasing Ca^{2+}. (C) Combined exposure to ryanodine and Xestospongin C (30 μmol/L) completely eliminated Ca^{2+} release. (D, E) Summary data indicating the rise time and peak Ca^{2+} of Ca^{2+} release in each condition. Note that the amount of exposure to TPFP required to induce release was significantly higher in the presence of ryanodine. $^bP<0.05$ *vs* control and Xestospongin C group. The numbers above the images indicate the time (ms) after exposure to TPFP.

Effect of Ca^{2+} release receptor inhibitors on Ca^{2+} release in intact smooth muscles As mentioned above, local uncaging of caged Ca^{2+} by exposure to UV laser light was capable of triggering Ca^{2+} release events in the presence of RYR or IP_3R alone in mouse bladder single cells. Next we further examined the role of Ca^{2+} release receptor (channel) inhibitors in the regulation of Ca^{2+} release channels in intact tissue strips. Figure 2A shows that exposed DMNP-EGTA and Furo-4 AP co-loaded tissues to UV laser light was also able to cause Ca^{2+} releases (sparks). But the time exposure to UV laser light to trigger Ca^{2+} release was much longer (100 ms) compared to that used in single cell experiments. Similarly, as observed in single cell experiments, Xesto-spongin C (10–30 μmol/L) or ryanodine (30 μmol/L) alone failed to block Ca^{2+} release triggered by uncaging of caged Ca^{2+} in intact tissue segments. Unlike Xestospongin C experiments, more flash times were needed to trigger Ca^{2+} release in the presence of ryanodine, as indicated in Figure 2B, suggesting the property of Ca^{2+} release was altered by ryanodine in intact tissues as well. Our results also indicated that co-application of ryanodine and Xestospongin C Ca^{2+} releases were completely inhibited in intact mouse bladder tissue segments (Figure 2C).

Effect of Ca^{2+} release receptor inhibitors on kinetics of Ca^{2+} induced Ca^{2+} release To further understand the role of specific intracellular Ca^{2+} release channels in locally elicited CICR, we analyzed the kinetics (rise time and time to release), Ca^{2+} wave propagation velocity, following TPFP. The rise

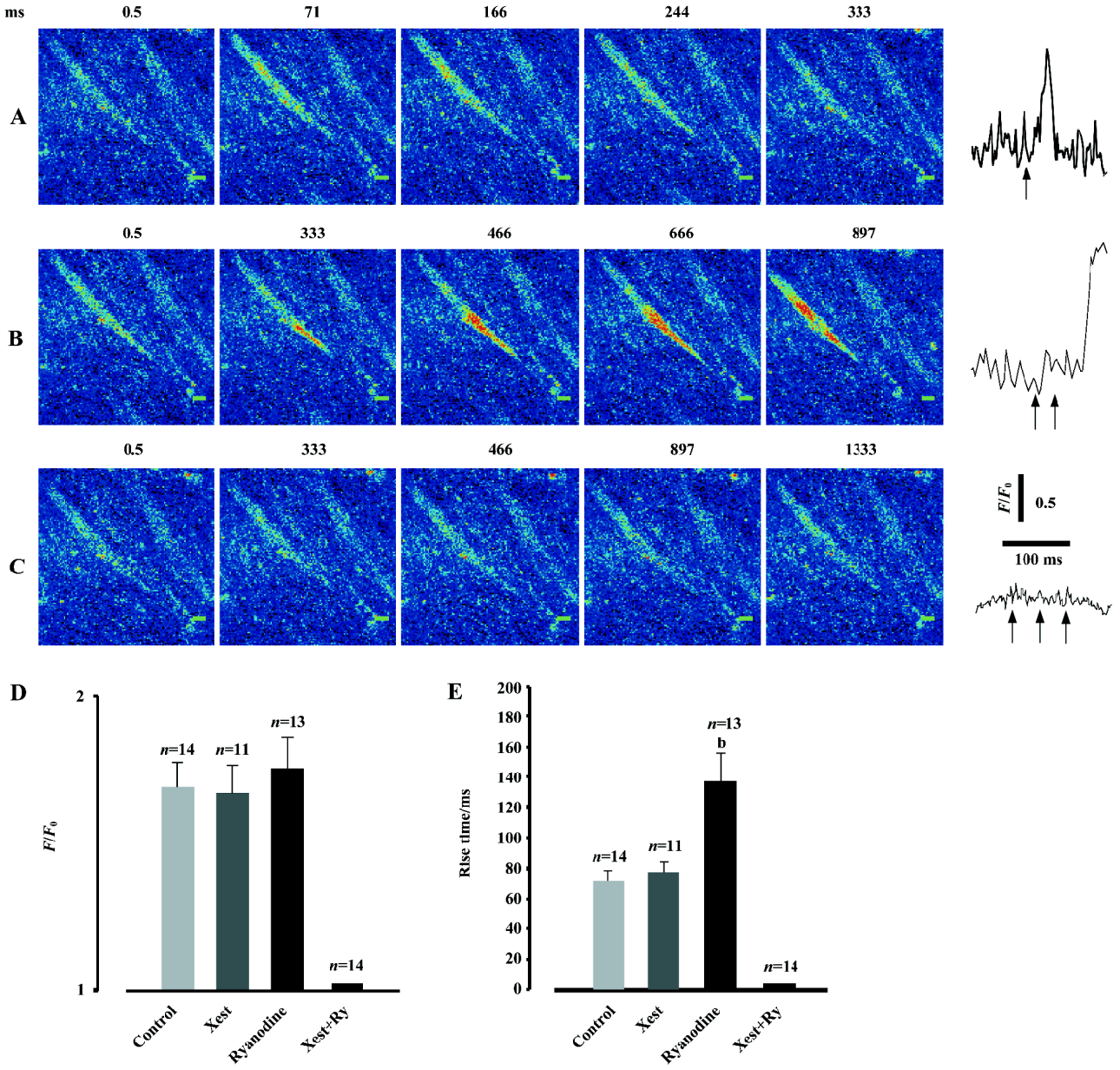

Figure 2. Inhibition of Ca^{2+} release by Ca^{2+} release channel inhibitors in intact tissue segments. (A) A representative experiment of x-y image from mouse bladder tissue strip in the absence of ryanodine and Xestospongin C. As observed in single cell experiment, TPFP was capable of triggering Ca^{2+} sparks in intact myocytes. (B) In the presence of 30 μmol/L ryanodine TPFP still induced Ca^{2+} release (shown here is Ca^{2+} wave) in tissue strip. Note more flash times were needed to trigger Ca^{2+} release, the time delay was much longer than that in control experiment (A). (C) Co-incubation of the tissue strip with ryanodine and Xestospongin C TPFP failed to trigger Ca^{2+} release, even more flash times were given. (D, E) Similarly to single cell experiments, the peak Ca^{2+} was not altered by Ca^{2+} release channel inhibitors, whereas the Ca^{2+} release rise time was significantly prolonged in the presence of ryanodine ($^bP<0.05$, $n=13$) compared to that in control and Xestospongin C groups.

time of Ca^{2+} release was markedly affected by RYR antagonism, likely associated with a decrease in the underlying rate of SR Ca^{2+} release. Ca^{2+} spark rise time was markedly prolonged in the presence of 10 μmol/L ryanodine (142.23±17.6 ms, $n=21$) compared to that in control experiments (64.22±6.21 ms, $n=17$, Figure 1F). Conversely, in the presence of 30 μmol/L xestospongin C the rate of rise of Ca^{2+} release following TPFP was not significantly different from control (60.6±4.33 ms, $n=38$). This could be observed in both single cell and intact tissue experiments (Figure 1D, 2E).

The hypothesis that CICR is the mechanism underlying Ca^{2+} spark/wave propagation is commonly accepted. In the present study, the speed of Ca^{2+} spark/wave propagation triggered by exposing caged Ca^{2+}, DMNP-EGTA, to UV laser light was determined in intact mouse bladder smooth muscle tissue strips by the time taken between the initiating point of Ca^{2+} sparks/waves and their propagating end in the cells. As shown in Figure 4, ryanodine receptor inhibition also markedly slowed the rate of Ca^{2+} wave propagation following TPFP. Wave propagation velocity decreased from 23.2± 3.6 μm/s (n=18) to 4.3±0.8 μm/s (n=16) in the presence of ryanodine, whereas in the presence of xestospongin C, wave propagation was not significantly different from control (P>0.05, n=12).

To more effectively quantify the time to release, intact muscle segments were exposed to UV light for 100 ms (4.3 mW) at the beginning of a prolonged line scan along the cell axis, and the time delay calculated as the time that from the beginning of the 730 nm pulse to the initiation of Ca^{2+} release. The time to release was 71.33± 6.8 ms for control group and 172.66±36.5 ms for ryanodine group, respectively. Whereas, inhibition of IP_3R with xestospongin C had no significant effect (80.32±7.41 ms, n=17, Figure 3B) observed .

Peak Ca^{2+} was not altered by Ca^{2+} release channel inhibitors As shown in Figures 1E, 2D, and 3B (right), the amplitude of the Ca^{2+} spark or Ca^{2+} wave was equivalent in control cells and those incubated with 10 (or 30) μmol/L ryanodine or 30 μmol/L xestospongin C, suggesting that efficient release of SR Ca^{2+} stores is achieved when CICR occurs through the activation of either SR release channel.

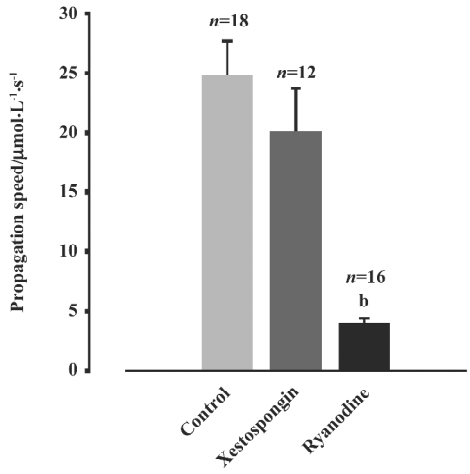

Figure 4. Summary data of Ca^{2+} release propagation. Propagation was significantly slowed in the presence of ryanodine, whereas in the presence of Xestospongin C, the propagation velocity was not significantly altered. ^bP<0.05 *vs* control and Xestospongin C group.

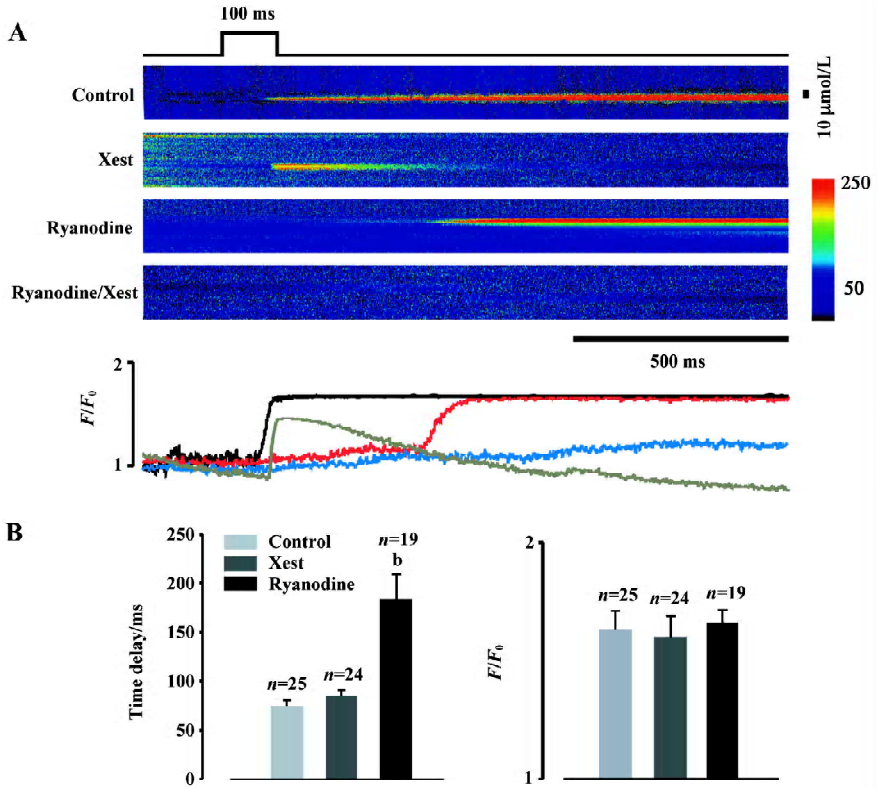

Figure 3. Increased delay to release by IP_3R. (A) Examples of linescan images obtained during TPFP. Plots below indicate continuous fluorescence at the peak of release for each condition. Ryanodine markedly increased the delay between initiation of the laser uncaging flash and initial Ca^{2+} release. Laser excitation pulse was 100 ms in all experiments. As observed in x-y experiments, rise time was markedly slowed in the presence of ryanodine. (B) Summary data from series of linescan experiments indicate a significant increase in delay in the presence of ryanodine. ^bP<0.05 *vs* control and Xestospongin C group.

Discussion

We report that localized uncaging of caged Ca^{2+} by two photon flash photolysis initiates CICR in urinary bladder myocyte. The most surprising finding in our study was the apparent ability of two photon flash photolysis to activate Ca^{2+} release through IP_3 receptors, as evidenced by the activation of Ca^{2+} that occurred with different kinetics (delay, rise time, and propagation) and required greater amounts of trigger Ca^{2+} in the presence of ryanodine (10–30 μmol/L). The results confirm that the kinetics of Ca^{2+} signals mediated by RyR and IP_3R are very different[15]. The activation of slowly propagating, asynchronous Ca^{2+} waves in the presence of ryanodine, the marked slowing of wave propagation velocity in the presence of ryanodine, and the complete inhibition of two photon flash photolysis in the presence of Xestospongin C and ryanodine, but not in the presence of ryanodine alone, strongly suggest that IP_3R Ca^{2+} release can be activated by local increases in $[Ca^{2+}]_i$. While substantial data indicate that the gating of IP_3R is augmented by increases in cytosolic Ca^{2+} [16,17], this process is thought to be insufficient to activate IP_3R gating in the absence of phospholipase C stimulation and a significant rise in cytosolic IP_3 concentration. The generation of ryanodine-insensitive, slowly propagating waves by two photon flash photolysis may reflect activation of IP_3R Ca^{2+} channels by non-physiologic increases in local $[Ca^{2+}]_i$, as Ca^{2+} release required multiple laser pulses in the presence of ryanodine (Figure 2), particularly as xestospongin C alone had little effect on Ca^{2+} release. While these findings suggest that ryanodine receptors dominate the pattern of Ca^{2+} release following local rises in $[Ca^{2+}]_i$, the ability to trigger release from IP_3R suggests the potential for some involvement of these release channels during CICR, as release through RYR gating would be expected to produce very high local concentrations of Ca^{2+}.

IP_3R, like RYR, is a Ca^{2+} release channel that specially responds to the second messenger IP_3. Three structurally and functionally different isoforms of the IP_3R that are expressed in a cell-type specific manner have been identified[8]. Our data indicate that all isoforms of IP_3R are expressed in mouse bladder smooth myocytes (data not shown). But the role of IP_3R, unlike RYR, in the regulation of Ca^{2+} release is not clear in smooth myocytes. In the present study, we, for the first time, demonstrated that high local cytosolic Ca^{2+} induced by local uncaging of DNMP-EGTA by TPFP triggers Ca^{2+} release through IP_3R in the absence of PLC activation and that this process, while kinetically distinct from RyR release, is capable of supporting robust CICR.

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