Cytosolic [Ca\(^{2+}\)] regulation of InsP\(_3\)-evoked puffs

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Introduction

The InsP\(_3\)R (InsP\(_3\) receptor) is a Ca\(^{2+}\)-permeable channel expressed in the ER (endoplasmic reticulum) which is gated by the binding of the second messenger InsP\(_3\), and by cytosolic Ca\(^{2+}\) itself [1–7]. Ca\(^{2+}\) liberation occurs at discrete functional release sites, formed by clusters of InsP\(_3\)R on the ER membrane. These clusters can release ions into the ER in a manner similar to how the InsP\(_3\)R releases Ca\(^{2+}\) at the cluster. The positive-feedback mechanism of CICR (Ca\(^{2+}\)-permeable channel released Ca\(^{2+}\) release), by which Ca\(^{2+}\) released from one InsP\(_3\)R channel promotes opening of neighboring channels, underlies these processes, and factors including cytosolic Ca\(^{2+}\) buffering, [InsP\(_3\)] and basal cytosolic [Ca\(^{2+}\)] determine the transition between local and global signaling patterns.

The role of cytosolic Ca\(^{2+}\) in modulating InsP\(_3\)R channel gating has been extensively studied by single-channel recordings from InsP\(_3\)R in excised nuclei and after reconstitution in lipid bilayers, revealing the well-known ‘bell-shaped’ curve of Ca\(^{2+}\) facilitation and inhibition [1,2,13,14]. However, less is known about how cytosolic Ca\(^{2+}\) modulates local InsP\(_3\)-mediated signals in the intact cell, although imaging studies in X. laevis oocytes demonstrate a profound potentiation of global Ca\(^{2+}\) waves [13,15–17].

In the present study, we expressed Ca\(^{2+}\)-permeable nAChRs (nicotinic acetylcholine receptor/channels) in the plasma membrane of X. laevis oocytes so as to experimentally regulate basal cytosolic [Ca\(^{2+}\)] concentration [18], and examined how elevations in cytosolic [Ca\(^{2+}\)] affected the dynamics of local and global Ca\(^{2+}\) signals evoked by photorelease InsP\(_3\). We show that an increased probability of triggering local Ca\(^{2+}\) release at puff sites underlies the strong augmentation of global InsP\(_3\)-mediated Ca\(^{2+}\) waves, whereas puff amplitudes and durations were unaffected.

Experimental

Oocyte preparation and expression of nAChRs

X. laevis were purchased from Nasco International and the oocytes were surgically removed [19] following protocols approved by the UC Irvine Institutional Animal Care and Use committee. Stage V–VI oocytes were isolated and treated with collagenase (1 mg/ml collagenase type A1 for 30 min) to remove the follicular cell layers. At 1 day after isolation the oocytes were injected with a cRNA mixture for nAChR expression {α, β, γ and δ subunits at a ratio of 2:1:1:1; 50 nl at a final concentration of 0.1–1 mg/ml} and were then maintained in modified Barth’s solution [88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO\(_3\), 0.82 mM MgSO\(_4\), 0.33 mM Ca(NO\(_3\))\(_2\), 0.41 mM CaCl\(_2\), 5 mM Hepes and 1 mg/ml gentamicin (pH 7.4)] for 1–3 days at 16°C before use. Expression of nAChRs was evaluated using a voltage clamp to measure the currents evoked by 500 nM ACh: oocytes showing currents >1 μA at −80 mV were selected for the experiments.

Microinjection of oocytes

Intracellular microinjections were performed using a Drummond microinjector. Approximately 1 h before the Ca\(^{2+}\) imaging experiments, oocytes in Ca\(^{2+}\)-free Barth’s solution were injected with Fluo-4 dextran (high affinity; \(K_d = 800 \text{ nM}\)) to a final concentration of 40 μM, assuming equal distribution throughout the oocyte. Intracellular microinjections were performed using a Drummond microinjector. Approximately 1 h before the Ca\(^{2+}\) imaging experiments, oocytes in Ca\(^{2+}\)-free Barth’s solution were injected with Fluo-4 dextran (high affinity; \(K_d = 800 \text{ nM}\)) to a final concentration of 40 μM, assuming equal distribution throughout the oocyte.
Ca\textsuperscript{2+} imaging and flash photolysis

Oocytes were voltage-clamped using a conventional two-microelectrode technique. The membrane potential was held at 0 mV during superfusion with a non-desensitizing concentration of ACh (100–500 nM) in Ringer’s solution and was briefly stepped to −120 mV to strongly increase the electrical driving force for Ca\textsuperscript{2+} influx [20]. Global Ca\textsuperscript{2+} signals were imaged at room temperature (18–20°C) by a custom-build confocal line scanner [21] interfaced to an Olympus inverted microscope IX 70, and fluorescence excitation was provided by the 488 nm line of an argon ion laser, with the laser spot focused by a ×40 oil-immersion objective [NA (numerical aperture) = 1.35] and scanned along at a rate of 10 ms/50 μm line. To image puffs, we used wide-field fluorescence microscopy using an Olympus IX 71 inverted microscope equipped with a ×40 oil-immersion objective, a 488 nm argon ion laser for fluorescence excitation and an electron-multiplied CCD (charge-coupled device) camera (Cascade 128+; Roper Scientific) for imaging fluorescence emission (510–600 nm) at a frame rate of 500 s−1. Fluorescence was imaged from a 40 μm × 40 μm (128 × 128 pixel) region within the animal hemisphere of the oocyte. Fluorescence measurements made by line-scan and camera imaging are expressed as a ratio (ΔF/F\textsubscript{0}) of the mean change in fluorescence at that pixel before stimulation (F\textsubscript{0}). The mean values of F\textsubscript{0} were obtained by averaging over several scans/frames before stimulation. To calibrate the changes in ΔF/F\textsubscript{0} values in terms of nanomolar increases of free [Ca\textsuperscript{2+}] we determined maximal (F\textsubscript{max}) and minimal (F\textsubscript{min}) fluorescence values by injecting fluo-4 dextran-loaded oocytes (n = 5) with 30 nl of 100 mM CaCl\textsubscript{2} or 100 mM EGTA from a micropipette located close to the imaging site. After correcting for oocyte autofluorescence, the mean values were F\textsubscript{max} = 8.52 ± 1.16 and F\textsubscript{min} = 0.857 ± 0.024 relative to the resting fluorescence F\textsubscript{0} before injection. We assumed a K\textsubscript{d} value for fluo-4 dextran of 2400 nM, based on measurements of ~800 nM in free solution [22] and a roughly 3-fold reduction in affinity in the cytoplasmic environment [22]. A fluorescence increase of ΔF/F\textsubscript{0} = 1 above baseline would then correspond to an increase of [Ca\textsuperscript{2+}]\textsubscript{0} of about 360 nM. Photolysis of caged InsP\textsubscript{3} was evoked by flashes of UV light (350–400 nm) from a mercury arc lamp, delivered through the microscope objective and adjusted to uniformly irradiate a circular region slightly larger than the imaging frame or scan line. Flash durations were set using a Uniblitz shutter and digital controller.

Reagents

Fluo-4 dextran (high affinity; K\textsubscript{d} ~ 800 nM), and caged InsP\textsubscript{3} were purchased from Invitrogen. All other reagents were from Sigma–Aldrich.

Data analysis

Custom routines written in the IDL programming environment (Research Systems) were used for linescan image processing and measurements. MetaMorph (Molecular Devices) was used to process and measure data obtained from wide-field camera-based imaging. Further analysis and graphing was accomplished using Microcal Origin version 6.0 (OriginLab). Results are means ± S.E.M. and significance was assessed by Student’s t test.

RESULTS

Elevated basal cytosolic [Ca\textsuperscript{2+}] enhances InsP\textsubscript{3}-evoked Ca\textsuperscript{2+} waves

In order to evoke cytosolic [Ca\textsuperscript{2+}] elevations, Ca\textsuperscript{2+} influx was induced through nAChRs expressed in the oocyte plasma membrane. Oocytes were continuously superfused with Ringer’s solution containing 1.8 mM Ca\textsuperscript{2+}, together with a low non-desensitizing concentration (100–500 nM) of acetylcholine, and were voltage-clamped to control the electrochemical gradient for Ca\textsuperscript{2+} entry. The membrane potential was held at 0 mV to minimize Ca\textsuperscript{2+} influx, and was then stepped to more negative values to promote Ca\textsuperscript{2+} influx beginning 2.5 s before the delivery of a UV flash to photorelease InsP\textsubscript{3} from a caged precursor loaded into the oocyte (Figure 1A). The resulting changes in fluo-4 fluorescence were imaged to compare InsP\textsubscript{3}-evoked Ca\textsuperscript{2+} responses evoked by identical UV flashes during cytosolic [Ca\textsuperscript{2+}] elevation with control records when the voltage pulse was not applied.

We first examined global Ca\textsuperscript{2+} signals evoked in oocytes that were not loaded with EGTA. The panels on the left-hand side of Figure 1(B) show representative linescan images of fluorescence signals evoked in oocytes that were not loaded with EGTA. The panels on the left-hand side of Figure 1(B) show representative linescan images of fluorescence signals evoked in oocytes that were not loaded with EGTA. The panels on the left-hand side of Figure 1(B) show representative linescan images of fluorescence signals evoked in oocytes that were not loaded with EGTA. The panels on the left-hand side of Figure 1(B) show representative linescan images of fluorescence signals evoked in oocytes that were not loaded with EGTA.

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changes evoked by photoreleased Ins\(_P_3\) without (upper panel) and with (lower panel) Ca\(^{2+}\) influx; the corresponding fluorescence profiles are presented on the right-hand side. We compared the latencies and peak amplitude of Ins\(_P_3\)-evoked Ca\(^{2+}\) signals under resting cytosolic [Ca\(^{2+}\)], and during Ca\(^{2+}\) influx that increased the basal fluorescence signal by a mean of 0.60 ± 0.06 ΔF/F\(_o\) (six oocytes from three different frogs). Latencies (time from the UV flash to the initial rise in fluorescence) were significantly shorter during cytosolic [Ca\(^{2+}\)] elevation (Figure 1C; control latency = 284 ± 14 ms and during Ca\(^{2+}\) influx latency = 177 ± 30 ms; P < 0.05). The mean peak amplitude of Ca\(^{2+}\) waves was profoundly augmented by cytosolic Ca\(^{2+}\) elevation (Figure 1D; control ΔF/F\(_o\) = 1.68 ± 0.23 and during Ca\(^{2+}\) influx ΔF/F\(_o\) = 3.50 ± 0.45; P < 0.05, n = 6). These results are consistent with previous observations showing facilitation of Ins\(_P_3\)-evoked Ca\(^{2+}\) signals by cytosolic [Ca\(^{2+}\)] [1–7].

Elevated basal cytosolic [Ca\(^{2+}\)] promotes Ins\(_P_3\)-evoked Ca\(^{2+}\) puffs

We next examined the effects of basal cytosolic [Ca\(^{2+}\)] elevations on local Ca\(^{2+}\) puffs. For this purpose oocytes were loaded with EGTA (final intracellular concentration 300 μM) to suppress generation of Ca\(^{2+}\) waves by inhibiting inter-cluster diffusion of Ca\(^{2+}\) ions [23]. We further employed wide-field fluorescence microscopy to image a 40 μm × 40 μm field of view with a fast (500 frames per s) electron-multiplied CCD camera, so as to sample many more puff sites than possible by one-dimensional linescan imaging. Figure 2 shows the experimental protocol (Figure 2A, a), and representative fluorescence traces monitored from small regions of interest centred on puff sites illustrating the responses evoked by photoreleased Ins\(_P_3\), at resting cytosolic Ca\(^{2+}\) (Figure 2A, b) and when Ca\(^{2+}\) was elevated by Ca\(^{2+}\) influx (Figure 2A, c and d). The photolysis flash was delivered 4 s after the onset of the hyperpolarizing pulse so as to allow cytosolic [Ca\(^{2+}\)] to equilibrate, and puffs were then recorded for 6 s while the hyperpolarization was maintained. We varied the duration of the photolysis flash to evoke differing numbers of puffs at resting cytosolic [Ca\(^{2+}\)]; ‘weak’ flashes (25–50 ms) were chosen to evoke on average about a single puff in the entire imaging field 40 μm × 40 μm, and ‘strong’ flashes (50–100 ms) to evoke up to four puffs. Even the ‘strong’ stimulus was chosen to evoke responses well below the maximal, so as to avoid possible saturation effects when responses were further potentiated by Ca\(^{2+}\) influx.

Figure 2(B) shows a scatter plot of the relationship between the numbers of individual sites in the imaging field where puffs were observed during 6 s following photorelease of Ins\(_P_3\), as a function of the elevation of cytosolic [Ca\(^{2+}\)] evoked by hyperpolarizing pulses. We express the [Ca\(^{2+}\)] elevation in terms of fluorescence ratio change, without correction for oocyte autofluorescence (about 50% of resting fluo-4 fluorescence). On the

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**Figure 2** Cytosolic [Ca\(^{2+}\)]-dependent potentiation of Ins\(_P_3\)-evoked Ca\(^{2+}\) puffs

(A) a, Schematic diagram of the experimental protocol. b and c, Representative fluorescence profiles of puffs evoked without (control) and with (+ influx) basal cytosolic [Ca\(^{2+}\)] elevation, obtained from the same oocyte. The record in b was obtained from the single responding site within the imaging field, whereas the one in c shows superimposed traces from seven responding sites. d, Zoomed version of c on an expanded timescale to illustrate more clearly the variation in puff latencies following photorelease of Ins\(_P_3\). The traces in b–d are blanked out during the photolysis flash. (B) Scatter plot showing the numbers of sites within the imaging field that showed puffs following weak (O, 25–50 ms flash duration) or strong (■, 50–100 ms) photorelease of Ins\(_P_3\) as a function of cytosolic Ca\(^{2+}\) elevation during influx (ΔF/F\(_o\)). (C) Mean numbers of responding puff sites within imaging field, grouped by photolysis strength and by elevation of basal cytosolic [Ca\(^{2+}\)] (ΔF/F\(_o\) < 0.1 or > 0.1).

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basis of the calibration described in the Experimental section, an increase in $\Delta F/F_0$ of 0.1 corresponds to a rise in $[\text{Ca}^{2+}]$ of about 36 nM. With both weak and strong photolysis flashes the number of responding puff sites increased steeply with increasing basal $[\text{Ca}^{2+}]$, with strong flashes giving greater numbers at any given basal $[\text{Ca}^{2+}]$. Figure 2(C) shows mean data, grouped according to flash duration and whether basal cytosolic levels just before the photolysis flash were at or close to the resting level ($\Delta F/F_0 = 0–0.1$) or were appreciably elevated ($\Delta F/F_0 > 0.1$).

**Elevated cytosolic $[\text{Ca}^{2+}]$ shortens puff latency**

Figures 3(A) and 3(B) show scatter plots of individual and mean latencies of puffs, grouped according to the cytosolic $[\text{Ca}^{2+}]$ elevation at the time of the photolysis flash. Puffs evoked by weak stimuli arose with relatively long (2–3 s) latencies, which tended to shorten with increasing cytosolic $[\text{Ca}^{2+}]$, but did not show a statistically significant correlation (Figure 3A). On the other hand, mean puff latencies were shorter with stronger photorelease of InsP$_3$ (Figure 3B) and showed a marked dependence on cytosolic $[\text{Ca}^{2+}]$, reducing from 2133 ± 200 ms at near resting level ($\Delta F/F_0 = 0.0$) to 1240 ± 174 ms when the fluorescence was elevated to $>0.1$ $\Delta F/F_0$ during $\text{Ca}^{2+}$ influx ($P < 0.01$).

Puff latencies followed roughly exponential distributions at both relatively low and high cytosolic $[\text{Ca}^{2+}]$ (Figures 3C and 3D respectively), with a markedly shorter time constant at higher $[\text{Ca}^{2+}]$. Puff durations are independent of basal cytosolic $[\text{Ca}^{2+}]$

We had observed previously a prolongation of puff duration when puffs were evoked after loading ER $\text{Ca}^{2+}$ stores by inducing a prior $\text{Ca}^{2+}$ influx in oocytes transfected to overexpress SERCA (sarcoplasmic/ER $\text{Ca}^{2+}$-ATPase), but not in control (non-expressing) oocytes. We now examined the effect of elevated $[\text{Ca}^{2+}]_{\text{cyt}}$ on puff duration. Puffs evoked by strong photorelease of InsP$_3$ were compared in the same imaging field at basal $[\text{Ca}^{2+}]_{\text{cyt}}$ and during induction of $\text{Ca}^{2+}$ influx. Figure 5(A) shows a scatter plot of durations of puffs [measured as FDHM (full duration at half-maximal amplitude)] against the latency of the puffs.
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DISCUSSION

The aim of the present study was to investigate how elevated basal cytosolic [Ca\(^{2+}\)] would affect InsP\(_3\)-evoked Ca\(^{2+}\) signals. We utilized the expression of Ca\(^{2+}\)-permeable nicotinic receptor/channels in the plasma membrane as a means to evoke controlled entry of extracellular Ca\(^{2+}\) into the cell during hyperpolarizing voltage-clamped pulses. Consistent with previous observations [4,5], we confirmed that cytosolic [Ca\(^{2+}\)] elevations powerfully facilitated InsP\(_3\)-mediated Ca\(^{2+}\) waves in terms of increased peak amplitude and shortened latency (Figures 1A and 1B). We further investigated the effect of elevated basal [Ca\(^{2+}\)]\(_{c}\) on the local InsP\(_3\)-mediated Ca\(^{2+}\) puffs that are the triggers and fundamental building blocks of Ca\(^{2+}\) waves, as well as serving signalling functions in their own right. Our results show that the numbers of puff sites that respond to a given [InsP\(_3\)] are strongly potentiated in a graded manner with increasing [Ca\(^{2+}\)]\(_{c}\) and that the mean latency of puffs markedly shortens. In contrast, puff amplitudes were little affected except at high [Ca\(^{2+}\)]\(_{c}\), and we observed no significant effects of [Ca\(^{2+}\)]\(_{c}\) on puff duration.

The effects we describe on InsP\(_3\)-evoked Ca\(^{2+}\) liberation from the ER can be directly attributed to changes in basal [Ca\(^{2+}\)]\(_{c}\), and not to any increase in Ca\(^{2+}\) store filling within the ER. We had previously utilized Ca\(^{2+}\) influx through nicotinic receptors as a means to increase ER Ca\(^{2+}\) store by applying a transient hyperpolarizing pulse and then allowing Ca\(^{2+}\) influx to subside to the resting level before examining responses to photorelease InsP\(_3\). However, changes in puff properties were observed only when SERCA activity was accelerated by cADP ribose [20,24] or when SERCA 2b was overexpressed [18]. With basal SERCA activity, no significant changes in puff triggering, kinetics or amplitude were apparent following even strong Ca\(^{2+}\) influx.

We have proposed that the puff is itself triggered by the stochastic opening of a single Ins\(_{3,1}\)R channel within the cluster [25,26]. Factors that determine the occurrence of puffs thus include the number of channels present in the cluster and the open probability of each channel. The latter, in turn, is a function of the concentrations of InsP\(_3\) and Ca\(^{2+}\), acting as co-agonists to open the channel [3,7,27]. Concordant with this mechanism, increasing [InsP\(_3\)] results in an increased frequency of puffs and a shortening of the latency to the first puff evoked at a site following photorelease of InsP\(_3\) [26,28]. Similarly, modest
elevations of [Ca\textsuperscript{2+}]\textsubscript{cyt} will increase the open channel probability at a given [InsP\textsubscript{3}], and hence increase the probability of puff triggering leading to a greater number of sites that generate puffs following photorelease of InsP\textsubscript{3}, and a shortening in mean latency of these puffs. Although gating of the InsP\textsubscript{3}\textsubscript{-}R channel is biphasically regulated by [Ca\textsuperscript{2+}][29], inhibition of the native Xenopus InsP\textsubscript{3}\textsubscript{-}R arises only when [Ca\textsuperscript{2+}] exceeds a concentration of several hundred micromolar [27] and thus would not be expected to be apparent in our experiments, where we estimate that the maximal Ca\textsuperscript{2+} influx (\Delta F/\Delta t \sim 0.3) corresponded to an increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} of <100 nM.

Because the resting [Ca\textsuperscript{2+}]\textsubscript{cyt} is very low, small elevations above this level will strongly potentiate puff triggering. On the other hand, once an initial ‘trigger’ channel opens, the Ca\textsuperscript{2+} flux passing through it will elevate the local free [Ca\textsuperscript{2+}] at the puff site to much higher levels, predicted to reach a concentration of a few hundred micromolar at the mouth of the open channel and at least several micromolar at the neighbouring InsP\textsubscript{3}\textsubscript{-}R within the cluster [29]. This will effectively ‘swamp’ the effect of any smaller elevation of basal [Ca\textsuperscript{2+}]. Once triggered, the puff thus becomes a self-regenerative process and its subsequent evolution is expected to be substantially independent of the preceding conditions; this is the probable explanation as to why we found little dependence of puff amplitudes and kinetics on basal [Ca\textsuperscript{2+}]\textsubscript{cyt}.

The sensitization of global Ca\textsuperscript{2+} waves by elevated basal [Ca\textsuperscript{2+}]\textsubscript{cyt} may similarly be explained by enhanced coupling between neighbouring release sites. Ca\textsuperscript{2+} waves propagate because Ca\textsuperscript{2+} released from one site diffuses to evoke CICR from adjacent sites [11,12], and this triggering will be facilitated if [Ca\textsuperscript{2+}]\textsubscript{cyt} is already elevated. The results in Figure 1 were obtained using relatively weak photorelease of InsP\textsubscript{3}, that evoked only abortive Ca\textsuperscript{2+} waves, and basal [Ca\textsuperscript{2+}] elevation promoted a more robust propagation by CICR resulting in strong potentiation of the spatially averaged Ca\textsuperscript{2+} signal. With stronger stimulation by InsP\textsubscript{3}, the amplitude of repetitive Ca\textsuperscript{2+} waves is not potentiated by Ca\textsuperscript{2+} influx [17], presumably because the more substantial Ca\textsuperscript{2+} release through InsP\textsubscript{3}\textsubscript{-}R swamps any effect of the elevated basal [Ca\textsuperscript{2+}][30], but wave velocities and frequency of repetitive spikes are increased [17].

InsP\textsubscript{3}-mediated Ca\textsuperscript{2+} signalling can function as a coincidence detector, whereby release of Ca\textsuperscript{2+} from intracellular stores is potentiated by extracellular Ca\textsuperscript{2+} entering through plasmalemmal ligand- or voltage-operated channels. This interaction may arise through two different mechanisms, operating on different timescales. Most directly, as we describe in the present paper, elevation of basal [Ca\textsuperscript{2+}]\textsubscript{cyt} enhances the probability of triggering local and global [Ca\textsuperscript{2+}] signals by binding to activating sites on the cytosolic face of the InsP\textsubscript{3}\textsubscript{-}R. In addition, we have described a more circuitous mechanism, whereby extracellular [Ca\textsuperscript{2+}] entering the cytosol is taken up by the action of SERCA pumps, leading to enhanced filling of ER Ca\textsuperscript{2+} stores [18]. That, in turn, promotes Ca\textsuperscript{2+} puffs and waves, probably because increased Ca\textsuperscript{2+} flux through the InsP\textsubscript{3}\textsubscript{-}R channel enhances CICR via the cytosolic activating sites on the InsP\textsubscript{3}\textsubscript{-}R, and possibly also through luminal regulation of InsP\textsubscript{3}\textsubscript{-}R function [30–32]. The direct action of Ca\textsuperscript{2+} influx on InsP\textsubscript{3}\textsubscript{-}R is immediate and short lasting, depending on clearance rate from the cytosol. In contrast, potentiation via ER store filling is slower to develop, more persistent and subject to potential modulation by other messenger pathways, such as CADPR, that affect SERCA activity either directly or indirectly [20,33,34]. Interactions between these different modulatory mechanisms are likely to be of particular importance for Ca\textsuperscript{2+} signalling in neurons with regard to activity-dependent synaptic plasticity as well as gene expression and protein synthesis [35–37].
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