Using two dyes to observe the competition of Ca\(^{2+}\) trapping mechanisms and their effect on intracellular Ca\(^{2+}\) signals

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Abstract
The specificity and universality of intracellular Ca\(^{2+}\) signals rely on the variety of spatio-temporal patterns that the Ca\(^{2+}\) concentration can display. Ca\(^{2+}\) liberation through inositol 1,4,5-trisphosphate receptors (IP\(_3\)Rs) is key for this variety. In this paper, we study how the competition between buffers of different kinetics affects Ca\(^{2+}\) signals that involve Ca\(^{2+}\) release through IP\(_3\)Rs. The study also provides insight into the underlying spatial distribution of the channels that participate in the signals. Previous works on the effects of Ca\(^{2+}\) buffers have drawn conclusions ‘indirectly’ by observing the Ca\(^{2+}\)-bound dye distributions in the presence of varying concentrations of exogenous buffers and using simulations to interpret the results. In this paper, we make visible the invisible by observing the signals simultaneously with two dyes, Rhod-2 and Fluo-4, each of which plays the role of a slow or fast Ca\(^{2+}\) buffer, respectively. Our observations obtained for different concentrations of Fluo-4 highlight the dual role that fast buffers exert on the dynamics, either reducing the intracell cluster channel coupling or preventing channel inhibition and allowing the occurrence of relatively long cycles of Ca\(^{2+}\) release. Our experiments also show that signals with relatively high Ca\(^{2+}\) release rates remain localized in the presence of large Rhod-2 concentrations, while the mean speed of the elicited waves increases. We interpret this as a consequence of the more effective uncoupling between IP\(_3\)R clusters as the slow dye concentration increases. Combining the analysis of the experiments with numerical simulations, we also conclude that Ca\(^{2+}\) release not only occurs within the close vicinity of the centers of the clearly identifiable release sites (IP\(_3\)R clusters) but there are also functional IP\(_3\)Rs in between them.

1. Introduction
Calcium (Ca\(^{2+}\)) signaling is involved in many physiological processes [1, 2]. Ca\(^{2+}\) release from the endoplasmic reticulum (ER) through inositol 1,4,5-trisphosphate receptors (IP\(_3\)Rs) is key: IP\(_3\)Rs are the primary cytosolic target for the initiation of intracellular Ca\(^{2+}\) signals [3]. IP\(_3\)Rs need to bind IP\(_3\) and Ca\(^{2+}\) to become open [4]. Thus, they are affected by the so called calcium-induced calcium release (CICR) [5]. IP\(_3\)-mediated Ca\(^{2+}\) signals start with the release of Ca\(^{2+}\) through a single open IP\(_3\)R and eventually propagate by means of CICR. In most cells, IP\(_3\)Rs are organized in clusters. The signals can then remain localized (i.e. involving the release through one or more IP\(_3\)Rs in a cluster), or become global depending on the efficiency of the released Ca\(^{2+}\) to elicit the opening of IP\(_3\)Rs from many clusters [6–8]. Localized signals in which Ca\(^{2+}\) is released from the ER through IP\(_3\)Rs that belong to a single cluster are known as puffs [9]. Ca\(^{2+}\) puffs are the building blocks of more global signals such as waves. The end responses that are eventually elicited can be different depending on the amplitude and dynamics of intracellular Ca\(^{2+}\) [10–12] and on whether the Ca\(^{2+}\) signal remains localized (puffs) or propagates (waves) throughout the cell [13, 14]. Thus, information is encoded in the spatio-temporal distribution of the intracellular Ca\(^{2+}\) concentration [15, 16]. Understanding the processes that modulate this distribution is key for the comprehension of the Ca\(^{2+}\) signaling toolkit.

Ca\(^{2+}\) signals can be reshaped through various mechanisms, among them, Ca\(^{2+}\) buffering [17, 18]. Cells contain different types of intracellular Ca\(^{2+}\) buff-
ers to keep the free cytosolic Ca\(^{2+}\) concentration low since prolonged high elevations lead to cell death [1]. Buffers not only decrease the Ca\(^{2+}\) concentration but also change its spatio-temporal distribution [17, 18]. The resulting reshaping depends on buffer kinetics. Thus, cells could modify buffer concentrations to reshape Ca\(^{2+}\) signals and, in this way, change the responses that arise upon a given stimulus. The modification of IP\(_3\)-R-mediated intracellular Ca\(^{2+}\) signals by buffers has been studied experimentally by observing the Ca\(^{2+}\) distribution that arises upon a controlled stimulus in the presence of various amounts of a slow (e.g. EGTA) or fast (e.g. BAPTA) buffer [17,18]. In this way, it was observed that EGTA speeds up Ca\(^{2+}\) signals, induces their spatial localization and can lead to signal amplitude potentiation. The addition of BAPTA, on the other hand, slows down the Ca\(^{2+}\) response and promotes signals that spread all over the cell [17]. In these experiments, the interaction of the added buffers with Ca\(^{2+}\) was observed indirectly using a single-wavelength Ca\(^{2+}\) dye that increases its fluorescence intensity upon Ca\(^{2+}\)-binding [19]. The fluorescent images of [17] then reflected the Ca\(^{2+}\)-bound dye distribution which was the result of the combined effect of various competing processes, one of which was the binding of Ca\(^{2+}\) to the added exogenous buffer. Interpreting the observations was difficult, not just because they depended on the competition between the various unobservable buffers and the dye for Ca\(^{2+}\), but also because different patterns of Ca\(^{2+}\) release could occur due to the effect of the exogenous buffer [20] or of the dye itself [21] on CICR. Having a good mathematical model of the Ca\(^{2+}\) dynamics with realistic biophysical parameters was then unavoidable, not only to quantify the properties of the Ca\(^{2+}\) release underlying the observations [22, 23], but also to interpret the effect that the added buffers had on the signals [24,25].

In the present paper, we take a somewhat different approach to study the role of buffers on IP\(_3\)-R-mediated Ca\(^{2+}\) signals, which provides more direct information. Namely, we observe the signals simultaneously with two Ca\(^{2+}\) dyes of different kinetics, Rhod-2 and Fluo-4, each of which plays the role of a slow or a fast Ca\(^{2+}\) buffer whose distribution is observed directly when bound to Ca\(^{2+}\). Even if Fluo-4 is only slightly faster than Rhod-2, the analysis of the changes that are induced when the concentration of one of the two dyes is varied allows us to infer trends. Comparing the images obtained with different dye concentrations is not completely straightforward. In particular, it is difficult to infer whether the changes observed are due to differences in the Ca\(^{2+}\) release rate or only in the amount of Ca\(^{2+}\) that is bound to the observed dye. In order to overcome this problem, we use numerical simulations to identify a quantity that increases with the underlying Ca\(^{2+}\) current, regardless of the concentrations of dye used. Briefly, given a localized Ca\(^{2+}\) current, this quantity is the relative variation of the concentration of Ca\(^{2+}\) bound to one of the dyes that would have been obtained for that given current in the absence of the other dye. As explained later in the paper and in the supplementary material (available online at (stacks.iop.org/PhysBio/15/066006/mmedia)), this quantity, which is meaningful in the case of localized release, can be estimated for each observed puff. In this way, we can associate with each observed puff a value that is an increasing function of the underlying Ca\(^{2+}\) current. A comparison of the values we obtain for the various puffs observed in our experiments leads to the conclusion that the largest (peak) Ca\(^{2+}\) current that underlies the observed puffs increases when the slow dye concentration increases and that the mean Ca\(^{2+}\) current decreases, while the release duration increases when the concentration of the fast dye is increased. Applying the approach we introduced in [26], in this paper, we derive from the observed fluorescence the distribution of Ca\(^{2+}\) bound to each of the dyes used. We then compare how the spatio-temporal dynamics of these distributions vary with the dye concentrations. In this way, we are able to observe directly that slow buffers cannot compete with fast ones in the regions of fastest Ca\(^{2+}\) release and bind Ca\(^{2+}\) around those regions.

Given that Ca\(^{2+}\) signals are the result of various processes, varying Ca\(^{2+}\) buffering also allows us to study some of the other factors that shape the signals. In particular, the time and spatial range of IP\(_3\)-R-mediated signals depends strongly on the ability of the released Ca\(^{2+}\) to elicit further Ca\(^{2+}\) release. The results we obtain for the increasing amounts of the slow dye support the interpretation that the presence of slow buffers disrupts the Ca\(^{2+}\)-mediated coupling between IP\(_3\)-R-clusters [21,27]. Our experiments show, on the other hand, that the addition of fast dye can decrease the localized Ca\(^{2+}\) current and allow the occurrence of long cycles of Ca\(^{2+}\) release. This result illustrates the dual role of fast buffers as moderators of the Ca\(^{2+}\)-mediated coupling between IP\(_3\)Rs in a cluster and as moderators of channel inhibition. The last observation agrees with the results of the analytic study of [28], which showed that the addition of a fast Ca\(^{2+}\) buffer could decrease the mean interpuff time interval. The dual role that fast buffers can play has also been observed in numerical and modeling studies of the dynamics of isolated and clustered IP\(_3\)Rs [24, 25, 29]. Namely, these studies showed that the mean Ca\(^{2+}\) release duration increased in the case of clusters with closely packed IP\(_3\)Rs where the fast buffer was able to moderate the inhibiting effect of high Ca\(^{2+}\) concentrations on the channels. They showed, on the other hand, that the release duration decreased when the net effect of the buffer was to moderate Ca\(^{2+}\) coupling via CICR—an effect that took place in clusters where the mean inter-channel separation was large enough. These studies and others [30–32] also illustrate the relevance of the intra-cluster spatial organization on the emergent behavior of the cluster as a
whole. Even if the channels are not directly observed in our experiments, our results provide hints on their distribution. Using numerical simulations to interpret our experimental observations, we conclude that Ca$^{2+}$ is not only released from clusters that are 1.4–2 μm apart from one another, as could be inferred from the observation of puffs. Namely, our studies suggest the need for ‘loose’ functional IP$_3$Rs in between clusters to explain several of our observations; among them, the non-co-localization of the Ca$^{2+}$-bound Fluo-4 and Ca$^{2+}$-bound Rhod-2 maxima for low Rhod-2 concentrations and the disruption of the intercluster coupling by slow buffers.

2. Materials and methods

2.1. Oocyte preparation

Experiments were performed on *Xenopus laevis* immature oocytes previously treated with collagenase. Oocytes were loaded intracellularly with the concentrations of Fluo-4 and Rhod-2 dextran high affinity ($K_d = 772$ nm) and Rhod-2 dextran ($K_d = 2000$ nm), which were used to probe cytosolic Ca$^{2+}$. Caged InsP$_3$ (d-myo-inositol 1,4,5-triphosphate, P4(5)-1-(2-nitrophenyl)ethyl ester) was used to induce IP$_3$R opening. The exogenous Ca$^{2+}$ buffer EGTA (ethylene glycol-bis-(2-aminoethyl)ether)-N,N,N′,N′-tetraacetinic acid) was also used. Final intracellular concentrations of the different compounds were calculated assuming a 1 μl cytosolic volume. The final intracellular concentration of InsP$_3$ was 9 μM in all the experiments. Fluo-4, Rhod-2, and InsP$_3$ were from Molecular Probes Inc.; EGTA was from Sigma Aldrich. Recordings were made at room temperature.

2.2. Confocal microscopy

Confocal imaging was performed using a spectral confocal scanning microscope Olympus FluoView1000 with a spectral scan unit connected to an inverted microscope IX81. The caged compound was photolyzed with the UV part of the spectrum of a mercury lamp that comes with the microscope using the modification introduced in [33]. Fluo-4 was excited with the 488 nm line of a multiline argon laser, and Rhod-2 was excited using the 543 nm line of a He–Ne laser. Both lasers were focused on the oocyte with a 60× oil immersion objective (NA 1.35). The emitted fluorescence was detected in the 500–600 nm and the 600–630 nm ranges, respectively, with PMT detectors. All the experiments were performed in the linescan imaging mode to improve the temporal resolution. Linescan images were obtained by scanning along a fixed line (250 px) within the oocyte. The acquisition rate was fixed at 10 μs per pixel, resulting in a scan rate of 3.26 ms per line. The caged compound was photo-released approximately 3 s after the linescan acquisition started. The UV uncing pulse was ~100–200 ms long.

2.3. Image analysis

All images were analyzed using routines written in MATLAB [34]. In the experiments, where we simultaneously acquired both fluorescence channels (around 510 nm for Fluo-4 and 570 nm for Rhod-2), we used a linear unmixing method with coefficient 0.1626 to minimize the effect of the spectral bleed-through, as in [35]. The images were also smoothed by averaging over the eight nearest pixels. This smoothing procedure gave the fluorescence, $f_D(x_1, t_1)$, at time $t_1$ and point $x_1$ of the linescan for each dye ($D = F$ for the wavelengths of emission of Fluo-4 and $D = R$ for those of Rhod-2).

2.3.1. Puff characterization

In figures 1(a) and (b), we show a typical linescan image with several puffs observed simultaneously with Fluo-4 and Rhod-2. In these figures, we plot the fluorescence distribution, $f_D(x_1, t_1)$, as a function of space (vertical) and time (horizontal) for $D = F$ in (a) and $D = R$ in (b) using a color code as indicated in the accompanying bars. In order to identify Ca$^{2+}$ puffs in the images, we first determined, by visual inspection, regions that contained a localized connected set of pixels where the intensity was sufficiently above the basal level in the Fluo-4 and in the Rhod-2 channels (marked with arrows). Rectangular regions enclosing the connected sets were then extracted. An example of a rectangular region enclosing a puff that could be observed simultaneously in the Fluo-4 and Rhod-2 channels is shown in figures 1(c) (for Fluo-4) and (d) (for Rhod-2). For further processing, we identified the position and time, $(x_1, t_1)$, of the rectangular region with the largest fluorescence, $f_D$, as illustrated in figures 1(c) and (d). To compute the different puff parameters, we worked with the ratios $\Delta f_D$:

$$\Delta f_D(x_1, t_1) = \frac{f_D(x_1, t_1) - f_D(x_1)}{f_D(x_1)}$$

where $f_D(x_1)$ is the fluorescence at spatial point $x_1$ averaged over time before the photolysis flash (i.e. for $t \leq t_{UV}$). To determine the amplitude and the temporal parameters of the puff located around the point $(x_1, t_1)$ of the image, we averaged $\Delta f_D$ over 11 horizontal lines (the line that corresponded to $x = x_1$), five below and five above it, as in [21]. In figures 1(c) and (f), we show the time trace of the averages obtained, $\Delta f_D$ for Fluo-4 and Rhod-2, respectively, in the case of the puff depicted in figures 1(c) and (d).

We used these traces to compute the amplitude of the puffs, $A_D$ (i.e. the maximum over time of $\Delta f_D$), and the rise, $t_{1/D}$, and decay, $t_{2/D}$, times for each dye, $D$, as conducted in [21] and illustrated in figures 1(c) and (f). We also computed the amplitude growth rate as $GR_D = 0.9 + \frac{\Delta u}{t_{0.5}}$.

2.3.2. Wave characterization

Waves were identified by visual inspection as events where Ca$^{2+}$ release occurred at neighboring sites
subsequently in time. Wave velocities for Fluo-4 were determined by selecting two points, \((x_i, t_i)\), \(i = 1, 2\), on the corresponding image, as explained in what follows, and then computing \(V_F = |x_1 - x_2| / |t_1 - t_2|\). The points were chosen so that \(x_1\) and \(x_2\) corresponded to the space location of the neighboring sites and \(t_1\) and \(t_2\) were the times at which the fluorescence started to increase at each site (see figure 2(a)).

2.3.3. \(\text{Ca}^{2+}\)-bound dye distributions and mask images

We derived from the fluorescence distributions, \(f_D\) and \(\Delta f_D\), the corresponding \(\text{Ca}^{2+}\)-bound dye and relative \(\text{Ca}^{2+}\)-bound dye concentrations, \([\text{Ca}D]\) and \(\Delta[\text{Ca}D]\), respectively, where \(D\) is the dye \((D = F\) for Fluo-4 and \(D = R\) for Rhod-2) and \([\text{Ca}D]_0\) is the basal \(\text{Ca}^{2+}\)-bound dye concentration. For this derivation, we followed \([26]\), neglecting fluctuations in the number of dye molecules that contribute to the fluorescence at each pixel (we assumed it was the same value, \(\langle N_D \rangle\), for each dye concentration everywhere):

\[
[\text{Ca}D] = \frac{[D]_T - q_{LD}}{q_{LD} - q_{LD}} \left(\frac{f_D}{\gamma_D \langle N_D \rangle} - q_{LD}\right), \tag{2}
\]

from which we obtained (see the supplementary material):

\[
\Delta[\text{Ca}D]_t(x, t) = \Delta f_D(x, t) \times \left(1 + \frac{q_{LD}}{q_{LD} - q_{LD}} \frac{[\text{Ca}D]_0 + K_{d,D}}{[\text{Ca}D]_0}\right). \tag{3}
\]

In equations (2) and (3), \(q_{LD}\) and \(q_{LD}\) represent the (apparent) brightness of the free and the \(\text{Ca}^{2+}\)-bound...
dye molecules, respectively, \( \gamma_D \) is an amplification factor introduced by the detectors and \( K_{4,D} \) is the dissociation constant, \( K_{4,D} = k_{\text{off}-D}/k_{\text{on}-D} \), of the corresponding dye \((D = F, R)\)—Ca\(^{2+}\) reaction. To compute the quantities in these equations, we used numerical values of \( q_{1,D}, q_{2,D} \), \( \gamma_D \), and \( \langle N_D \rangle \) that we derived from the estimates of [26]. \( q_{1,F} = 0.45 \), \( q_{2,F} = 0.01 \), \( \gamma_F = 5 \) and \( \langle N_F \rangle = 32 \) for Fluo-4 and \( q_{1,R} = 0.36 \), \( q_{2,R} = 0.02 \), \( \gamma_R = 6 \) and \( \langle N_R \rangle = 32 \) for \([R]_0 = 36 \) and \( 90 \) \( \mu \)M, respectively) and used \([Ca]_b = 100 \) nM. More details may be found in the supplementary material.

We also generated mask images of the Ca\(^{2+}\)-bound dye distributions using a 0.6 CaD\(_{\text{max}} \) threshold where CaD\(_{\text{max}} \) is the maximum of \([CaD] \) over the whole image. In figure 2, we illustrate the processing performed with an image observed in the Fluo-4 channel. From the fluorescence, \( f_F \) (figure 2(a)), observed as a function of space and time, we derived the corresponding \([CaF] \) (figure 2(b)) using equation (2) for \( D = F \). We then computed the maximum, CaF\(_{\text{max}} \), over the image in figure 2(b) and generated the mask (figure 2(c)) using 0.6 CaF\(_{\text{max}} \) as the threshold.

### 2.4. Numerically simulated reaction–diffusion model

We performed numerical simulations of the cytosolic Ca\(^{2+}\) dynamics solving a set of reaction–diffusion equations in a spherical volume (assuming spherical symmetry with \( r \) the radial coordinate) for: Ca\(^{2+}\), an immobile endogenous buffer (S), two cytosolic indicators (F and R) and an exogenous mobile buffer (EGTA). A point source located at the origin and pumps (P) that removed Ca\(^{2+}\) uniformly in space were also included. The Ca\(^{2+}\) sources that we are dealing with in the experiments are IP\(_3\)R clusters. Our simulations do not resolve the intra-cluster dynamics as conducted in [20, 28, 30, 32]. The aim of our simulations is to describe, as simply as possible, how the Ca\(^{2+}\) bound-dye distribution varies with the dye concentrations around a localized Ca\(^{2+}\) source which is simply represented by a point. For the source, we assumed that it consisted of \( n_c \) channels that opened simultaneously at \( t = 0 \) s, each of which released a constant current, \( I_{Ca} = 0.1 \) pA. We assumed that the channels closed after a random time that was drawn from an exponential distribution with mean \( t_{\text{open}} = 20 \) ms [36]. For the Ca\(^{2+}\)-buffer or dye reactions, we considered that a single Ca\(^{2+}\)-ion was bound to a single buffer or dye molecule (X) according to:

\[
\frac{k_{\text{off}-X}}{k_{\text{on}-X}} \text{Ca}^{2+} + X \xrightarrow{\text{CaX.}}
\]

In equation (4), \( X \) represents F, R, EGTA, or S and \( k_{\text{off}-X} \) and \( k_{\text{on}-X} \) are the forward and backward binding rate constants of the corresponding reaction, respectively. We assumed that each species, \( X \), diffused with the same coefficient, \( D_X = D_X \) in its free and Ca\(^{2+}\)-bound forms and that their total concentration, \([X]_T\), was spatially uniform at \( t = 0 \). Thus, \([F]_T\), \([R]_T\), \([\text{EGTA}]_T\), and \([S]_T\) remained uniform and constant for all times and we calculated the free concentrations, \([F]_T\), \([R]_T\), \([\text{EGTA}]_T\), and \([S]_T\), by subtracting the concentration of their Ca\(^{2+}\) bound forms to their total concentrations. The dynamical equations of the model were then given by:

\[
\frac{\partial [Ca^{2+}]}{\partial t} = D_Ca \nabla^2 [Ca^{2+}] - \sum_{X = F, R, S, \text{EGTA}} R_{\text{CaX}}
+ \sigma \delta (r) - \nu_p \frac{[Ca^{2+}]^2}{[Ca^{2+}]^2 + k_p^2},
\]

(5)

\[
\frac{\partial [X]}{\partial t} = D_X \nabla^2 [X] + R_{\text{CaX}}; \quad X = F, R, S, \text{EGTA}
\]

(6)

with \( \sigma = 20758 \cdot N_0(t) \cdot I_{Ca} \mu \)M \( \mu \)M \(^{-1}\) \( s^{-1}\); \( N_0(t) \), the number of simultaneously open channels at time \( t \); \( D_Ca \), the free diffusion coefficient of Ca\(^{2+}\) and the reaction terms:

\[
R_{\text{CaX}} = k_{\text{on}-X}[Ca^{2+}][X]_T - [CaX] - k_{\text{off}-X}[CaX],
\]

(7)
Table 1. Parameter values used to solve the simulations.

| Parameter                             | Value     | Units  |
|---------------------------------------|-----------|--------|
| Free calcium                          | 220       | µm³ s⁻¹|
| [Ca]₀                                | 0.05–0.1  | µM     |
| Calcium dye Fluo-4-dextran            | 15        | µm³ s⁻¹|
| kₘᵦ₋₉                                | 240       | µM⁻¹ s⁻¹|
| k₉ᵦ₋₉                                | 180       | s⁻¹    |
| [F]₀                                 | 0, 36     | µM     |
| Calcium dye Rhod-2-dextran            | 15        | µm³ s⁻¹|
| kₘᵦ₋₉                                | 70        | µM⁻¹ s⁻¹|
| k₉ᵦ₋₉                                | 130       | s⁻¹    |
| [R]₀                                 | 0, 36, 90 | µM     |
| Exogenous buffer EGTA                 | 0.8       | µM⁻¹ s⁻¹|
| kₘᵦ₋EGTA                            | 0.75      | s⁻¹    |
| k₉ᵦ₋EGTA                            | 45, 90    | µM     |
| Endogenous immobile buffer            | 0         | µm³ s⁻¹|
| kₘᵦ₋₅                                | 400       | µM⁻¹ s⁻¹|
| k₉ᵦ₋₅                                | 800       | s⁻¹    |
| [S]₀                                 | 300       | µM     |
| Source                                |           |        |
| n₀                                    | 3, 6, 10, 15, 30 |        |
| t_open                                | 20 ms     |        |
| I₀                                    | 0.1 pA    |        |

The reaction–diffusion of equations (5) and (6) were solved with no flux boundary conditions at \( r = 20.5 \) µm using a backward Euler method in time and an explicit finite-difference formula in space with a 2nd order expression (first neighbors) for the Laplacian. The spatial grid size was \( dr = 0.041 \) µm and the time step \( dt = 10 \) µs. For the initial condition, we assumed that all concentrations were in equilibrium with \( \text{Ca}^{2+} \) at its (uniform) basal concentration, \([\text{Ca}]₀\).

The values of the parameters used in the simulations (taken from [21]) are listed in table 1.

To compare the results with the experimental confocal images, we calculated a weighted average of \([\text{CaD}]\) for each dye \( (D = F, R) \) along the linescan \( r = (x, 0, 0) \) according to the confocal microscope point spread function:

\[
[\text{CaD}] (x, t) = \frac{1}{V} \int [\text{CaD}] (r', t') \exp \left( -2 \left( \frac{(x-x')^2 + y'^2 + z'^2}{w_x^2 + w_y^2 + w_z^2} \right) \right) dx' dy' dz',
\]

where \( w_x = 0.23 \) µm, \( w_z = 1.15 \) µm and \( V = \frac{w_x^2 w_z^2}{2} \). This is the blurred version of the \( \text{Ca}^{2+} \)-bound dye concentration \(([\text{CaD}]\)). In figure 3, we show an example of a numerical simulation performed using the parameters of table 1 with \([F]₀ = 36 \) µM, \([\text{EGTA}]₀ = 45 \) µM and \([R]₀ = 90 \) µM and with a source of \( nᵢ = 10 \) channels that opened simultaneously at \( t = 0 \), each of which released a constant current, \( I₀ = 0.1 \) pA, and closed after a random time that was drawn from an exponential distribution of mean \( t_{open} = 20 \) ms. We plot in figure 3(a) the \( \text{Ca}^{2+} \) current, \( I(t) \), used in the simulation, and in figures 3(b) and (c), the obtained blurred distributions of \( \text{Ca}^{2+} \)-bound to Fluo-4, \([\text{CaF}]\), and to Rhod-2, \([\text{CaR}]\), respectively.

To compare the results with the experimental images, we calculated a weighted average of \([\text{CaD}]\) at its (uniform) basal concentration, \([\text{Ca}]₀\).

\[
\Delta[\text{CaD}] = \frac{[\text{CaD}] - [\text{CaD}]₀}{[\text{CaD}]₀} \equiv \Delta[\text{CaD}]_{|D = 0},
\]

with \([\text{CaD}]_{|D = 0}\) as in equation (8) for the \( \text{Ca}^{2+} \)-bound dye concentration at basal conditions.

We also relied on the numerical simulations to assess the rate of CICR-mediated coupling between neighboring clusters. To this end, we used the concentration \([\text{Ca}^{2+}]_{|D = 0} (d, \tau) \) obtained numerically at distance \( |r| = d \) from the \( \text{Ca}^{2+} \) source and time \( \tau \) after the initiation of the release to calculate the probability \( P₀(d, \tau) \) that an IP₃R within a cluster with \( nᵢ \) IP₃-bound IP₃Rs located at a distance \( d \) from the source becomes open by time \( \tau \). We computed:

\[
P₀(d, \tau) = 1 - \exp \left( -\int_0^\tau dr \, nᵢ kₖₙ₁[\text{Ca}^{2+}] (d, \tau') \right)
\]

with \( kₙ₁ = 20 \) µM⁻¹ s⁻¹ the rate of \( \text{Ca}^{2+} \) binding to the IP₃R activating site of the DeYoung–Keizer model [36]. We illustrate this computation in figures 3(d) and (e). In figure 3(d), we show three snapshots of the free \( \text{Ca}^{2+} \) distribution as a function of the distance to the source for the same simulation as in figures 3(a)–(c). We indicate in this figure the ‘sensing’ distance \( d \). In figure 3(e), we plot the free \( \text{Ca}^{2+} \) concentration at this distance as a function of time \( \tau \). Integrating this concentration up to a given time, \( \tau \), as indicated in the figure, we compute the open probability, \( P₀(d, \tau) \), given by equation (10).

2.5. Comparison of \( \text{Ca}^{2+} \) release amplitudes between experiments performed under different conditions

The comparison of \( \text{Ca}^{2+} \) images obtained from experiments performed under different conditions is not completely straightforward since the changes observed could be due to differences in the \( \text{Ca}^{2+} \) release rate or in the amount of \( \text{Ca}^{2+} \) that is bound to the dye. Numerical studies can provide a hint as to how images of the same \( \text{Ca}^{2+} \) release event change when observed under varying concentrations of the dyes. This is what we analyzed in the supplementary material. Here we explain how we processed the experimental images to obtain a quantity that,
According to the simulations, increases with the Ca$^{2+}$ release rate regardless of the two dye concentrations used in each experiment. We first computed $\Delta[\text{CaF}]_{r,M}$ and $\Delta[\text{CaR}]_{r,M}$ for each Ca$^{2+}$-release event observed experimentally, as explained in section 2.3.3. We then computed their maximum values over the observed region, $\Delta[\text{CaF}]_{r,M}$ and $\Delta[\text{CaR}]_{r,M}$. We subsequently used the total Rhod-2 concentration, $[R]_T$ of the corresponding experiment to obtain an estimate of the maximum value of $\Delta[\text{CaF}]$, that would have been attained for the same release event if only Fluo-4 had been present. We repeated the procedure using $[F]_T$ to estimate the maximum value of $\Delta[\text{CaR}]$, that would have been attained if only Rhod-2 had been present. As shown in the supplementary material, these estimates that we refer to as $A_{\text{lib}--F}$ and $A_{\text{lib}--R}$, respectively, are increasing functions of the underlying Ca$^{2+}$ current independent of the total dye concentrations that are used if the current arises in a very localized region. The results of the simulations presented in the supplementary material further illustrate that, for a given (localized) Ca$^{2+}$ current, the difference $A_{\text{lib}--F} - \Delta[\text{CaF}]_{r,M}$ is linearly proportional to the concentration, $[R]_T$ used in the simulations and that the constant of proportionality is weakly dependent on the current. Analogously, $A_{\text{lib}--R} - \Delta[\text{CaR}]_{r,M}$ is linearly proportional to $[F]_T$. Based on these results, in the experiments we estimate $A_{\text{lib}--F}$ and $A_{\text{lib}--R}$ from $\Delta[\text{CaF}]_{r,M}$ and $\Delta[\text{CaR}]_{r,M}$, respectively, as:

$$
\begin{align*}
A_{\text{lib}--F} & \approx \Delta[\text{CaF}]_{r,M} + \alpha_{F,R} [R]_T, \\
A_{\text{lib}--R} & \approx \Delta[\text{CaR}]_{r,M} + \alpha_{R,F} [F]_T
\end{align*}
$$

with $\alpha_{F,R} = 4.58 \times 10^{-3}$ μM$^{-1}$ and $\alpha_{R,F} = 1.16 \times 10^{-3}$ μM$^{-1}$.

To obtain quantities that reflect more directly the underlying Ca$^{2+}$ current and the total Ca$^{2+}$ released during the event, we computed, respectively, the growth rate, $GR - A_{\text{lib}--D} = 0.9 \times \frac{A_{\text{lib}--D}}{t_D}$ and $I_{\text{lib}--D} = A_{\text{lib}--D} \cdot t_D$ for each dye ($D = F$ for Fluo-4 and $D = R$ for Rhod-2), $A_{\text{lib}--D}, GR - A_{\text{lib}--D}$ and $I_{\text{lib}--D}$ are reporters of the maximum Ca$^{2+}$ concentration, of the Ca$^{2+}$ current and of the total amount of Ca$^{2+}$ released during the time course of the event, respectively.

### 3. Results

In this section, we show some of the results of the Ca$^{2+}$ imaging experiments performed as described in section 2. The different concentrations of dyes and EGTA used in each experiment are detailed in table 2 where we classify the experiments into six sets. In particular, we focus on how the signals change with the dye concentrations, $[F]_T$ and $[R]_T$, as a way to understand how fast (in this case, Fluo-4) or slow (in this case, Rhod-2) buffers affect the spatiotemporal dynamics of cytosolic Ca$^{2+}$. To this end, we first validate the assumption that Rhod-2 acts as
a slow buffer and Fluo-4 as a fast one by looking at how the distributions of the puff properties observed in the Fluo-4 or the Rhod-2 fluorescence channel change, respectively, with [R] or [F]. This analysis is contained in the supplementary material, where, as shown, the mean rise time of the puffs decreases and their growth rate increases with an increase in [R] which is consistent with the previous results obtained when increasing slow buffer concentration [17, 18]. Although the results obtained with varying [F] are not conclusive, the fraction of small amplitude and large rise time puffs observed with Rhod-2 increased with increasing Fluo-4 while their mean amplitude decreased. This agrees with previous observations [17, 18] that demonstrate a more continuous Ca2+ release, albeit of smaller amplitude, when adding a fast buffer. We then analyze whether the changes correspond solely to variations in the observed fluorescence or if the underlying Ca2+ current is also modified when the dye concentrations are varied. We describe, in what follows, the results obtained when doing this and when analyzing the spatio-temporal distribution of [CaF] and [CaR]. We end the section showing the results of numerical simulations that suggest the need for functional IP3Rs between IP3R-clusters to explain the observations.

3.1. Changes in Ca2+ release with varying buffer concentrations observed in the experiments

In order to determine whether the underlying Ca2+ current changes when the dye concentrations are varied, we compute the quantities, Alib−→Fluo−D with D = F for Fluo-4 or D = R for Rhod-2, as described in section 2.5. We show in figures 4(a) and (b) the CDFs of Alib−→F (i.e. Alib computed from the fluorescence observed in the Fluo-4 channel) and of its growth rate, GR−Alib−→F, for experiments with [F] = 36 μM, [EGTA] = 45 μM and [R] = 36 μM (i.e. Set V of table 2, with a dotted line) or [R] = 90 μM (i.e. set IV, with a solid line). The mean values of Alib−→F and GR−Alib−→F as [R] is increased are [Avb−→F] = 2.6, (GR−Alib−→F) = 38 s−1 for Set V and [Avb−→F] = 3.1, (GR−Alib−→F) = 60 s−1 for Set IV). As discussed later, the differences obtained indicate that the largest Ca2+ current that underlies the observed puffs increases as the concentration of the slow dye, Rhod-2, is increased. The change in the Alib−→F and GR−Alib−→F distributions illustrated in figures 4(a) and (b) is accompanied by a change in the mean velocity of the waves observed in the same two experiment types, as shown in figure 4(c), where we have plotted the CDFs of the velocity of the waves observed in the Fluo-4 channel (see Vp in section 2.3.2) in the experiments with [F] = 36 μM, [EGTA] = 45 μM and [R] = 36 μM (i.e. Set V of table 2, with a dotted line) or [R] = 90 μM (i.e. set IV, with a solid line). The mean velocity increases when [R] is increased (from ⟨Vp⟩ = 22 μm s−1 to ⟨Vp⟩ = 27 μm s−1 for [R] = 36 and 90 μM, respectively). As discussed in more detail later, the changes analyzed so far can be interpreted within a unified framework by assuming that larger values of [R] disrupt the CICR mediated inter-cluster coupling more efficiently.

In figures 4(d) and (e), we present the CDFs of Alib−→R (i.e. Alib computed from the fluorescence observed in the Rhod-2 channel) and the growth rate of GR−Alib−→R, respectively, for experiments with [R] = 36 μM, [EGTA] = 45 μM and [F] = 0 μM (i.e. Set VI of table 2, dashed line) and [F] = 36 μM (i.e. Set IV, solid line). In this case, (Alib−→R) decreases when Fluo-4 is added (from 3.0 for Set VI to 2.8 for Set IV) and events with the largest Alib−→R observed in Set VI are not observed in Set IV. The distribution of GR−Alib−→R, the quantity that is more directly linked to the underlying current, presents a similar behavior. The fraction of puffs with the largest rise times observed with Rhod-2, on the other hand, increases with increasing [F] (see supplementary material). The changes observed in the distributions of Alib−→R, GR−Aib−→R and rise time seem to indicate that there is a more continuous release at a slower rate when the fast dye concentration, [F], is increased. This conclusion is consistent with the fact that the distribution of Ilib−R shown in figure 4(f) and the mean value, ⟨Ilib−R⟩, remain almost unchanged with an increase in [F].

3.2. Differences in the spatial distributions of CaF and CaR in experimentally observed puffs

We now analyze the spatial distributions of CaF and CaR, focusing on the points where their concentrations are maximal. To this end, we first compute the Ca2+-bound dye distributions for each image as explained in section 2. We then compute, for each puff that could be observed simultaneously with both dyes, the distance, δ, between the location of the maximum (in space and time) of [CaF] and the maximum of [CaR]. In figure 5, we show the distributions of the values of δ obtained in the experiments performed with [F] = 36 μM (Set V, Set IV, Set II and Set I of table 2). Figures 5(a) and (b) correspond to [EGTA] = 45 μM and figures 5(c) and (d) to [EGTA] = 90 μM. [R] increases from 36 to 90 μM when going from (a) to (b) or from (c) to (d). We observe that for both [EGTA] = 45 μM and

| Table 2. Combinations of the dyes and EGTA concentrations used in the different experiments. We classify the experiments into six sets. |
|-----------------|--------|--------|
| Experiment      | [F] (μM) | [R] (μM) | [EGTA] (μM) |
| Set I           | 36     | 90     | 90       |
| Set II          | 36     | 36     | 90       |
| Set III         | 36     | 0      | 90       |
| Set IV          | 36     | 90     | 45       |
| Set V           | 36     | 36     | 45       |
| Set VI          | 0      | 90     | 45       |
90 \mu M, the distributions of \(\delta\) are concentrated around smaller values as \([R]_T\) is increased. This means that for \([R]_T=36\ \mu M\), Rhod-2 is not able to follow the dynamics of the signal near the source, while for larger \([R]_T (90\ \mu M)\), Rhod-2 competes more efficiently with Fluo-4 for \(Ca^{2+}\) so that both maxima tend to co-localize. This different spatial distribution of \(Ca^{2+}\)-bound Rhod-2 and Fluo-4 is also reflected in figure 6, where
we show the mask images obtained as described in section 3 for the case of four examples. In the figure, we observe that for \([R]_T = 36 \mu M\), \([\text{CaR}]\) attains its largest values (shown in red) in regions that surround those where \([\text{CaF}]\) is largest (shown in green). For \([R]_T = 90 \mu M\), the region with the largest \([\text{CaR}]\) overlaps with that of \([\text{CaF}]\) (yellow pixels) extending further around it in the case of small \([\text{EGTA}]_T\) (see the red pixels surrounding the green/yellow region in figure 6(b) which are absent in figure 6(d)). This analysis not only has implications for how the two dyes compete for \([\text{Ca}^{2+}]\) (see section 4) but also leads to the conclusion that the correction that is implicit in the computation of \(A_{\text{lib} - F}\) may be unnecessary for the experiments with \([R]_T = 36 \mu M\); namely, the assumption that \(A_{\text{lib} - F}\) or \(A_{\text{lib} - R}\) are increasing functions of the \([\text{Ca}^{2+}]\) release rate derives from simulations with a \([\text{Ca}^{2+}]\) point source. If the maxima of \([\text{CaR}]\) and \([\text{CaF}]\) approximately co-localize, then the competition of Rhod-2 and Fluo-4 for the same \([\text{Ca}^{2+}]\) occurs similarly to the case of a point source. Therefore, we expect the computation of \(A_{\text{lib}}\) to still provide an increasing function of the underlying \([\text{Ca}^{2+}]\) current. If the maxima do not co-localize, however, the correction may not be necessary. Recomputing \(A_{\text{lib} - F}\) with \(\alpha_{F,R} = 0 \mu M^{-1}\) for the experiments with \([R]_T = 36 \mu M\), we obtain CDFs that are slightly shifted to the left with respect to those of figure 4(a) or the equivalent one for \([\text{EGTA}]_T = 90 \mu M\), but the basic trends remain unchanged (data not shown). Based on this, we still conclude that there are (more) events with larger \([\text{Ca}^{2+}]\) currents that remain spatially localized as \([R]_T\) is increased.

3.3. Differences in the spatial distributions of \([\text{CaF}]\) and \([\text{CaR}]\) in experimental events involving multiple release sites

We now analyze the competition between Rhod-2 and Fluo-4 for \([\text{Ca}^{2+}]\), comparing the spatio-temporal distributions of \([\text{CaR}]\) and \([\text{CaF}]\) in experimentally observed events that involve \([\text{Ca}^{2+}]\) release from multiple sites. As in the previous section, \([\text{CaR}]\) and \([\text{CaF}]\) were derived from the observed fluorescence as explained in section 2.3.3. In figure 7, we show two examples observed in the experiments with \([F]_T = 36 \mu M, [R]_T = 90 \mu M\) and \([\text{EGTA}]_T = 45 \mu M\) (Set IV of table 2). The \([\text{Ca}^{2+}]\)-bound Fluo-4 and the \([\text{Ca}^{2+}]\)-bound Rhod-2 distributions are shown, respectively, in figures 7(a) and (b) for the first example and in figures 7(c) and (d) for the second one. The spatial averages of \([\text{CaF}]\) (gray) and \([\text{CaR}]\) (black) over regions 1 and 2 defined in figure 7(a) are shown in figures 7(e) and (f), and the corresponding averages over regions 1 and 4 defined in figure 7(c) are shown in figures 7(g) and (h). The bar to the right of figure 7(d) displays the spatial locations of the pixels where, at some instant during the time course spanned by the images in figures 7(c) and (d), \([\text{CaF}]\) (green) or \([\text{CaR}]\) (red) are higher than 60% of \([\text{CaF}]_{\text{max}}\) and \([\text{CaR}]_{\text{max}}\), respectively, with yellow indicating the pixels that meet both the conditions for \([\text{CaF}]\) and \([\text{CaR}]\). In the first example, \([\text{CaR}]\) is initially larger in region 2 than in region 1. This results in a much slower increase of \([\text{CaF}]\) in region 2 which is also delayed with respect to the increase in \([\text{CaF}]\) in region 3. Rhod-2 captures \([\text{Ca}^{2+}]\) more efficiently in region 2 than in the others,
3.4. Numerical simulations: the need for functional
IP$_3$Rs between clusters to explain the observations

We here use numerical simulations to interpret how the changes in the relative location of the maxima in [CaR] and [CaF] obtained in the experiments and shown in figures 5 and 6 could be produced when varying $[R]_T$. To this end, we simulate the reaction–diffusion model described in section 2.4 using the same (point) Ca$^{2+}$ source (with $n_t = 10$) and basal Ca$^{2+}$ ($[Ca]_b = 0.1 \mu M$). We then generate mask images of the Ca$^{2+}$-bound dye, $D$, distributions ($D = F$ for Fluo-4 and $D = R$ for Rhod-2) obtained in the simulations using the same criterion that we applied to the experimental images. Namely, we used a 0.6 $Ca_{D_{max}}$ threshold with $Ca_{D_{max}}$ the maximum value of [CaD] over the whole numerically simulated image (i.e. maximum value in space and time). We show two examples in figure 8, the one for Set V (sets described in table 2) in figure 8(a) and the one for Set IV in figure 8(b). The only difference between these two simulations is the value, $[R]_T$, which is 36 $\mu M$ in (a) and 90 $\mu M$ in (b). In both figures, the mask derived from [CaF] is shown in green, the one derived from [CaR] in red and the overlapping region in yellow. We observe in the figures that the two masks overlap almost everywhere. In particular, the local (and global) maxima of both [CaF] and [CaR] occur at the location of the Ca$^{2+}$-point source. We also observe that, different from our findings in the experiments, [CaF] is spread over a (slightly) wider region than [CaR]. Both the overlapping and the [CaF] dominated regions seem to be a little wider (both in space and time) in (a) than in (b), but their difference is minuscule. Similar regions were obtained in the simulations for Set II and Set I. If we increase the underlying Ca$^{2+}$ current, we obtain qualitatively similar images as well. We thus conclude that the mask images do not display noticeable differences when the concentrations of dye and EGTA are varied, as in the experiments, if the underlying Ca$^{2+}$ current remains localized.

The comparison of figures 6 and 8 puts into question the fact that functional IP$_3$Rs are limited to clusters that are $\sim 1.4 \mu M$ apart. We now look further into this problem by analyzing the changes in Ca$^{2+}$ release with $[R]_T$ that we inferred from figures 4(a)–(c) and the equivalent ones obtained for the experiments with [EGTA] = 90 $\mu M$. Based on these results, we concluded that the presence of larger amounts of a slow buffer

![Figure 7](image.jpg)

**Figure 7.** Dynamics of Ca$^{2+}$-bound Fluo-4 and Ca$^{2+}$-bound Rhod-2 in events that involve Ca$^{2+}$ release from multiple sites. (a), (b), (c) and (f) correspond to one example, (c), (d), (g) and (h) to the other. They were both observed during Set IV experiments. (a) and (c) [CaF], (b) and (d) [CaR], (e) and (f) Averaged profiles of [CaF] (gray) and [CaR] (black) computed over regions 1 and 4, respectively. As observed in figure 7(c), we show the values of [CaF] (gray) and [CaR] (black) obtained in the experiments and shown in figures 5 and 6 could be produced when varying $[R]_T$. (e) and (g) Averaged profiles of [CaF] (gray) and [CaR] (black) averaged over regions 1 and 4, respectively. The difference is minuscule. Similar regions were obtained for the experiments with $[R]_T$ of [CaF] and [CaR] in $\mu M$. The bar to the right of (d) corresponds to the location of the pixels that, at any given time during the time-course of the images in (c) and (d), [CaF] (green) or [CaR] (red), exceed 60% of their corresponding maxima. (h) shows the color bars representing levels of [CaF] and [CaR] in M. The bar to the right of (d) corresponds to one example, (e) and (f) correspond to another one. They were both observed during Set V in experiments. (a) and (c) [CaF], (b) and (d) [CaR], (e) and (f) Averaged profiles of [CaF] (gray) and [CaR] (black) computed over regions 1 and 4, respectively. The color bars represent levels of [CaF] and [CaR] in M. The bar to the right of (d) corresponds to one example, (e) and (f) correspond to another one.
allowed the occurrence of puffs with larger underlying $Ca^{2+}$ currents. As discussed in [21], this counter-intuitive result can, in principle, be explained in terms of a more efficient uncoupling between IP$_3$R clusters due to the presence of the slow buffer. We here use numerical simulations to determine whether increasing $[R]_T$ as in the experiments in figures 4(a) and (b) can disrupt the CICR mediated coupling between IP$_3$R clusters that are $\sim 1.4 \, \mu m$ apart. To this end, we perform numerical simulations as before with a point $Ca^{2+}$ source with $n_i$ open channels at $t = 0$. We then compute the probability, $P_0(d, t)$, that a channel within an IP$_3$R cluster with $n_i$ IP$_3$-bound IP$_3$Rs located at a distance $d$ from the original source becomes open by time $t$ (see section 2). We show, in figures 9(a)–(c), the open probability, $P_0(d, t)$, for experiments with $[F]_T = 36 \, \mu M$, $[EGTA]_T = 45 \, \mu M$ and $[R]_T = 36 \, \mu M$ (i.e. set V of table 2, with a dotted line) or $[R]_T = 90 \, \mu M$ (i.e. set IV, with a solid line). In figure 9(a), we show the results obtained for a source with $n_i = 10$ open IP$_3$Rs and an isolated ‘sensing’ IP$_3$R ($n_i = 1$) at $d = 0.6 \, \mu m$. In figures 9(b) and (c), we show the results obtained for a sensing cluster with $n_i = 5$ IP$_3$Rs located at a distance $d = 1.4 \, \mu m$ from the source which has $n_i = 10$ simultaneously open IP$_3$Rs in (b) and $n_i = 50$ in (c). The change in $P_0$ with an increase in $[R]_T$ is unobservable at $d = 1.4 \, \mu m$ for both $n_i = 10$ ($\approx 0.004$) and $n_i = 50$ while it can be $\sim -0.045$ at $d = 0.6 \, \mu m$ when $[R]_T$ varies from 36–90 $\mu M$. In particular, the difference in the open probability between these two simulations is $\Delta P_0(d = 0.6 \, \mu m, t) \approx -0.034$ at $t = d/V$ with $V \sim 10 \, \mu m \, s^{-1}$, a typical wave velocity.

4. Discussion and conclusions

We have studied the role of buffers on IP$_3$R-mediated $Ca^{2+}$ signals observing the signals simultaneously with two dyes of different kinetics. Previous works on the effects of $Ca^{2+}$ buffers drew conclusions ‘indirectly’ by observing the $Ca^{2+}$-bound dye distributions in the presence of varying concentrations of exogenous buffers [17, 18, 27]. Different modeling strategies and numerical simulations were then used to interpret the results [24, 25, 28, 29]. In the present paper, we have made visible the invisible by observing the signals simultaneously with two dyes, Rhod-2 and Fluo-4, of different kinetics. In this way, we could study the role of slow and fast buffers on the emerging signal more directly from the observations. Most dyes are designed and/or chosen so that they only minimally perturb the signals and yet can report on fast $Ca^{2+}$ changes. This implies that their kinetics is usually fast. In fact, Fluo-4 is only slightly faster than Rhod-2. In spite of this, the analysis of the changes induced when the
concentration of one of these two dyes was varied allowed us to infer trends.

We first focused on how some properties that characterize the release of Ca\(^{2+}\) during localized signals (puffs) changed when varying the dyes and EGTA concentrations. As a first step, we produced a proof of concept by checking that the observed puff properties varied as expected for increasing concentrations of a slow or fast buffer when increasing \([R]_T\) or \([F]_T\), respectively (see the supplementary material). This allowed us to identify the variations produced by either one of the dyes with those produced by slow or fast buffers. We subsequently investigated whether the observed changes were merely observational or could be attributed to modifications in the underlying Ca\(^{2+}\) release. To this end, we used numerical simulations to identify quantities, \(A_{\text{lib} - f}\) and \(A_{\text{lib} - R}\), that could compensate for the different amounts of Ca\(^{2+}\) that are trapped by one of the dyes when the concentration of the other is varied (see the supplementary material). These quantities require the knowledge of the Ca\(^{2+}\)-bound dye concentration to be computed. Our previous work on the analysis of Ca\(^{2+}\) images obtained with single wavelength dyes [26] prescribes how to estimate these concentrations. Using this tool, we computed, for our experiments, \([\text{CaF}]\) and \([\text{CaR}]\) and then used equation (11) to derive \(A_{\text{lib} - f}\) and \(A_{\text{lib} - R}\). We further calculated the corresponding growth rates, \(GR_{A_{\text{lib} - f}}\) and \(GR_{A_{\text{lib} - R}}\), that reflect more directly the events that turn into waves be, on average, of larger velocity as \([\text{CaF}]\) and \([\text{CaR}]\) increased. The KS test, however, does not reject the hypothesis that the CDFs of \(A_{\text{lib} - f}\) and \(GR_{A_{\text{lib} - f}}\) plotted in figures 4(d) and (e), showed a slight decrease of \(\langle A_{\text{lib} - R}\rangle\) and of \(\langle GR_{A_{\text{lib} - R}}\rangle\) with increasing \([F]_T\). In the experiments performed with no Fluo-4 (Set VI), the lack of events with the largest \(A_{\text{lib} - R}\) observed when Fluo-4 was present (Set V) was also apparent in figure 4(d). The distribution of the \(A_{\text{lib} - R}\) growth rate (figure 4(e)) also shifted towards smaller values as \([F]_T\) increased. The KS test, however, does not reject the hypotheses that the CDFs of \(A_{\text{lib} - R}\) and of \(GR_{A_{\text{lib} - R}}\) compared in these figures come from the same distribution (\(P_{\text{value}} = 0.42\) in (d) and 0.24 in (e)). Although mild, the observed changes are an indication that the Ca\(^{2+}\) current, not just the Ca\(^{2+}\)-bound Rhod-2 concentration, decreased when the concentration of the fast dye, Fluo-4, increased. This seems to contradict the idea that the presence of a fast buffer (Fluo-4) alleviates IP\(_{3}\)-R-inhibition, allowing more Ca\(^{2+}\) to be released. We must remember, however, that \(A_{\text{lib} - D}\) and \(GR_{A_{\text{lib} - D}}\) are reporters of the maximum Ca\(^{2+}\) concentration and of the Ca\(^{2+}\) current, not of the total Ca\(^{2+}\) released during the time course of the event. In order to estimate the latter, we computed \(I_{\text{lib} - R}\) (see section 2). As shown in figure 4(f), the \(I_{\text{lib} - R}\)
distribution remained more or less unchanged with varying Fluo-4. The results on $A_{\text{Rhod}}$, $B_{\text{Rhod}}$, $A_{\text{Fluo}}$, and $B_{\text{Fluo}}$ together with the larger values of the time, $t_{\text{open}}$, during which there is Ca$^{2+}$ release as $|F|_T$ is increased (figure SM4(b)) support the hypothesis that fast buffers may play different roles during IP$_3$-induced Ca$^{2+}$ signals. Depending on their concentration and on the IP$_3$, spatial organization, they can either moderate CICR within the cluster leading to signals with smaller Ca$^{2+}$ currents, or they can buffer enough Ca$^{2+}$ so as to prevent IP$_3$-inhibition allowing the almost immediate re-opening of IP$_3$Rs and a longer cycle of Ca$^{2+}$ release. The combination of a longer release at a slower rate can eventually lead to an almost invariant total released [Ca$^{2+}$] with varying buffer (in this case, Fluo-4) concentration as observed in our experiments.

4.3. Differences in the spatial distribution of Ca$^{2+}$ bound to Rhod-2 and Fluo-4 indicate that Ca$^{2+}$ is released from relatively wide regions

We then compared the spatio-temporal distributions of [CaR] and [CaF] for the various experiments. To this end, we derived estimates of these concentrations from the observed fluorescence distributions as explained in section 2.3.3. The analysis of the distance, $\delta$, between the locations of the maxima of [CaR] and [CaF] for the Ca$^{2+}$ puffs that we could observe simultaneously with both dyes showed distributions that were concentrated around small $\delta$ values for the experiments with $|R|_T = 90$ µM (about 85% of the observations had $\delta \leq 0.2$ µm (see figures 5(b) and (d)) and that embraced a larger range (up to $\sim 1$ µm) for those with $|R|_T = 36$ µM (figures 5(a) and (c)). As illustrated in the simulations of figure 8, if the release came from a single point, the maxima of [CaR] and [CaF] should occur at that point. The large $\delta$ values observed in figures 5(a) and (c) are an indication that Ca$^{2+}$ can be released from a relatively wide spatial region during puffs and reflect the differential ability of both dyes to trap Ca$^{2+}$. Figure 6 further illustrates these aspects. There, we observed that for $|R|_T = 36$ µM (figures 6(a) and (c)), the points where [CaR] was above 60% of its corresponding maximum value over the whole image, CaR$_{\text{max}}$, hardly ever coincided with those where [CaF] > 0.6CaF$_{\text{max}}$. In this case, Rhod-2 was most likely trapping Ca$^{2+}$ in regions where Fluo-4 was locally depleted. For $|R|_T = 90$ µM (figures 6(b) and (d)) there was more co-localization as reflected by the larger overlapping areas. Here, Rhod-2 was almost as efficient as Fluo-4 in trapping Ca$^{2+}$ where the release occurred at the fastest pace. In either case, this competition was different depending on EGTA$|T$. For low EGTA$|T$ (figure 6(b)), [CaR] was relatively large over a wider spatial and temporal region than [CaF]. For EGTA$|T = 90$ µM (figure 6(d)), the space and time extent of CaR and CaF were similar. It seems as if, in figure 6(d), EGTA (as it was Rhod-2 in figures 6(a) and (c)) was the slow buffer that could only trap Ca$^{2+}$ away from the regions of fastest release. This was further supported by the fact that the maximum variation in [CaR] was almost 40% larger in figure 6(d) than in figures 6(b) and (c) and 60% larger than in figure 6(a), while the maximum variation in [CaF] remained almost unchanged in all four subfigures. The results of figures 5 and 6 indicate that the slow buffers that restrict the spatial extent of the distribution of Ca$^{2+}$-bound to faster buffers do it by trapping the Ca$^{2+}$ that is released at different spatial points from those that are clearly identified when looking at the distribution of Ca$^{2+}$-bound to fast dyes. This assumption is consistent with the results of the numerical simulations. As illustrated in figure 8, there is no difference in the numerically simulated versions of figures 6(a)–(d) as the concentrations of Rhod-2 or EGTA are varied if Ca$^{2+}$ is released from a single point and the Ca$^{2+}$ current is not changed. No changes were observed either in simulations with other values of the (localized) Ca$^{2+}$ current (data not shown).

In order to further study the differences in the spatio-temporal distributions of [CaR] and [CaF], we analyzed examples of the experimental observations where, apparently, Ca$^{2+}$ was released from various separate sites (figure 7). The examples showed that where Rhod-2 captured Ca$^{2+}$ more efficiently (site 2 of figure 7(a)) the rise in [CaF] was slowed down. Although Rhod-2 did not prevent the propagation of the signal between sites in this particular case, it illustrated how it could do it in cases with less Ca$^{2+}$ release (at sites 1 or 3). This same behavior was also observed in the wave illustrated in figures 7(c) and (d). In this figure, we also looked at the region over which the Ca$^{2+}$-bound dye distributions were above 60% of their maximum values. We observed that this occurred over spatially contiguous regions that spanned up to 2.5 µm. Similar regions were obtained if the threshold was defined as 75% of the maximum fluorescence in each channel. The results of figures 5–7 seem to indicate that Ca$^{2+}$ release can occur over contiguous regions that can be wider than what is supposed to be the typical inter-cluster separation (~1.4–2 µm). We then hypothesize that even though active IP$_3$Rs are largely clustered in sites 1.5–2 µm apart from one another, there could also be active IP$_3$Rs distributed in between.

4.4. Numerical simulations to interpret the experimental observations suggest the presence of ‘loose’ functional IP$_3$Rs in between clusters

In order to look, in more detail, into the possibility of having active IP$_3$Rs between clusters, we performed numerical simulations of a model of the Ca$^{2+}$ dynamics that included the presence of the two dyes and the exogenous buffer (EGTA) used in the experiments, an immobile endogenous buffer, Ca$^{2+}$ pumps and a point source with $n_s$ open channels at $t = 0$ that closed stochastically with mean time, $t_{\text{open}} = 20$ ms. In particular, we studied the probability, $P_0(d, t)$, that an IP$_3$R within a cluster with $n_s$ ‘sensing'
explain how slow buffers disrupt the analyses suggest that there are at least one functional experiments, they further show that the presence of apart from one another. In combination with our change of $P_0$ with varying $[R]_T$ was unobservable at $d = 1.4 \mu m$ for both $n_t = 10$ and $n_t = 50$ while it could be $\sim -0.045$ at $d = 0.6 \mu m$ when increasing $[R]_T$ from 36 to 90 $\mu m$, even if there was only one sensing IP$_3$R in the latter while there were five in the simulations at $d = 1.4 \mu m$. Furthermore, the variation in $P_0(d,t)$ between the cases with $[R]_T = 36$ $\mu m$ and $[R]_T = 90$ $\mu m$ was $\sim -0.034$ at $d = 0.6 \mu m$ for the simulation with $n_t = 10$ and $n_t = 1$ at time $t$ of the order of the time that it would take for a typical Ca$^{2+}$ wave to travel a distance $d = 0.6 \mu m$. This difference, $\sim -0.034$, implies a reduction of the order of 14% in the open probability with respect to the value obtained for $[R]_T = 36$ $\mu m$. Similar simulations performed for the conditions of the experiments with $[\text{EGTA}]_T = 90$ $\mu m$ gave a 22% change in the open probability between the cases with $[R]_T = 0$ $\mu m$ and $[R]_T = 90$ $\mu m$ at $d = 0.6 \mu m$, $t = 60$ ms with $n_t = 1$ sensing IP$_3$R and of 0.6% at $d = 1.4 \mu m$, $t = 140$ ms with $n_t = 5$ sensing IP$_3$Rs. We also repeated the simulations in the presence of only one dye, Fluo-4, with $[\text{EGTA}]_T = 0$ $\mu m$ and with $[\text{EGTA}]_T = 300$ $\mu m$ to mimic some of the conditions of [17] that showed, respectively, global and localized only Ca$^{2+}$ signals upon the same release of IP$_3$. The relative change of $P_0$ obtained with respect to the situation with no EGTA was less than 1% at $d = 1.4 \mu m$, $t = 140$ ms, with $n_t = 5$ sensing channels, while it was $\sim 14\%$ at $d = 0.6 \mu m$, $t = 60$ ms with $n_t = 1$ IP$_3$R (data not shown). These simulations indicate that the changes observed in the signals with increasing concentrations of slow buffers cannot be understood under the assumption that Ca$^{2+}$ is released solely from IP$_3$R-clusters that are $\sim 1.4 \mu m$ apart from one another. In combination with our experiments, they further show that the presence of at least one functional IP$_3$R in between clusters may explain how slow buffers disrupt the Ca$^{2+}$-mediated coupling between IP$_3$R-clusters. The presence of (diffusing) IP$_3$Rs outside clusters has been observed for quite some time [37]. Single-particle tracking experiments revealed the coexistence of motile and immotile IP$_3$Rs with most of the latter organized in clusters [38]. Given that Ca$^{2+}$ puffs have been observed to arise from fixed locations [39], the results of [38] suggested the identification between functional and immobile IP$_3$Rs. Our observations and analyses suggest that there are ‘loose’ IP$_3$Rs between clusters that are functional (and probably immobile) and that they are key for the CICR coupling between IP$_3$R clusters.

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Author contributions

EP and LFL performed the experiments. EP performed the simulations. EP, LFL and SPD analyzed the experiments and simulations and wrote the paper. SPD conceived the work.

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