Sarcoplasmic reticulum vesicles embedded in agarose gel exhibit propagating calcium waves

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Abstract In different cell types, activation of signal transduction pathways leads to the generation of calcium oscillations and/or waves. Due to this important impact for cellular function, calcium waves are the subject of intensive investigations. To study interactions of cell organelles with no influence of the cell membrane, sarcoplasmic reticulum (SR) vesicles and well-coupled mitochondria were reconstituted. For the first time, we demonstrate the generation and propagation of calcium waves in a suspension of sarcoplasmic reticulum vesicles, embedded in an agarose gel. The propagation dynamics resemble those of calcium waves in living cells. Moreover, the addition of well-coupled mitochondria leads to more pronounced and significantly faster propagating waves, demonstrating the importance of the mitochondrial Ca^{2+} transport. The experimental and simulation results indicate the resemblance of the in vitro system to an excitable medium.

Key words: In vitro system; SR vesicle; Mitochondrion; Calcium wave pattern; Excitable medium

1. Introduction

Biological self-organization is a widespread phenomenon that has been investigated in biochemical systems [1], multicellular tissue [2], non-excitable, and excitable isolated single cells [3]. In several types of living cells, mechanisms of the intracellular calcium signaling have been issues of central interest, not only under physiological conditions, but also at elevated intracellular calcium, where spontaneous calcium oscillations may easily occur (e.g. in cardiac myocytes [4,5]). Spatiotemporal fluctuations of the cytosolic calcium concentration (calcium waves) involve (i) Ca^{2+} release from intracellular stores (through IP_3-receptors [6,7] or ryanodine receptors [8]), (ii) the coupling of Ca^{2+} release sites by diffusing Ca^{2+} [9], and (iii) Ca^{2+} reuptake into the endoplasmic reticulum by SERCA pumps [8]. Not dependent on how propagating calcium waves become initiated, their patterns can reveal features of non-linear diffusion-reaction system (such as excitability and refractoriness).

To study interactions of cell organelles with no influence of the outer cell membrane, we reconstituted sarcoplasmic reticulum (SR) vesicles (isolated from the *Mm. longissimi dorsi* of German landrace pigs [10]) in an agarose gel where disturbing thermal or mechanical influences may be excluded. An advantage of the novel in vitro system compared to an intact cell is that it can be easily extended by the addition of other cell constituents and drugs. This study demonstrates the occurrence of calcium oscillations and waves in such a simple system, and provides proof that the observed phenomena can be influenced by several interventions. Mitochondria are presumed to be one of the most interesting candidates modulating calcium wave patterns. Therefore, features of the simple system are compared with those of the extended one (addition of well-coupled mitochondria). Agarose gel preparations with the Ca^{2+}-sensitive dye Fluo-3 were investigated by confocal fluorescence microscopy.

2. Materials and methods

2.1. Agarose gel system

SR vesicles were isolated from the *Mm. longissimi dorsi* of German landrace pigs [10]. A microsomal SR fraction stored at −80°C in a suspension with 300 mM saccharose and 10 mM PIPES (30–40 mg protein/ml) was calcium-loaded at room temperature for 10 min in a solution with the following composition (mM): KCl 100, $MgCl_2$ 5, Na_2-ATP 4, creatinphosphate 10, $CaCl_2$ 1, PIPES 20; pH 7.2. After centrifugation (20 min with $700\times g$), a suspension of SR vesicles was mixed at 37°C with an 0.66% agarose gel (type VII: low gelling temperature, Sigma) of the composition (mM): KCl 100, $MgCl_2$ 5, Na_2-ATP 4, creatinphosphate 10, EGTA 0.04, PIPES 20; Fluo-3 0.01; pH 7.2. An aliquot of 8 μl of the gel was transferred to a cover glass. To obtain a relatively thin and stable layer, a second cover glass of 10 mm in diameter was pressed on the gel (room temperature) so that an area of 70–80 mm^2 was covered by the gel. When mitochondria were reconstituted additionally, a second cover glass was not used to avoid hypoxia. The preparation of mitochondria is described previously [11]. For calcium stimulation we either used a glass tip or a small stripe of paper soaked with calcium solution (200 μM). Drugs were added by pipettes.

2.2. SR vesicle size

An environmental scanning electron microscope (Philips XL 30 ESEM-FEG) was used to estimate the size of ultra-centrifuged SR vesicles (30 min with $120\,000\times g$) which was found to be Gaussian distributed with 1.63 ± 0.42 μm in diameter (mean ± S.D., $n=250$).

2.3. Assessment of calcium wave patterns

A confocal laser scanning fluorescence microscope was used (INSIGHT-PLUS, Meridian Instruments). Its scan system consists of a galvanometer-driven bidirectional mirror (system Brakenhoff) which

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allows a scanning rate of 100 scans/s (512×480 pixel). Images were displayed with 25/s (CCD camera). The light source is an argon ion laser with emission wave lengths of 488 and 514 nm. The Z-drive accessory provides computerized control of optical sectioning with a vertical step size of 0.6 μm at minimum. Image series showing calcium waves were saved on videotape. For the digitization of video frames we used a frame-grabber board with software package QuickCapture (Data Translation, Marlboro, MA, USA) and a Macintosh IIci computer. The software used for both the calculation of wavespeed and the presentation of images in this paper was IPLab-Spectrum QC (Signal Analytics, Vienna, VA, USA) and NIH Image 1.43 (Microsoft).

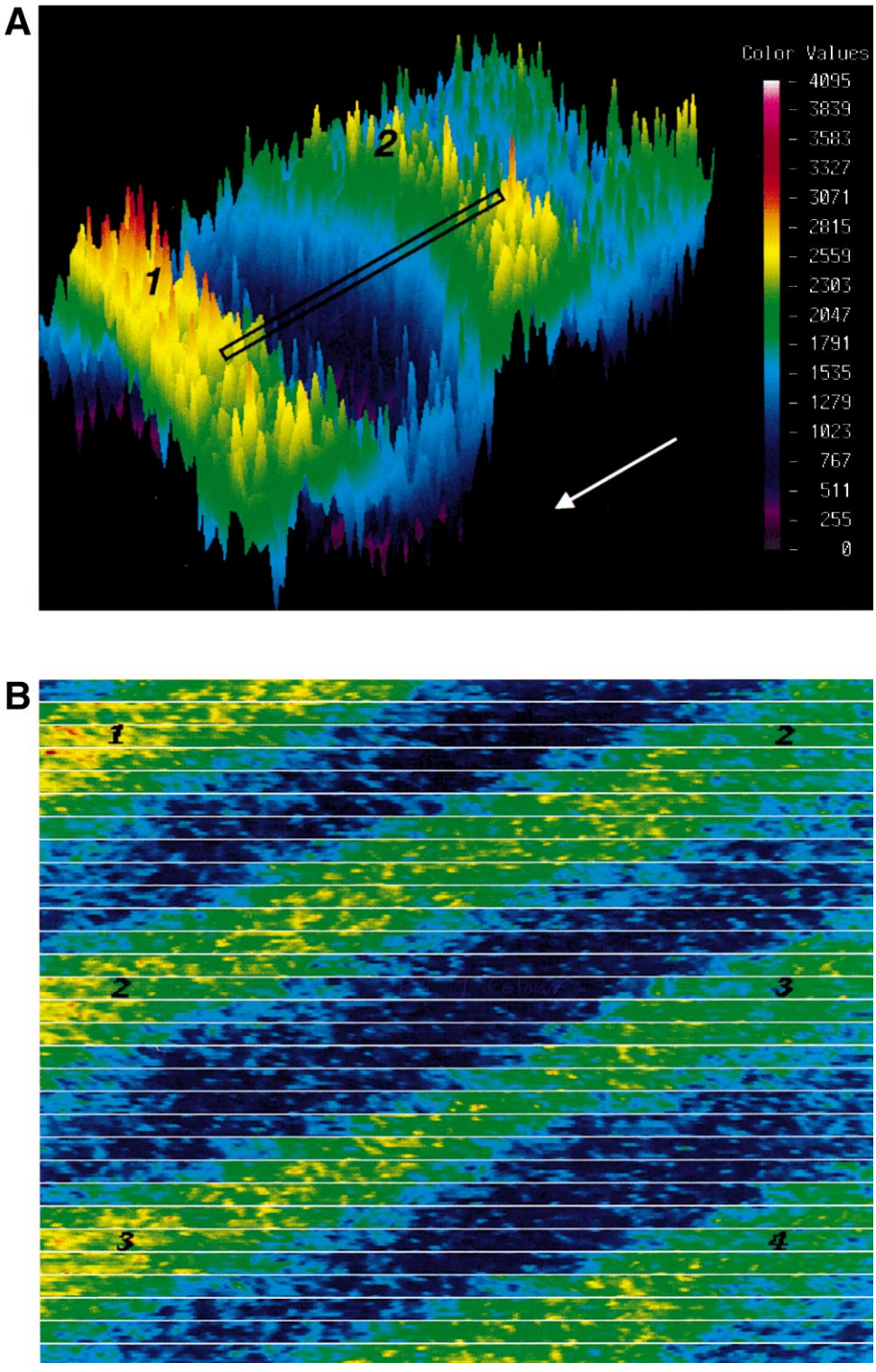

Fig. 1. Spontaneous calcium waves in a SR vesicle agarose gel assessed by confocal scanning fluorescence microscopy. Ca^{2+} indicator Fluo-3 (10 μM). A: Three-dimensional view of the fluorescence intensity within a cluster of SR vesicles (edge lengths 242 μm×413 μm). Region of interest (ROI, 7.4 μm×250 μm) is marked. Arrow indicates direction of wave propagation. B: Time-space plot of propagating wavefronts. ROIs (1/s) showing four waves during 30 s: wavespeed ~17 μm/s, wave length ~200 μm, interwave period ~11 s.

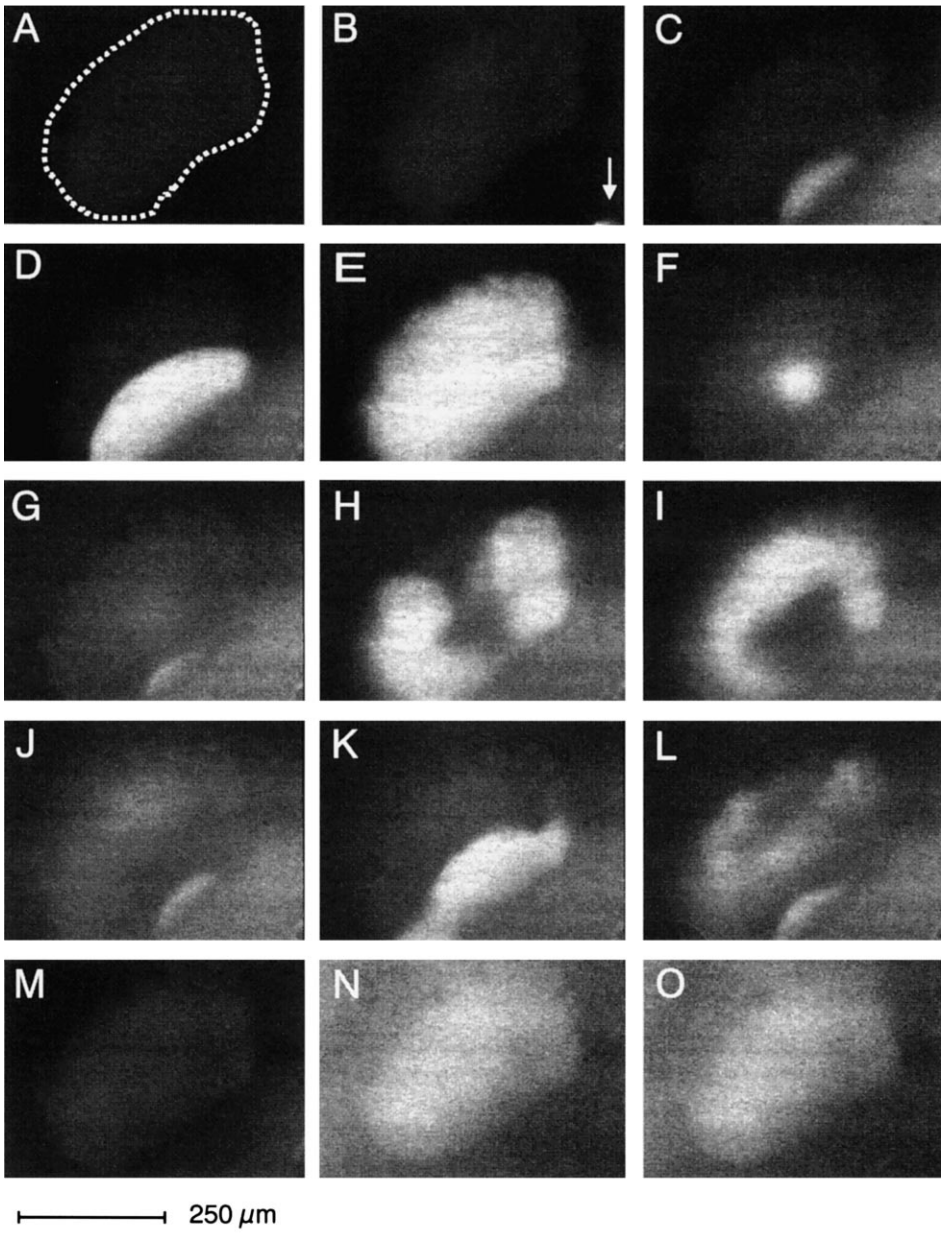

Fig. 2. Calcium wave patterns in the SR vesicle agarose gel with added mitochondria. SR vesicles, isolated mitochondria (ratio of protein concentrations 5:1), substrate (10 mM pyruvate/2 mM malate), and agarose gel with 10 μM Fluo-3 were mixed. A (prior to stimulation): cluster of SR vesicles (dotted outline) with low, non-fluctuating fluorescence; B (0 s): stimulation by Ca^{2+} near the cluster (arrow); C (13 s): initiation of a wave after diffusion of Ca^{2+} to the cluster; D (14 s): spreading of a positively curved wavefront; E (15 s): excitation of the whole cluster; F (29 s): phase of decreasing fluorescence; G (31 s): development of a second wave; H (34 s) and I (35 s): horseshoe-shaped wavefronts due to refractoriness (compare with position of bright spot in F); J (38 s): development of a third wave; K (40 s): wavefront positively curved in the center and negatively in the periphery of the cluster (compare with fluorescence in I); L (43 s): disappearance of the third and development of a fourth wave; M (5 min): low, non-fluctuating fluorescence after decrease of calcium concentration by diffusion; N (5.5 min) and O (8 min): increase of fluorescence after application of 1 mM ionomycin; lack of calcium waves.

Table 1
Characteristic parameters of calcium waves in the SR vesicle agarose gel (controls) and after various interventions

Excitable medium	Velocity (μm/s) (mean ± S.D.)	$\Delta F/F$ (mean ± S.D.)
SR vesicles in agarose gel (controls)	$39.2 \pm 16.2^{\#}$ ($n = 22$)	1.0 ± 0.2 ($n = 8$)
SR vesicles in agarose gel+10 nM thapsigargin	$19.6 \pm 4.4^{\#}$ ($n = 8$)	1.0 ± 0.2 ($n = 8$)
SR vesicles in agarose gel+mitochondria	57.9 ± 12.9* ($n = 20$)	2.8 ± 1.7** ($n = 8$)
SR vesicles in agarose gel+mitochondria+60 μM antimycin A	40.9 ± 10.1* ($n = 20$)	0.9 ± 0.3** ($n = 8$)

$\Delta F/F$ = relative fluorescence intensity = $(F_{peak} - F_{rest})/F_{rest}$. Fluorescence intensities measured in ROIs of 10×10 pixels.

3. Results and discussion

3.1. Calcium waves in the SR vesicle agarose gel system

In clusters of SR vesicles (vesicle size between 1 and 4 μm), which emerged from the mixing of the liquid agarose gel with the vesicle suspension, local Ca^{2+} oscillations and waves occurred either spontaneously or after stimulation with calcium ions. Spontaneous Ca^{2+} waves were propagated with velocities varying between 10 and 60 μm/s (Table 1, controls). Fig. 1A shows a typical three-dimensional view of the fluorescence intensity of two simultaneously propagating Ca^{2+} waves. Once stimulated, waves propagated repetitively during some minutes. Fig. 1B shows 30 consecutive frames obtained from a selected region of Fig. 1A (7.4 μm × 250 μm), where four calcium waves became visible during 30 s (wavespeed ∼ 17 μm/s, interwave period ∼ 11 s, and interwave length ∼ 200 μm).

To interfere with the function of the SR vesicles as calcium stores and to demonstrate that the system resembles features of living cells, several drugs were applied to the SR vesicle agarose gel. Under the influence of 10 nM thapsigargin, an inhibitor of the SR Ca-ATPase (SERCA), the velocity of calcium waves decreased significantly compared to the controls (Table 1, means ± S.D.[#], $P < 0.01$). Even if no drug was applied, the propagation velocity of repetitive waves was not exactly constant. This was probably due to the dispersion relation which predicts that the wavespeed is a function of the interwave period [12–14]. Therefore, the effect of thapsigargin was determined at roughly constant interwave periods (∼27 s), both in controls and after drug intervention. In the presence of thapsigargin (0.1 to 20 nM), a decrease of the calcium wavespeed was also observed in rat cardiac myocytes (unpublished results of the authors). In the SR vesicle agarose gel, calcium waves were abolished both by applying caffeine (which opens the Ca^{2+} release channel/ryanodine receptor and releases sarcoreticular calcium) and ryanodine (which blocks the Ca^{2+} release channel/ryanodine receptor), each in the millimolar range (not shown).

3.2. Effects of mitochondria on spatiotemporal calcium patterns including wavefronts

In oocytes of *Xenopus laevis*, the energization of mitochondria (enhancement of the electrochemical proton gradient) by injection of oxidizable substrates was reported to strengthen the Ca^{2+} wave activity [15]. To prove whether Ca^{2+} signaling in clusters of SR vesicles is modulated by energized mitochondria, freshly prepared and well-coupled mitochondria (respiratory control index > 8) were added to SR vesicles (ratio with respect to the protein concentration was 1:5) and mixed with agarose gel. The composition of the agarose gel was completed by substrate (pyruvate/malate) with a final concentration of ∼10 mM/∼2 mM. In such a system, bright Ca^{2+} waves with smooth wavefront edges and long decay of the fluorescence intensity were observed (Fig. 2C–L). The spreading velocity of the wavefronts was higher than in SR vesicle agarose gels with no mitochondria (Fig. 2D: ∼100 μm/s; Table 1: compare effect of mitochondria with controls). Fig. 2H and I show horseshoe-shaped wave patterns which are likely due to refractory zones in the excitable medium. Calcium waves completely disappeared after a few minutes (Fig. 2M). This might be caused by a diffusion-induced decrease of the calcium concentration within the cluster. Fig. 2N and O finally show an increase of the fluorescence intensity after the application of the non-fluorescent Ca^{2+}-ionophore ionomycin (∼1 mM) but no more calcium wave. To suppress the influence of mitochondria on calcium wave patterns, antimycin A (60 μM), an inhibitor of the electron transport system, was applied. This resulted in a significant retardation of the wave spreading and a significant decrease of the relative fluorescence intensity $\Delta F/F$ (see Table 1*, $P < 0.01$, and **, $P < 0.01$). Additionally, numerous local oscillations of the fluorescence intensity became visible (not shown). Antimycin A seems to transform the extended agarose gel system to one which lacks mitochondria (compare data in presence of antimycin A with controls).

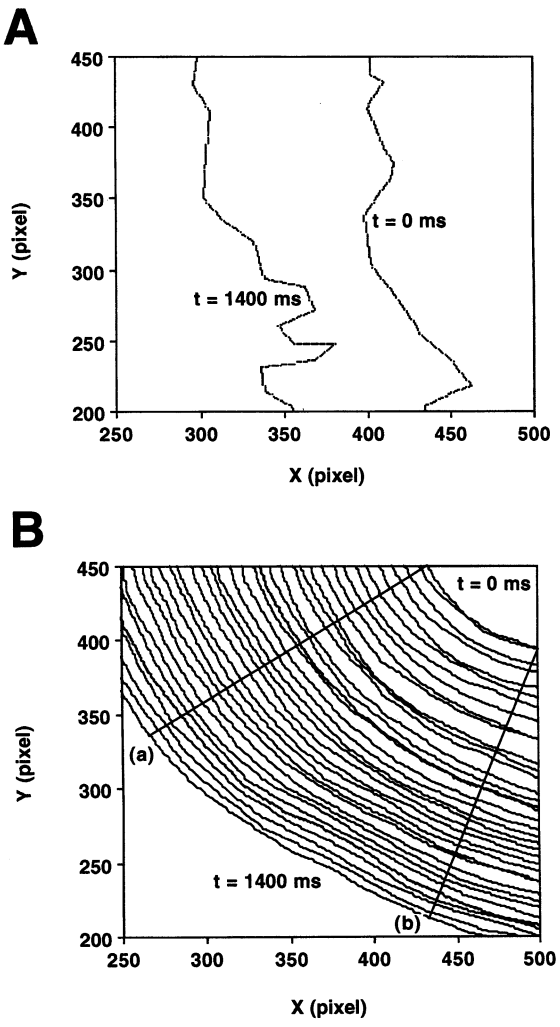

Fig. 3. Spreading of calcium wavefronts representing iso-fluorescence data (constant extravesicular [Ca^{2+}]). Calibration: 100 pixel = 74.15 μm. A: SR vesicle agarose gel; note clefts and indents of the wavefronts at $t = 0$ and 1400 ms. Velocity ∼ 50 μm/s. B: SR vesicle agarose gel with mitochondria; note smooth wavefronts. Velocity ∼ 100 μm/s with a slight difference when measured along (a) or (b). Time resolution 40 ms.

Spatial inhomogeneity has been reported to modulate spatiotemporal patterns, both in chemical [16] and biological systems [17]. It appears to be obvious that calcium waves cannot develop in media with distances that are too large between Ca^{2+} release sites. This might explain the occurrence of cal-

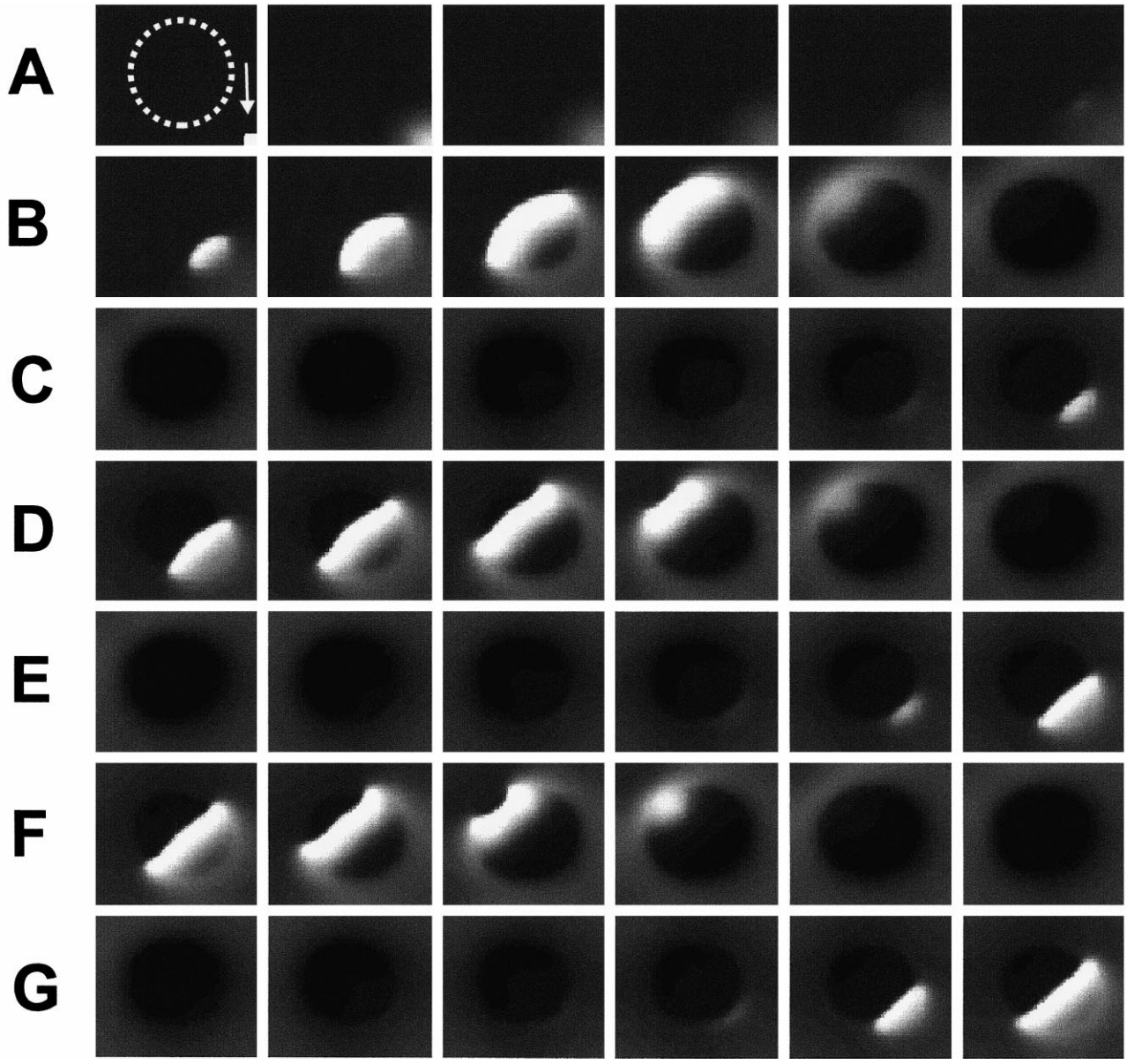

Fig. 4. Simulation by a modified Tang-Othmer model [20] of repetitive calcium waves. Changes of concentration proportional to intensity. Time between consecutive frames 2.5 s. Excitable medium is assumed to be 200 μm in diameter (dotted outline). A stimulus is applied out of circle (arrow). Note that first frames (panel A) exclusively show diffusion. The first wave becomes visible in panel B. Altogether, four waves occur in B–G. For parameters see Table 2.

cium waves exclusively in the clusters of SR vesicles and not in neighboring unclustered regions where the coupling between Ca^{2+} release sites is assumed to be too small to maintain a propagating excitation. On the other hand, it may be supposed that the excitability spatially also varies within the clustered structures. Fig. 3 shows calcium wavefronts (iso-fluorescence data) in clusters of two different preparations, (A) without and (B) with mitochondria, both with the same spatial but different time resolution during 1.4 s. The wavefront in Fig. 3A appears non-uniform which is likely due to both inhomogeneous distribution of SR vesicles and local changes of excitability. Inhomogeneity, however, is essentially reduced in the presence of well-coupled mitochondria (Fig. 3B). A residual inhomogeneity remains and results in small direction-dependent changes of the propagation velocity (Fig. 3B, lines (a), (b)).

Taken together, these data indicate that the novel SR vesicle agarose gel system exhibits the fundamental properties of an excitable medium with self-organization. The occurrence of calcium waves is obviously due to autoregenerative Ca^{2+} release from Ca^{2+} release sites coupled by diffusing Ca^{2+} in the clusters of reconstituted SR vesicles. Well-coupled mitochondria strengthen calcium wave patterns in a typical manner. The observed modulations well agree with those in intact cells, e.g. oocytes of *X. laevis* [15,18] or isolated rat cardiac myocytes [19].

3.3. Simulation of spatiotemporal calcium patterns

To simulate the spreading of calcium waves in the novel in vitro system, a simplified mathematical model is proposed, similar to but not identical with the model of Tang and Othmer (1994), who simulated calcium waves in cardiac myocytes

[20]. We consider the calcium flux of the SR vesicles (J_s), only, instead of two fluxes (through the sarcolemma and the membranes of the SR), as in the original model [20]. The parameters used are listed in Table 2. To obtain wave patterns, which resemble experimental data with similar periods, we adopted the kinetic parameters of Tang and Othmer [20] with an altered time scale. Fig. 4 shows calculated waves over a period of 120 s, where the time between consecutive frames amounts to 2.5 s. Diffusion is initiated by a stimulus (panel A, arrow) and leads to the generation of a wave which clearly becomes visible after 15 s (panel B). Panels B–G show repetitive waves propagating within a circular 'cluster' of 200 μm in diameter (see first frame, dotted outline).

3.4. Summary

In summary, a novel excitable medium of reconstituted SR vesicles in an agarose gel was established. The in vitro system appears to be a powerful tool for studying regenerative Ca^{2+} release from diffusion-coupled ryanodine receptors/SR calcium channels. The patterns of calcium waves in such a simple system resemble those of living cells. Feedback mechanisms between mitochondria and SR calcium channels seem to play an important physiological but not completely understood role in the Ca^{2+} signaling. The presented in vitro system opens new possibilities to study modulating effects of isolated mitochondria (via phosphorylation potential and/or calcium transport) on vesicles of the sarcoplasmic reticulum.

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Table 2
Parameters (following [20]) assumed to numerically simulate Ca^{2+} waves

Ryanodine receptor (RyR):	
k_1	1.5 μM^{-1} s^{-1} (binding rate)
k_{-1}	0.76 s^{-1} (dissociation rate)
k_2	0.08 μM^{-1} s^{-1} (binding rate)
k_{-2}	0.084 s^{-1} (dissociation rate)
k_f	8 s^{-1} (constant which determines the rate of Ca^{2+} release through RyR)
g_1	0.04015 s^{-1} (leakage coefficient)
D	200 μm^2 s^{-1} (apparent diffusion coefficient)
SR pump:	
p_1	117 μM s^{-1} (maximal rate)
p_2	0.12 μM (threshold concentration)
Steady state initial values:	
x_1	0.6489
x_2	0.2139
x_3	0.03403
c	0.1670 μM
c_s	44.23 μM

Appendix

Let c be the calcium concentration in the agarose gel and c_s the calcium concentration in the SR vesicles, then

$$dc/dt = \nu J_s + D\Delta c,$$

$$dc_s/dt = -J_s$$

with the only flux

$$J_s = (k_f x_2 + g_1)(c_s - c) - p_1 c^2/(p_2^2 + c^2).$$

The states of the ryanodine-sensitive calcium channel are described by

$$dx_1/dt = k_{-1}x_2 + k_{-2}\left(1 - \sum_{i=1}^{3} x_i\right) - (k_1 + k_2)x_1 c$$

$$dx_2/dt = -k_{-1}x_2 + k_{-2}x_3 + (k_1 x_1 - k_2 x_2)c$$

$$dx_3/dt = \left[k_2 x_2 + k_1\left(1 - \sum_{i=1}^{3} x_i\right)\right]c - (k_{-2} + k_{-1})x_3.$$

The equations of the model are solved in a two-dimensional domain $\Omega = [0–300\ \mu m] \times [0–300\ \mu m]$ with zero flux BC, i.e. $\partial c/\partial n = 0$ on $\partial\Omega$. We consider a circular cluster with 200 μm in diameter and describe the receptor's density ν by $\nu = \nu(x) = 0.185$ if x in Ω is located within the cluster and $\nu = \nu(x) = 0$ else.

For the numerical solution we use the parameters listed in Table 2 and approximate the diffusion by second order finite differences on a grid with 50×50 points and get an ordinary differential equation system of the dimension 12500. This system has been solved in FORTRAN by using the Krylov-integration method ROWMAP [21]. The complete program source can be requested from the authors.

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