Nano Communication Networks 1 (2010) 43–49

Contents lists available at ScienceDirect

Nano Communication Networks

journal homepage: www.elsevier.com/locate/nanocomnet

Biological excitable media based on non-excitable cells and calcium signaling

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ARTICLE INFO

Article history:
Received 18 January 2010
Received in revised form 25 February 2010
Accepted 14 March 2010
Available online 2 May 2010

Keywords:
Biological computing and communication
Biological excitable media
Non-excitable cells
Calcium signaling

ABSTRACT

In this paper, we investigate a design of biological excitable media based on non-excitable cells and intercellular calcium signaling mechanisms. The calcium induced calcium release mechanism in non-excitable cells is exploited to transform the non-excitable cells into excitable media that propagate calcium signals cell-to-cell. The biological excitable media investigated in this paper represent versatile media for controlling biological systems owing to the nature and function of calcium signals as the universal second messenger for the cell. The enhanced calcium excitability of non-excitable cells is experimentally demonstrated and a mathematical model is developed to investigate the condition for non-excitable cells to increase the calcium excitability.

1. Introduction

A grand challenge to next generation computing and communication technology is to utilize materials and mechanisms from biological systems to establish a new computing and communication paradigm [17]. Unlike silicon-based devices, hardware made of biological materials and mechanisms is inherently biocompatible and presents unique features such as self-organization, massive parallelization, and functional complexity. These features are expected to allow for design and development of *in vivo* information processing systems applicable to prosthetic devices, tissue engineering and regenerative medicine.

A promising approach to engineering biological computing and communication media is to use living cells and cell–cell communication mechanisms [1]. Living cells in biological systems use numerous networking mechanisms to establish cell–cell communication while they are self-organized into highly functional structures such as tissues and organs. In addition, living cells possess the ability to perform massively parallel information processing in Avogadro-scale complexity. Naturally occurring cells as well as genetically engineered cells are therefore potential materials for the design and engineering of biological computing and communication media.

In this paper, we investigate a design of excitable media based on living cells and intercellular calcium signaling mechanisms — a common form of cell–cell communication

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doi:10.1016/j.nancom.2010.03.002

mechanisms. The biological mechanism exploited is the calcium induced calcium release (CICR) [18] found in non-excitable cells. In non-excitable cells, calcium signals (Ca^{2+}) are released via binding of Ca^{2+} and inositol 1,4,5-trisphosphate (IP_3) to IP_3 receptors (IP_3Rs) located on the endoplasmic reticulum (ER) surface. An IP_3R has calcium binding sites for activation to potentially enable CICR, yet such behavior is not commonly observed in experiments with non-excitable cells [12]. However, IP_3 dictates the sensitivity of IP_3Rs to Ca^{2+} [18], and thus a CICR-like behavior may be induced in non-excitable cells under the condition that the intracelular IP_3 concentrations are elevated. To test this hypothesis, we perform experiments using non-excitable cells and a peptide hormone known to increase intracellular IP_3 concentrations, which demonstrate that the non-excitable cells increase the calcium excitability to propagate Ca^{2+} cell-to-cell when the IP_3 concentrations are elevated. We also perform mathematical modeling and simulation to identify the required IP_3 concentration that can transform non-excitable cells into excitable media that propagate Ca^{2+} cell-to-cell via a CICR-like mechanism.

The rest of the paper is organized as follows. Section 2 experimentally demonstrates that a monolayer of non-excitable cells can form highly excitable media to propagate Ca^{2+} when intracellular IP_3 concentrations are increased. Section 3 describes a theoretical study including a mathematical model and simulation results on the calcium excitability of non-excitable cells, and Section 4 concludes this paper.

2. Experimental studies

Non-excitable cells exhibit low calcium excitability due to the lack of the CICR mechanism typically found in excitable cells. In non-excitable cells, an intracellular increase of Ca^{2+} is affected by various *off*-mechanisms [4] and diminishes quickly. In this section, we perform experiments to show that non-excitable cells increase the calcium excitability when the intracellular IP_3 concentration is elevated. In our experiments, we use HeLa cells constitutively expressing connexin 43 proteins (i.e., non-excitable cells forming gap junction channels) and vasopressin, a peptide hormone known to increase the intracellular IP_3 concentration, to demonstrate that the transiently elevated Ca^{2+} in one cell by flash-photolysis of caged-Ca^{2+} propagates cell-to-cell in a CICR like manner in the presence of vasopressin.

2.1. Materials and methods

2.1.1. Cell culture

HeLa cells constitutively expressing connexin 43 (designated HeLa Cx43 cells) (a gift from Dr. K. Willecke of Institut fur Genetik, Germany) [6] were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (Gibco), 100 i.u./ml penicillin and 100 μg/ml streptomycin (Sigma). The cells were incubated in 10 cm diameter culture dishes at 37 °C under 5% CO_2. Prior to flash-photolysis experiments, cells were transferred to 35 mm glass-bottom dishes and cultured for 1–3 days under the same culture conditions.

2.1.2. Fluo-4 and caged-Ca^{2+} loading

The cytosolic calcium concentrations ($[Ca^{2+}]$) were monitored using the calcium indicator, Fluo4/AM (Molecular Probes). Cells were first loaded with 2.0 μM Fluo4/AM in Hank's balanced salt solution buffered with 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid containing Ca^{2+} [HBSS-HEPES (+)] for 40 min at room temperature. The cells were subsequently loaded with 2.5 μM caged-Ca^{2+} (o-nitrophenynl EGTA, AM) (Invitrogen) for 10 min at room temperature. Cells were then washed twice with the HBSS-HEPES (+) and were left for 20 min prior to flash-photolysis experiments to allow for de-esterification of the AM esters.

2.1.3. Flash-photolysis of caged-Ca^{2+} and Ca^{2+} imaging

Flash-photolysis of caged-Ca^{2+} and Ca^{2+} imaging was performed using a fluorescence microscope system (DeltaVision, Applied Precision, Inc., Seattle, WA) equipped with a 406 nm diode laser module (30 mW). In the flash-photolysis experiments, Ca^{2+} was photo-released by laser spot illumination (excitation wavelength 406 nm). The center of a cell was selected and the selected spot was exposed for 1.0 s. Before and after the Ca^{2+} photo-release event, $[Ca^{2+}]$ was determined by the fluorescence intensity of Fluo4 at 488/528 nm wavelengths for excitation/emission. Fluorescence images were obtained using a 40x oil lens objective (UApo40, NA = 1.35, Olympus) or a 60x oil lens objective (PlanApo60, NA = 1.4, Olympus) with a 0.2–2.0 s image time interval. Cytosolic Ca^{2+} concentrations in figures are expressed as the relative fluorescence intensity; i.e., $(Ft - F_0)/F_0$, where F_t is the fluorescence intensity measured at time t after the flash is applied and F_0 the resting fluorescence intensity measured before the flash is applied.

2.1.4. Vasopressin application

A subthreshold concentration (0.001 i.u./ml) of vasopressin (Sigma) was added to increase intracellular IP_3 concentrations prior to flash-photolysis of caged Ca^{2+} and Ca^{2+} imaging. This concentration of vasopressin caused no significant changes in resting $[Ca^{2+}]$, inducing only slight increases in a small number of cells (∼10%), under which changes in $[Ca^{2+}]$ in response to a flash-photolysis event are measurable.

2.2. Results

Flash-photolysis and Ca^{2+} imaging were performed in the presence of the subthreshold concentration (0.001 i.u./ml) of vasopressin, which intercellularly propagated a locally induced increase of $[Ca^{2+}]$ in the flashed cell (Fig. 1 A). An increased $[Ca^{2+}]$ was observed in the cell as far as five cells away from the flashed cell. These cells show a peak mode of calcium responses as shown in Fig. 1B. An averaged propagation distance over 10 independent experiments was 2.0 ± 0.5 cells (excluding the flashed cell) (Fig. 1C), whereas the achieved propagation distance in control experiments reported in [16] is 0.2 ± 0.1 ($n = 19$) where the same experimental methods were employed in the absence of vasopressin.

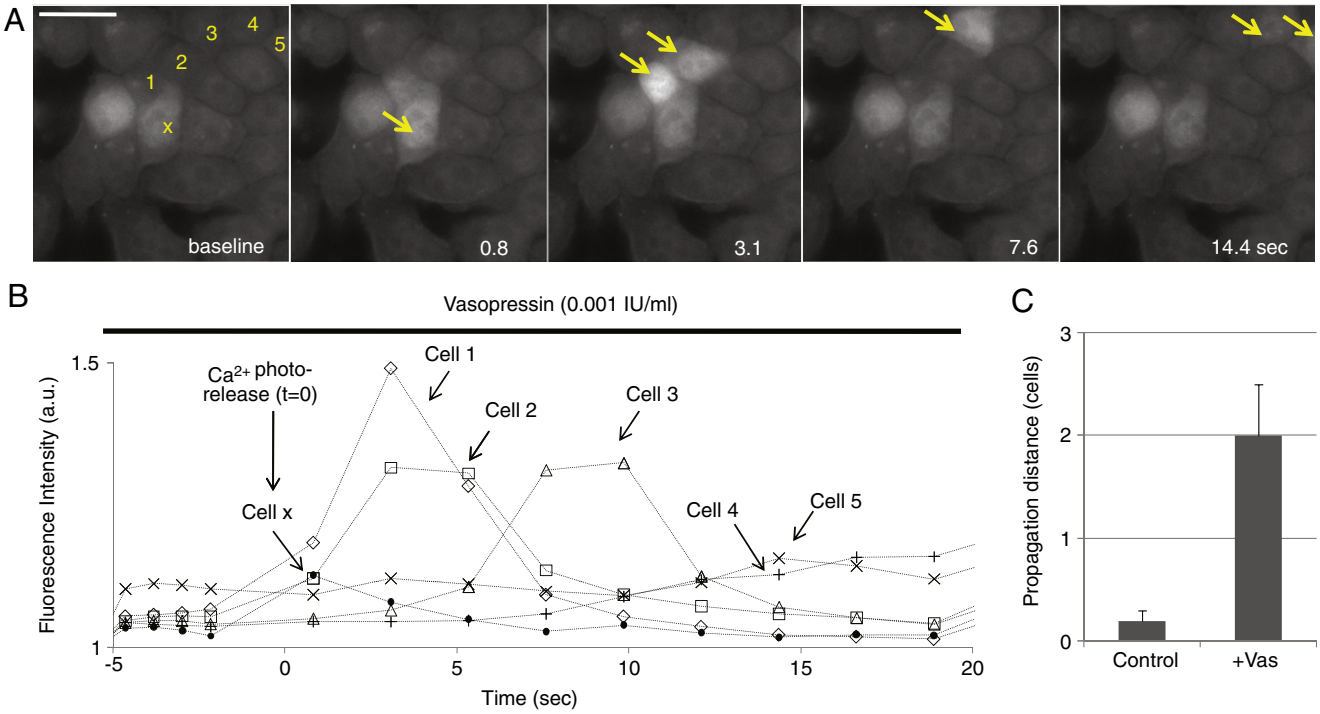

Fig. 1. Flash-photolysis of caged-Ca^{2+} in the presence of a subthreshold concentration of vasopressin (0.001 i.u./ml). (A) A series of fluo-4 fluorescence images demonstrates that a locally induced increase in the intercellular calcium propagated over the five cells in the presence of vasopressin; cell x was flashed, and the $[Ca^{2+}]_i$ increase was subsequently propagated from cells 1 to 5. The arrows indicate the cell that increased $[Ca^{2+}]_i$ during the wave propagation. (Scale bar, 20 μm) (B) Averaged fluo-4 fluorescence intensity measured at each cell from (A) are plotted with respect to time. (C) The average propagation distance was increased from 0.2 ± 0.1 ($n = 19$) in the absence of vasopressin [16] to 2.0 ± 0.5 cells ($n = 10$) in the presence of vasopressin.

The above experimental results indicate that non-excitable cells can establish a highly excitable medium to propagate locally sensed information to distant cells when the IP_3 concentration is elevated. The above results are also consistent with previous studies based on other types of non-excitable cells (SKHep1 [10], pancreatic acini [21], and salivary gland cells [22]), in which a local Ca^{2+} increase induced by either flash-photolysis of caged-Ca^{2+} [10] or microinjection of Ca^{2+} [21,22] was able to generate a Ca^{2+} wave that propagated cell-to-cell after cells were stimulated with subthreshold concentrations of agonistic substances (vasopressin [10], cholecystokinin [21], or serotonin [22]).

3. Theoretical studies

In this section, we use a mathematical model to investigate the conditions to induce CICR-like behavior in non-excitable cells.

3.1. The model

A non-excitable cell is modeled on a $L \times L$ square lattice in which the cytosolic Ca^{2+} concentration ($[Ca^{2+}]$) is controlled by the three calcium fluxes: the channel flux that releases Ca^{2+} from the ER to the cytosol through the IP_3R ($J_{Channel}$), the pump flux that transports Ca^{2+} from the cytosol into the ER with the ATP (Adenosine TriPhosphate)-dependent pumps (J_{Pump}), and the leakage flux that releases Ca^{2+} from the ER to the cytosol ($J_{Leakage}$). All the three fluxes exist on the ER membrane homogeneously extended in the cytosolic space. The extrusion and entry of Ca^{2+} across the plasma membrane are ignored in the model. The diffusion of Ca^{2+} in the cytosol is modelled with the effective diffusion coefficient (D) accounting the buffering effects of Ca^{2+} in the cytosol. Here the dynamics of the cytosolic Ca^{2+} concentration is written as

$$\frac{\partial [Ca^{2+}]}{\partial t} = D\nabla^2[Ca^{2+}] + J_{Channel} - J_{Pump} + J_{Leakage}. \quad (1)$$

A theoretical model for IP_3Rs was proposed by De Young and Keizer [5]. The model assumes that three equivalent and independent subunits are involved in the conduction of an IP_3R. Each subunit has one IP_3 binding site (m-gate) and two Ca^{2+} binding sites: one for activation (n-gate) and the other for inhibition (h-gate). In our model, we use a simplified version of the DeYoung–Keizer model, which is proposed by Li and Rinzel [13]. In the Li–Rinzel model, m- and n-gates are substituted by the steady state values, m_∞ and n_∞; i.e., the channel flux is given as

$$J_{Channel} = v_c m_\infty^3 n_\infty^3 h^3([Ca^{2+}]_{ER} - [Ca^{2+}]), \quad (2)$$

with

$$m_\infty = \frac{[IP_3]}{[IP_3] + d_m}, \quad (3)$$

$$n_\infty = \frac{[Ca^{2+}]}{[Ca^{2+}] + d_n}, \quad (4)$$

where $[Ca^{2+}]_{ER}$ is the Ca^{2+} concentration in the ER and $[IP_3]$ the IP_3 concentration in the cytoplasm. Due to its slow

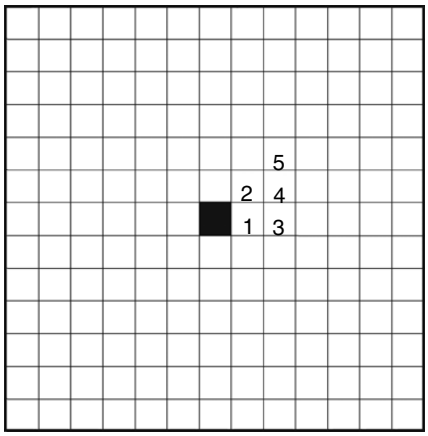

Fig. 2. Simulated regular grid network of cells.

time scale, the h-gate is considered as a variable as follows:

$$\frac{dh}{dt} = \alpha(1-h) - \beta h, \tag{5}$$

with

$$\alpha = a_1 \frac{[IP_3] + d_1}{[IP_3] + d_2}, \tag{6}$$

$$\beta = a_2[Ca^{2+}], \tag{7}$$

where d_m, d_n, d_1 and d_2 are receptor dissociation constants, and a_1 and a_2 are receptor binding and unbinding constants [5,13]. The pump flux J_{Pump} is given by

$$J_{Pump} = v_P \frac{[Ca^{2+}]^2}{k^2 + [Ca^{2+}]^2}, \tag{8}$$

where k is the activation constant for Ca^{2+}-ATPase pumps. The leakage flux $J_{Leakage}$ is given by

$$J_{Leakage} = v_L([Ca^{2+}]_{ER} - [Ca^{2+}]), \tag{9}$$

where the two parameters v_P and v_L respectively describe the maximum pump flux and leakage rate.

A 13 × 13 regular grid network of cells is considered (Fig. 2), representing a monolayer of cell culture. Neighboring cells in the network are coupled by gap junctions that permeate Ca^{2+}. Following the other models of intercellular Ca^{2+} waves [19,9], we simply assume that the gap junctions are homogeneously distributed throughout the whole boundaries of a cell, allowing the diffusion of Ca^{2+} between two neighboring cells across any part of the cell boundary. As a result, the condition for the intercellular Ca^{2+} flux at a cell boundary is represented as

$$D\frac{\partial[Ca^{2+}]}{\partial n} = P([Ca^{2+}]^+ - [Ca^{2+}]^-), \tag{10}$$

where P is the gap junctional permeability for Ca^{2+}, $[Ca^{2+}]^+$ and $[Ca^{2+}]^-$ are Ca^{2+} concentrations on either side of the boundary, and n the unit normal vector to the boundary [19].

To simulate the Ca^{2+} photo-release event in response to 1.0 s of laser spot illumination, the cytosolic Ca^{2+} concentration of the centered cell in the monolayer (i.e., the filled square in Fig. 2) is transiently increased by adding a stimulus flux J_{stim} to the right hand side of Eq. (1). The model parameters introduced above and parameter values used in numerical simulations are listed in Table 1.

Table 1
Model parameters.

Parameter	Value (Unit)
L	24 μm
D	20 $\mu m^2/s$
P	0.5
$[IP_3]$	0.21 μM
$[Ca^{2+}]_{ER}$	15 μM
v_C	0.6 /s
v_P	0.5 μM/s
v_L	0.001 /s
d_m	0.13 μM
d_n	0.08 μM
d_1	0.13 μM
d_2	0.94 μM
a_1	0.21 /s
a_2	0.2/μM/s
k	0.1 μM

3.2. Results

Consistent with the experimental design, all the cells are in the resting state at the beginning of simulation where $[IP_3] = 0.1$ μm. At $t = 0$ s, the IP_3 concentrations of all the cells are increased to a certain level ($[IP_3]_{stim}$), simulating the effect of vasopressin application. As observed in the experiments, this increase of $[IP_3]$ causes only slight increases of $[Ca^{2+}]$. At $t = 5$ s, the Ca^{2+} stimulus (J_{stim}) is turned on for the centered cell and turned off at $t = 6$ s, modeling the Ca^{2+} photo-release event induced by 1 s of laser spot illumination in the experiments.

The dynamics of the intracellular Ca^{2+} concentration of a cell in the grid network (Fig. 2) shows a fixed point when $[IP_3]$ <0.24 μM, giving the typical behavior of non-excitable cells observed in the experiments. Accordingly, we use $[IP_3]_{stim} = 0.21$ μM as a default value in simulations.

Fig. 3 illustrates a simulation result with time series images showing the intercellular spreading of Ca^{2+} waves responding to $[IP_3]_{stim} = 0.21$ μM and $J_{stim} = 5.0$ μM/s. As shown in the figure, the centered cell, upon stimulation, increases the intracellular Ca^{2+} concentration. Ca^{2+} ions generated in the centered cell then diffuse through gap junction channels to the four neighboring cells, which in turn respond by increasing the intracellular Ca^{2+} concentrations. Similarly, the next nearby 4 cells subsequently increase their intracellular Ca^{2+} concentrations. In this simulation, calcium waves spread out from the centered cell to the nearby 12 cells, each of which shows a large increase in their intracellular calcium concentration.

Fig. 4 shows the dynamics of cytosolic Ca^{2+} concentrations of cells 1–5 in response to the stimulus flux transiently added to the centered cell, where $J_{stim} = 0.01$, 0.1, 1 or 10 μM/s and $[IP_3]_{stim} = 0.21$ μM. The figures clearly show that the increase in J_{stim} causes a larger number of cells to increase their intracellular Ca^{2+} concentrations in a peak mode manner as observed in the experiments (Fig. 1 B). The number of responding cells as a function of J_{stim} is given in Fig. 5 at $[IP_3]_{stim} = 0.21$ μM, showing that the number of responding cells increases as the stimulus flux increases till the maximum number of responding cells (i.e., 20 in this case) is reached at $J_{stim} = 15$ μM/s. A further increase in J_{stim} (>15 μM/s) does not lead to an increase in the number of responding cells, which is consistent with experimental results (data not shown).

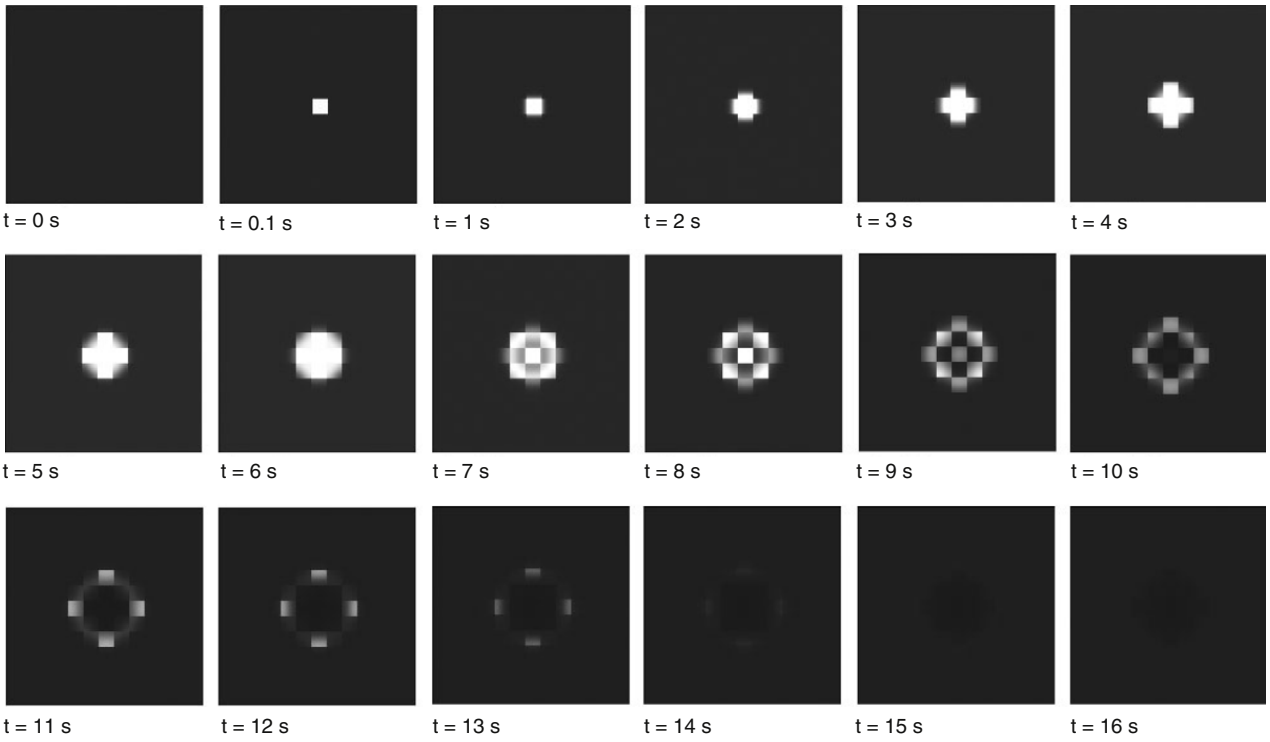

Fig. 3. Intercellular spreading of Ca^{2+} waves generated in response to the stimulus flux turned on at time $t = 0$ s, where $[IP_3]_{stim} = 0.21$ μM and $J_{stim} = 5.0$ μM/s. The black lattices represent $[Ca^{2+}] = 0$ μM and white lattices $[Ca^{2+}] = 0.6$ μM. The propagation distance is 2 cells in one direction, reproducing a typical experimental result presented in Section 2 (propagation distance, 2.0 ± 0.5 cells).

The number of responding cells as a function of the IP_3 concentration is given in Fig. 6 at $J_{stim} = 0.5$ μM/s, showing that the number of responding cell sharply increases around $[IP_3]_{stim} = 0.212$ μM, which indicates a condition for non-excitable cells to enable the CICR-like mechanism to propagate Ca^{2+} cell-to-cell.

4. Conclusion

In this paper, we have experimentally and theoretically investigated conditions under which non-excitable cells are transformed into excitable media that propagate Ca^{2+} cell-to-cell. The significance of the biological excitable media investigated in this paper is that it is potentially possible to perform logical computation in a manner similar to excitable chemical systems [20,15]. Here a group of cells represents computational units where the interconnection between computational units is mediated by the diffusion of Ca^{2+}. To transform the excitable media into a specific calcium signaling circuit, various properties related to calcium signaling (e.g., the calcium excitability, the releasable amount of calcium ions, gap junctional conductance between cells) can be modified, while modifying multiple properties should allow for many different designs for computation. Additionally, each cell can have a calcium signaling circuit inside itself based on the specific spatial arrangement of calcium channels [8] or clusters of calcium channels. The computational units constituting the calcium signaling circuit in a cytosol can be localized in calcium microdomains [3], which are loosely coupled by the diffusion of Ca^{2+}. In such cases, there exist multiple levels of hierarchy in calcium signaling circuits (e.g., microdomain, single cell and group level). This increases the scale and complexity of the biological excitable media while enhancing the ability to process information.

It is also noted that the biological excitable media investigated in this paper should be capable of generating spatiotemporal patterns of Ca^{2+}. It has been observed in experimental studies on Ca^{2+} signaling that Ca^{2+} waves can spread as rotating spiral waves in a variety of biological media (e.g., a cytosolic environment, a sheet of cells, and a three dimensional tissue [7]). A more complex pattern can in principle be formed by exploiting various properties of the cell, such as the spatial distribution of protein complexes involved in calcium signaling (e.g., calcium channels and pumps), the organization and structure of the cell, and the dynamics of calcium mobilizing molecules [11].

The ability of the biological excitable media to perform computation and pattern formation presents the potential to control biological systems. In biological systems, Ca^{2+} and its regulatory mechanisms control numerous gene expression and protein activations crucial for the survival of the cell [14]. Biological excitable media designed and integrated within a biological system potentially play functional roles in the biological system. Applications for prosthetic devices, tissue engineering, regenerative medicine [17] as well as *in vivo* medical diagnosis [2] are highly anticipated.

Acknowledgements

The authors would like to acknowledge Dr. K. Willecke of Institut fur Genetik, Germany for kindly providing HeLa Cx43 cells. This research has been carried out at the Frontier Research Base for Global Young Researchers, Osaka University, through the program Promotion of En-

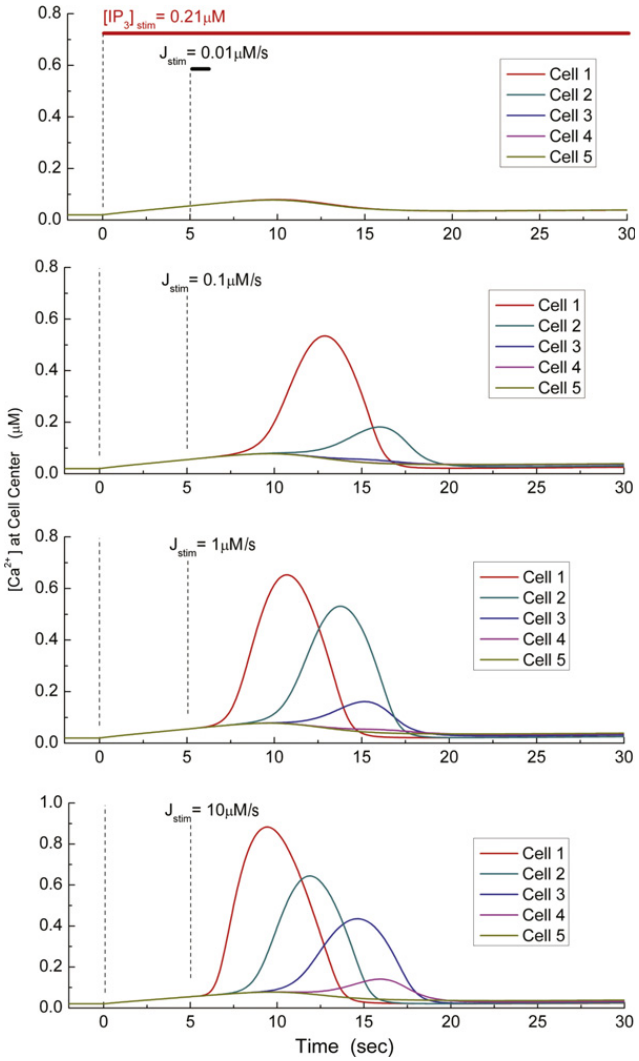

Fig. 4. The cytosolic Ca^{2+} concentrations of cells 1–5 in response to the stimulus flux. $[IP_3]_{stim} = 0.21\ \mu M$ and J_{stim} = (a) 0.01, (b) 0.1, (c) 1, (d) 10 μM/s.

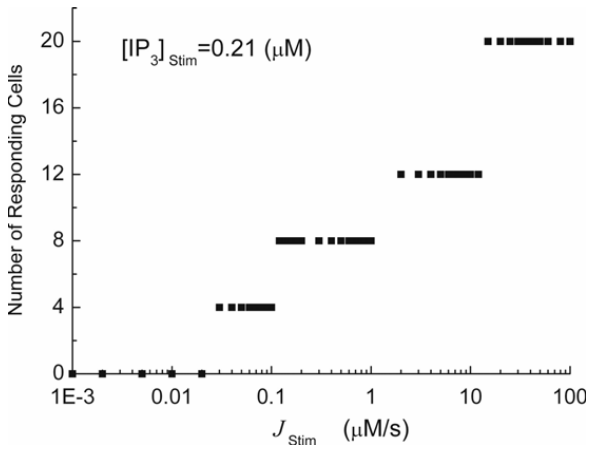

Fig. 5. The number of responding cells as a function of the stimulus flux (J_{stim}) where $[IP_3]_{stim} = 0.21\ \mu M$.

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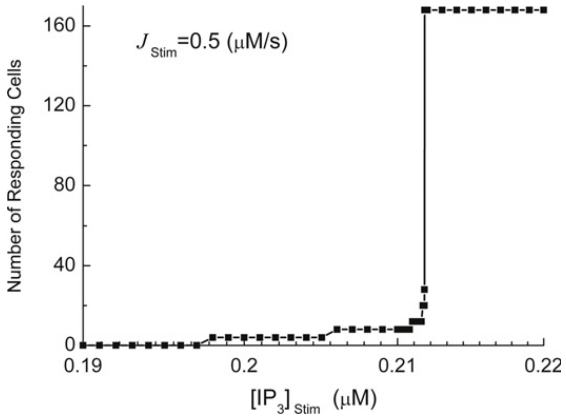

Fig. 6. The number of responding cells as a function of the IP_3 concentration where $J_{stim} = 0.5\ \mu M/s$.

was supported by KAKENHI 22680006. This work was also supported by a research grant to J.S. from the Science and Technology Foundation of the Ministry of Education of China under Grant 108075. This work was also supported by grants from the MEXT of Japan to T.H. and Y.H.

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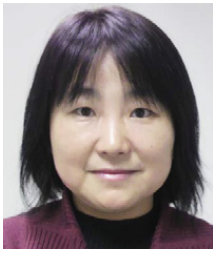

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