Biophysical Chemistry 148 (2010) 42–50

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

Importance of cell variability for calcium signaling in rat airway myocytes

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ARTICLE INFO

Article history:
Received 15 November 2009
Received in revised form 26 January 2010
Accepted 8 February 2010
Available online 11 February 2010

Keywords:
Calcium signaling
Airway smooth muscle cell
Stochastic effects
Cell variability
Sensitivity analysis

ABSTRACT

Calcium signaling controls several essential physiological functions in different cell types. Hence, it is not surprising that different aspects of Ca^{2+} dynamics are in the focus of in-depth and extensive investigations. Efforts concentrate on the development of proper theoretical models that would provide a unified description of Ca^{2+} signaling. Remarkably, experimentally recorded Ca^{2+} signals exhibit a rather large diversity, which can be observed irrespective of the cell type, measuring techniques, or the nature of the signal. Our goal in the present study therefore is to present a theoretical explanation for the variability observed in experiments, whereby we focus on caffeine-induced Ca^{2+} responses in isolated airway myocytes. By employing a stochastic model, we first test whether the observed variability can be attributed to intrinsic fluctuations that are a common feature of biochemical reactions that govern Ca^{2+} signalization. We find that stochastic effects, within ranges that correspond to actual conditions in the cell, are far too modest to explain the large diversity observed in experimental data. Foremost, we reveal that only cell variability in theoretical modeling can appropriately describe the observed diversity in single-cell responses.

1. Introduction

Mathematical modeling is a well-established method when studying complex biological systems. The method is useful and the predictive power of theoretical models is in many cases indispensable for catalyzing further experimental studies [1,2]. Modeling of Ca^{2+} signaling, as other cellular processes, consists in the mathematical description of the functional properties of the components of the cellular system, and the analysis of the predictions of the model about the behavior of the cell system. Validation of the model basically consists in the comparison of the predicted output of the model system with the experimental data. Though some scientific literature has been devoted to cellular diversity in several cell types [3–7], the experimental data that are typically considered are the mean values calculated from several recordings, with no or little interest in interindividual variability. Nevertheless, biological systems are under permanent influences of several internal and external protuberances and the variability in biological systems has to be taken into account. Interindividual variability is actually, as well as the central tendency usually estimated by the mean value, a physiological property, which can be quantified by the standard deviation. As a consequence, a realistic model should account not only for the mean tendency but also for the individual dispersion.

Additionally, one of the limitations of the theoretical modeling is the lack of precise experimental data for the parameters that describe the components of the system, so that they may be chosen in a given range. Hence, the biological relevance of the values chosen for the parameters should also be addressed. The problem is the fact that the correspondence between the model predictions and the experimental data may critically depend on the parameter values incorporated in the model. In that sense, in some cases, mathematical models are considered as “hypersensitive” to changes in model parameters. The general idea is that the cell, as a biological system, exhibits a relative robustness, i.e., its behavior is not deeply affected by small changes in biological parameters. If not, the biological system, which is always under permanent influences of several internal and external perturbances, would be unable to maintain its functional integrity. It is hence admitted that, to be realistic, the model should reflect this biological robustness and hence exhibits itself a relative robustness.

Usually, the robustness of the system is evaluated by the sensitivity analysis, which is a means to acquire insight about the importance of model parameters. Perhaps the most famous example is the metabolic control analysis, which represents in general a systematic method for analyzing the control of steady states. It quantifies the extent to which any parameter, but more notably all molecular processes, controls any steady-state variable within a metabolic pathway [8,9]. Methods, developed in the framework of metabolic control analysis have

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0301-4622/$ – see front matter
doi:10.1016/j.bpc.2010.02.006

afterward been successfully applied to the control analysis of various phenomena in biological systems, including non-steady states and oscillations [10–15]. However, in the modeling of signaling processes, it is difficult to objectify a correspondence between the effect of such arbitrary changes and experimental biological measurements.

The cellular dynamics results from a huge body of biochemical reactions, which are all subjects to thermal noise, and belongs in reality to non-deterministic processes, which require special treatments [16–18]. In the last decade a lot of stochastic models have been developed for describing and studying different cellular processes. In the modeling of gene expression and circadian rhythms, for example, where the number of reacting particles in the cell is very low (only few hundred in some cases), the implementation of stochastic algorithms has been emphasized and realized by several authors [19–23]. Furthermore, recent experimental and theoretical investigations indicate that Ca^{2+} signaling is clearly subjected to stochastic dynamics as well [24–29]. Consequently, stochastic modeling of intracellular Ca^{2+} signaling pathways is gaining increasingly more attention [30–36]. Recent findings indicate that the stochastic nature of Ca^{2+} signaling arises from the stochastic behavior of ion channels in the membranes. Local random opening and closing of these channels introduces stochasticity into global Ca^{2+} responses [28,37,38]. Moreover, it has been observed that the Ca^{2+} release channels are spatially organized in clusters [39,40]. Such an organization can on one hand enhance the sensitivity of the Ca^{2+} signaling pathway in the presence of internal fluctuations by weak stimulation levels (coherence resonance) [31,41], and on the other hand it can explain the intrinsic irregularity of Ca^{2+} oscillations by supra-threshold agonist concentrations observed in experiments [27,42].

Nevertheless, it is difficult to surely identify and to characterize perturbances to which biological systems are submitted. However, we have an objective measurement of their impact, since it is reasonable to consider that the observed cell-to-cell variability is the consequence of these changes. Hence, the cell-to-cell variability is a critical biological parameter that should be taken into account in the modeling of the functional properties of the components of the system and in the sensitivity analysis of the cellular system.

The aim of this study is to elucidate a rather large cell-to-cell variability in calcium responses as observed experimentally. First, we determine whether cell-to-cell variability can be explained by non-deterministic molecular processes. For that purpose, we have developed a stochastic version of a model of Ca^{2+} signaling we have already published [43], and we compare the individual cell response pattern and interindividual cell response dispersion predicted by the stochastic model with original Ca^{2+} response traces and observed cell-to-cell variability.

Second, we propose a “reverse” sensitivity analysis. In contrast to the well-known sensitivity analysis, where the model's behavior is studied in the context of variations in model parameters, we scrutinize the inversed problem. In particular, since the scattering of the output calcium signals is known from experiments, we determinate the range within a given parameter should vary, so that the subsequent variations in the predicted output correspond to the observed cell-to-cell variability of experimental recordings. In addition, we propose an extended version of such reverse sensitivity analysis, where all parameters are varied simultaneously and thus the overall dispersion of parameters is evaluated. In this way the actual variability of model parameters is estimated, at which theoretical predictions concur with experimental observations.

This study is based on the calcium response to caffeine stimulation in airway myocytes, a biological cellular system for which we have both a theoretical model that includes the major components involved in Ca^{2+} homeodynamics, and a large set of experimental measurements. The conclusions are extended to the general question of cellular Ca^{2+} signaling, on the basis of bibliographical data on the Ca^{2+} response to various agonists in different cell types.

2. Materials and methods

2.1. Experimental

2.1.1. Cell preparation and fluorescence measurement of $[Ca^{2+}]_i$

Measurements of the concentration of calcium ions ($[Ca^{2+}]_i$) in freshly isolated cells were performed as previously described [43]. Here summarizing briefly, rat tracheae were obtained from male Wistar rats 10–15 weeks old, weighing 300–400 g. Animals were sacrificed by CO_2 exposure, heart and lungs were removed en bloc, and the trachea was rapidly dissected out. The muscular strip located on the dorsal face of the trachea was further dissected, the epithelium-free muscular strip was cut into several pieces and the tissue was then incubated overnight (14 h) in low-Ca^{2+} (200 μM) physiological saline solution (PSS; composition given below) containing 0.5 mg ml^{-1} collagenase, 0.35 mg ml^{-1} pronase, 0.03 mg ml^{-1} elastase and 3 mg ml^{-1} bovine serum albumin at 4 °C. After this time, the muscle pieces were triturated in a fresh enzyme-free solution with a fire polished Pasteur pipette to release cells. Cells were stored for 1 to 3 h to attach on glass coverslips at 4 °C in PSS containing 0.8 mM Ca^{2+} and used on the same day. In control experiments, immunocytochemistry was performed using monoclonal mouse anti-smooth muscle α-actin antibodies and FITC-conjugated anti-mouse IgG antibodies to verify that the isolated cells obtained by dissociation were smooth muscle cells (data not shown). Cells were loaded with indo-1 by incubation in PSS containing 1 μM indo-1 acetoxymethylester for 25 min at room temperature and then washed in PSS for 25 min. Coverslips were then mounted in a perfusion chamber and continuously superfused at room temperature. A single cell was illuminated at 360 ± 10 nm. Emitted light from that cell was counted simultaneously at 405 nm and 480 nm by two photomultipliers (P100, Nikon). $[Ca^{2+}]_i$ was estimated from the 405/480 ratio using a calibration for indo-1 determined within the cells. Caffeine (5 mM) was applied to the tested cell by a pressure ejection from a glass pipette located close to the cell. No changes in $[Ca^{2+}]_i$ were observed during test ejections of PSS (data not shown). Each record of $[Ca^{2+}]_i$ response to caffeine was obtained from a different cell. Cytosolic calcium concentrations ($[Ca^{2+}]_i$) are expressed as mean ± SD, for the number of cells indicated in the text.

2.1.2. Solutions, chemicals and drugs

PSS contained (in mM): 130 NaCl, 5.6 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 11 glucose, 10 Hepes, pH 7.4 with NaOH. Collagenase (type CLS1) was from Worthington Biochemical Corp. (Freehold, NJ, USA). Bovine serum albumin, elastase, pronase and caffeine (CAF) were purchased from Sigma (Saint Quentin Fallavier, France). Indo-1 AM was from Calbiochem (France Biochem, Meudon, France). Indo-1 AM was dissolved in dimethyl sulphoxide in which the maximal concentration used in our experiments was <0.1% and had no effect on the resting value of the $[Ca^{2+}]_i$ nor on the variation of the $[Ca^{2+}]_i$ induced by caffeine (data not shown).

2.1.3. Bibliographical data

Data obtained from the literature were taken from publications selected via Pubmed database. To be selected, the articles should give original data on intracellular Ca^{2+} concentration at rest and upon stimulation that triggers $[Ca^{2+}]_i$ increase. Data should be presented as mean ± standard deviation (SD) and/or standard error of the mean (SE), with the number of measurements. Usually, SD is not given in the publications. In this case, it has been recalculated from SE and the sample size (n) using the formula $SD = SE \times \sqrt{n}$. SD was calculated as the absolute value, and as percent of the mean value.

3. Computational

3.1. Deterministic model

We employ the mathematical model for calcium dynamics upon airway smooth muscle cell stimulation by caffeine that has been proposed by Roux and Marhl [43]. The model considers Ca^{2+} exchange between cytosol (Ca_i) and intracellular calcium stores (the sarcoplasmic reticulum (Ca_{SR}), mitochondria (Ca_m) and signaling (SPr) and buffering (BPr) proteins). Here, the model equations are presented only briefly. For a more detailed presentation see the original paper [43]. The free Ca^{2+} concentrations in the cytoplasmic space, in the SR, and in the mitochondria are calculated by the following differential equations:

$$\frac{dCa_i}{dt} = \frac{1}{1 + K_{SPr}SPr_{tot}/(K_{SPr} + Ca_i)^2} \times \left(J_{RyR} - J_{SERCA} + J_{leak} + J_{out} - J_{in} + k_{off}CaBPr - k_{on}Ca_iBPr\right), \tag{1}$$

$$\frac{dCa_{SR}}{dt} = \frac{\beta_{SR}}{\rho_{SR}}\left(J_{SERCA} - J_{RyR} - J_{leak}\right), \tag{2}$$

$$\frac{dCa_m}{dt} = \frac{\beta_m}{\rho_m}(J_{in} - J_{out}), \tag{3}$$

where the fluxes are defined as follows:

$$J_{RyR} = k_{RyR}\frac{Caff^2}{K_{Caff}^2 + Caff^2}CICRMg(Ca_{SR} - Ca_i), \tag{4}$$

$$CICRMg = \frac{Ca_i}{\left(Ca_i + K_{A,Ca}\left[1 + Mg_i/K_{A,Mg}\right]\right)\left(1 + Ca_i/K_{I,Ca} + Mg_i/K_{I,Mg}\right)}, \tag{5}$$

$$J_{SERCA} = k_{SERCA}\frac{Ca_i^2}{K_{SERCA}^2 + Ca_i^2}, \tag{6}$$

$$J_{leak} = k_{leak}(Ca_{SR} - Ca_i), \tag{7}$$

$$J_{in} = k_{in}\frac{Ca_i^8}{K_m^8 + Ca_i^8}, \tag{8}$$

$$J_{out} = k_{out}Ca_m. \tag{9}$$

The concentrations of free and occupied signaling (SPr and CaSPr) and buffering (BPr and CaBPr) binding sites of proteins are calculated by the following equations, whereby the conservation relations for the total signaling (SPr_{tot}) and buffering (BPr_{tot}) protein binding sites and the total Ca^{2+} concentration (Ca_{tot}) in the cell are considered:

$$SPr = \frac{K_{SPr}SPr_{tot}}{K_{SPr} + Ca_i}, \tag{10}$$

$$CaSPr = SPr_{tot} - SPr, \tag{11}$$

$$CaBPr = Ca_{tot} - Ca_i - \frac{\rho_{SR}}{\beta_{SR}}Ca_{SR} - \frac{\rho_m}{\beta_m}Ca_m - CaSPr, \tag{12}$$

$$BPr = BPr_{tot} - CaBPr. \tag{13}$$

Cell stimulation with caffeine was simulated by raising the model parameter *Caff* to 5 mM between $t = 10$ s and $t = 40$ s. The parameter values used in our calculations are: $\rho_{SR} = 0.01$; $\rho_m = 0.01$; $\beta_{SR} = 0.0025$; $\beta_m = 0.0025$; $Ca_{tot} = 50\ \mu M$; $SPr_{tot} = 90\ \mu M$; $BPr_{tot} = 120\ \mu M$; $Mg_i = 500\ \mu M$; $k_{RyR} = 2000\ s^{-1}$; $k_{SERCA} = 1\ \mu M\ s^{-1}$; $k_{leak} = 0.02\ s^{-1}$; $k_{in} = 20\ \mu M\ s^{-1}$; $k_{out} = 0.1\ s^{-1}$; $k_{on} = 0.1\ \mu M\ s^{-1}$; $k_{off} = 0.01\ s^{-1}$; $K_{Caff} = 250\ \mu M$; $K_{A,Ca} = 2.5\ \mu M$; $K_{A,Mg} = 75\ \mu M$; $K_{I,Ca} = 400\ \mu M$; $K_{I,Mg} = 300\ \mu M$; $K_{SERCA} = 0.1\ \mu M$; $K_m = 1\ \mu M$; $K_{SPr} = 5\ \mu M$.

3.2. Stochastic model

Traditionally, reaction systems can be stochastically described as a birth–death process governed by the master equation. Because there is no practical procedure to solve this equation, many simulation algorithms have been developed and one of the widely used is the Monte Carlo simulation proposed by Gillespie [44,45]. Originally, the method has been intended for stochastic solving of chemical reaction schemes. However, as proposed by Gracheva et al. [30], a stochastic simulation can be performed on the basis of differential model equations, so that the decomposition of a known deterministic mechanism into detailed reaction steps is unnecessary. In this case, reaction rates are ascribed to fluxes, which constitute the partial differential equation.

All reaction rates are specified in Table 1, along with the corresponding stochastic processes. At each iteration step one of the events with a probability proportional to the transition rate a_j is randomly chosen. Accordingly, the j-th event is selected according to the following criteria:

$$\sum_{k=1}^{j-1}\frac{a_k}{a_0} < r_1 \le \sum_{k=1}^{j}\frac{a_k}{a_0}, \tag{14}$$

where $a_0 = \sum_{k=1}^{M} a_k$ is the sum of the $M = 12$ a_j values and r_1 is a uniformly distributed random number from the unit-interval. Along with that, the stochastic time increment is calculated:

$$\Delta t = \frac{1}{a_0}\ln\left(\frac{1}{r_2}\right), \tag{15}$$

where r_2 is a random number statistically equivalent to r_1. So, after each iteration a discrete change of concentration proportional to $\propto 1/V$ is performed in accordance with the chosen event j, as described in Table 1. The discrete change in the Ca^{2+} concentration refers to the change in the number of calcium ions. Importantly, V is the volume of the cytosolic space, which directly determinates the intensity of internal noise, since stochasticity is most noticeable for small system sizes and vanishes in the thermodynamic limit ($V \to \infty$).

In addition, we further evolve our stochastic model by taking into account clustering of RyR receptors on the sarcoplasmic reticulum.

Table 1
Reaction rates and stochastic processes. Reaction rates a_j and the corresponding stochastic processes entailed in the stochastic model. The meaning of symbols is as follows: N_A is the Avogadro's number, ρ_{SR} and ρ_m are the volume ratios between the SR and the cytosol and between the mitochondria and the cytosol, respectively, and $\beta_{cyt}\ (Ca_i) = (1 + K_{SPr}SPr_{tot}/[K_{SPr} + Ca_i]^2)^{-1}$. Note that $n_C = 1$ if the clustering of the ryanodine receptors is not considered.

Transition rate	Transition process
$a_1 = \frac{N_A V}{n_C}\beta_{cyt}(Ca_i)J_{RyR}$	$Ca_i \to Ca_i + n_C/N_A V$
$a_2 = N_A V\beta_{cyt}(Ca_i)J_{leak}$	$Ca_i \to Ca_i + 1/N_A V$
$a_3 = N_A V\beta_{cyt}(Ca_i)J_{SERCA}$	$Ca_i \to Ca_i - 1/N_A V$
$a_4 = N_A V\beta_{cyt}(Ca_i)J_{out}$	$Ca_i \to Ca_i + 1/N_A V$
$a_5 = N_A V\beta_{cyt}(Ca_i)J_{in}$	$Ca_i \to Ca_i - 1/N_A V$
$a_6 = N_A V\beta_{cyt}(Ca_i)k_{off}CaBPr$	$Ca_i \to Ca_i + 1/N_A V$
$a_7 = N_A V\beta_{cyt}(Ca_i)k_{on}Ca_iBPr$	$Ca_i \to Ca_i - 1/N_A V$
$a_8 = \frac{N_A V}{n_C}\beta_{SR}J_{RyR}$	$Ca_{SR} \to Ca_{SR} - n_C/\rho_{SR}N_A V$
$a_9 = N_A V\beta_{SR}J_{leak}$	$Ca_{SR} \to Ca_{SR} - 1/\rho_{SR}N_A V$
$a_{10} = N_A V\beta_{SR}J_{SERCA}$	$Ca_{SR} \to Ca_{SR} + 1/\rho_{SR}N_A V$
$a_{11} = N_A V\beta_m J_{out}$	$Ca_m \to Ca_m - 1/\rho_m N_A V$
$a_{12} = N_A V\beta_m J_{in}$	$Ca_m \to Ca_m + 1/\rho_m N_A V$

Previous studies about functioning of InsP3 or RyR receptors in different cell types have revealed, that those receptors are clustered into functional Ca^{2+} release units [39,40,46]. Similar as performed by Dupont et al. [27,42], a cluster with n_C receptors is modeled as 1 large channel with a n_C times larger conductance as an isolated receptor, whereby it is assumed that the dynamics of RyR receptors inside one cluster is completely synchronized, since the Ca^{2+} concentration around the cluster is supposed to be the same. For that reason, all receptors in a cluster will open simultaneously. Accordingly, the discrete change of the Ca^{2+} concentration that goes along with this event will be n_C times larger, as it is specified in Table 1 and thus the Ca^{2+} release through RyR channels is subjected to a greater extent of intrinsic fluctuations as n_C is increased.

3.3. Reverse sensitivity analysis and modeling of cell-to-cell variability

Sensitivity analysis is generally used to determinate the sensitivity of a mathematical model to changes in model parameters. Information gathered by studying how the model behavior responds to variations of parameters is a useful tool in model building as well as in model evaluation. Usually, the changes in the output variables provoked by parameter shift are quantified via response coefficients R^A_{Pi}, which are defined by the following equation (e.g. [8]):

$$R^A_{p_i} = \frac{\partial A / A}{\partial p_i / p_i} = \frac{p_i}{A}\frac{\partial A}{\partial p_i}, \tag{16}$$

where p_i is a given parameter and A is a dependant variable, like for example in our case peak or plateau value. Furthermore, in some cases a simplified version of the sensitivity analysis is used for the evaluation of the robustness of the system. Here, parameters are varied from 10% of the value of each parameter and the corresponding changes in the predicted output values are then quantified.

However, in our study, we consider an inverted situation. In particular, on the basis of a known distribution of the output variables, i.e. peak and plateau values, which are determined in experimental recordings, we wish to find out, what are the required variations of individual parameters that provoke the dispersion in the same extent. For this purpose, we make use of a different procedure, which we call a single reverse sensitivity analysis. In particular, we employ the deterministic model for our calculations, whereby the parameter being varied is considered to be randomly distributed according to the Gaussian distribution, whereby a cut-off at two times its standard deviation is employed to avoid using unreasonably small or large values. As a consequence, the predicted values for the output variables, peak and plateau Ca^{2+} concentrations, vary. For each parameter i, we determine the SD_i of the Gaussian distribution of this parameter that induces the distribution of the predicted peak and plateau values which SD is equal to the experimental SD. So, values of the individual parameters p_i are calculated as follows:

$$p_i = p_i\left(1 + \%SD_{i,\mathrm{peak}}\xi_i\right), \tag{17}$$

$$p_i = p_i\left(1 + \%SD_{i,\mathrm{plateau}}\xi_i\right), \tag{18}$$

where ξ_i is a random Gaussian number (cut-off at two times its standard deviation as previously explained) with zero mean and unit variance and $\%SD_{i,\mathrm{peak}}$ and $\%SD_{i,\mathrm{plateau}}$ represent the SD reverse sensitivity coefficients, which signify the required SD of individual parameters that provoke a distribution of the predicted peak and plateau values with a SD that is equal to the SD obtained in experiment, expressed as the percent of the mean value.

In the second procedure, which we call an overall reverse sensitivity analysis, we vary all parameters simultaneously. In this case the cell-to-cell variability is modeled by distributing the model parameters according to the Gaussian distribution:

$$p_i = p_i(1 + X\xi_i), \tag{19}$$

where X signifies the SD of the Gaussian distribution of all parameters and ξ_i is a random Gaussian number with zero mean and unit variance. Obviously, as X is increased, the dispersion of the predicted values for the Ca^{2+} output variables will increase too. In this manner we are able to examine the average SD off all parameters, which corresponds to the same dispersion of the output variables, as observed in experiment.

4. Experimental results

4.1. Experiments

Fig. 1A shows the effect of caffeine stimulation (5 mM; 30 s) on the free cytosolic calcium concentration. Calcium responses to caffeine are non-oscillatory. The initial peak is followed by a plateau level. Descriptive statistical analysis of the dispersion of the experimental data reveals that the spike amplitudes as well as the plateau levels largely differ between cells. Distribution of experimentally obtained values for the peak and plateau are presented in Fig. 1B and C. Mean values and standard deviation of the peak values for 30 cells was 0.69 μM and 0.28 μM, respectively, which corresponds to 41% of SD normalized to mean value ($\%SD_{\mathrm{peak}} = 41\%$). For the plateau, mean and SD values were 0.047 μM and 0.20 μM ($\%SD_{\mathrm{plateau}} = 43\%$), respectively. Tests of normality indicate that the distribution of both peak and plateau values can be considered as Gaussian.

4.2. Bibliographical data

In order to make our conclusions more general, we compare the dispersion of experimental data in this paper, being around 40%, with other results selected from the literature. The aim of this selection was not to be exhaustive, but to present values obtained by different research groups, with different techniques of $[Ca^{2+}]_i$ estimation, in response to different agonists, in different cell types from different taxons. Table with gathered data is available in the supplementary material. In particular, Table S1 compiles the mean values, standard error of the mean (SE) and the SD of different parameters of the Ca^{2+} homeodynamics at rest and upon stimulation measured on single cells in a variety of cell types and organisms, both animal and vegetal: baseline $[Ca^{2+}]_i$, amplitude of $[Ca^{2+}]_i$ rise upon stimulation (e. g. peak and plateau), and oscillation frequency or duration. In the compiled data, %SD ranges from 15% to 183%, the median being 48%. This variability is observed whatever the agonist, in different cell types, either muscle or non-muscle cells, obtained from distinct organs and organisms, including cells from animals and plants. The variability is observed with distinct fluorescent dyes, since several ones, e. g., indo-1, fura 2, have been used in the listed studies and, moreover, it was also seen when the Ca^{2+} signal was not analyzed by a fluorescent dye, but via the electrophysiological measurement of the Ca^{2+}-induced Cl^- current. It is observed both in isolated cells and cells in situ (on lung slides).

5. Computational results

5.1. Role of stochastic effects

To investigate the influences of stochastic effects on the dynamics of calcium responses to caffeine, we use a stochastic version of the model originally proposed as a deterministic model [43]. The stochastic simulation is implemented according to Gillespie [44,45] (see Materials and methods).

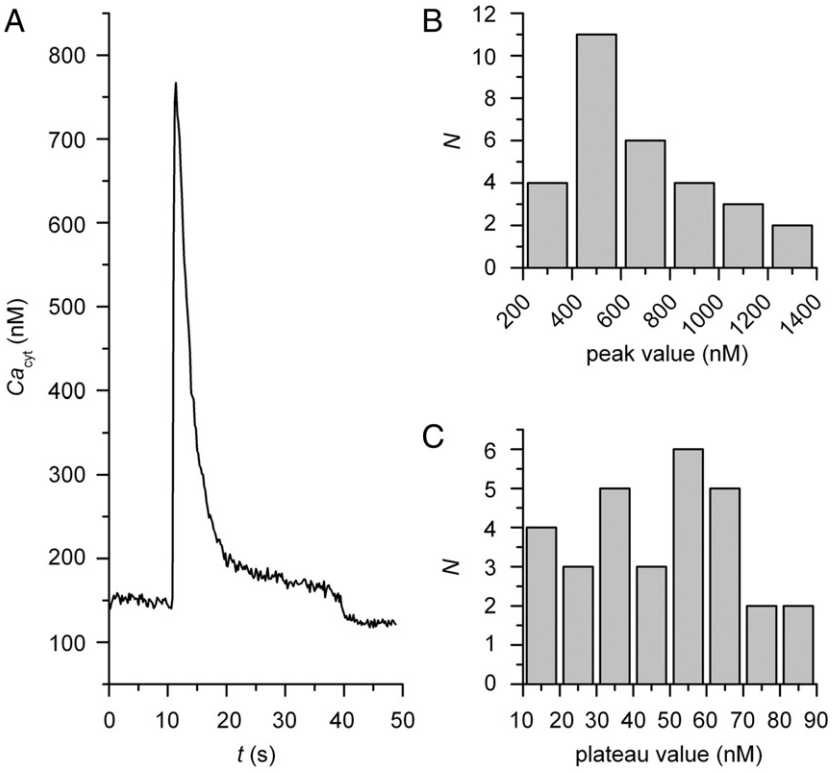

Fig. 1. Experimental measurements of Ca^{2+} responses to caffeine. (A) Typical traces of caffeine-induced Ca^{2+}-responses in isolated airway smooth muscle cells. (B) Histogram of experimental measured peak values for 30 cells; mean peak value 0.69 μM, SD = 0.28 μM. (C) Histogram of experimental measured plateau values for 30 cells; mean plateau value 0.047 μM, SD = 0.020 μM.

First, we have to evaluate the volume of the cytosolic space which appears in the stochastic model and is directly related to the intensity of the internal noise. As they were used in our experiments, isolated myocytes exhibit an ellipsoid shape around 30–40 μm long and 8–10 μm wide, which hence corresponds to an estimated cell volume of around 1000–2000 μm^3. Considering one third being the cytosolic volume, hence in our calculations we take $V = 500\ \mu m^3$. Fig. 2A shows a typical predicted trace of the stochastic solution. Clearly, the solution looks practically deterministic with only minute markers of stochasticity. To determine if the non-deterministic effects due to finite system sizes can explain the experimentally observed distribution of the peak and plateau levels as presented in Fig. 1B and C, we systematically scrutinize the influence of the system size variation on the dispersion of the values predicted by the stochastic model. Fig. 2B shows the standard deviation of peak values obtained for different cellular volumes ranging from 0.1 to 10,000 μm^3. The model predictions would approach the experimentally observed standard deviation of the peak value only for volumes less than 1 μm^3 (see the dashed line in Fig. 2B), much smaller than the genuine volume of the cytosolic space.

We proceed by analyzing the role of clustering of ryanodine receptors. Chen-Izu et al. [46] have studied the distribution of ryanodine receptor clusters in cardiac myocytes. They suggested that the number of receptors in one cluster is ~100. We suppose that a comparable distribution can be considered for airway myocytes, and show in Fig. 2C a typical predicted trace of the stochastic solution for $V = 500\ \mu m^3$ and $n_C = 100$. We can observe that in this case stochastic effects are clearly more expressive. In order to determine the influence of channel clustering more quantitatively, we show in Fig. 2D the standard deviation of the peak values obtained for different numbers of receptors in one cluster. It can be inferred that for physiologically relevant values of n_C the distribution of peak values can not exceed 10%.

At this point it should be emphasized, that Ca^{2+} signalization is in general a spatio-temporal process. Several recent studies [27,28,32,38] indicate that Ca^{2+} liberation occurs at discrete functional release sites in the ER membrane, where small localized Ca^{2+} release events are taking place. A coordination of these events via Ca^{2+} diffusion can lead to global Ca^{2+} oscillations. In view of that, a global Ca^{2+} signal can indeed be triggered by biochemical reactions, in which very few molecules are involved, much less than the number of molecules in the system [28,38]. Accordingly, the extent of stochasticity in the output signal can be quite substantial, which is especially perceivable at lower, particularly subthreshold stimulation levels [27,28]. Therefore, by neglecting the spatial dimension of the cell as well as the diffusional interactions between individual channels, one can indeed underestimate the level of fluctuations in the system. However, our experimental conditions consist of overall cell stimulation via caffeine. As shown in our previous paper (Ref. [43]) using the quenching properties of caffeine, this non-physiological agonist reaches the maximal intracellular concentration in less than 1 s so that the RyR receptors are activated quickly after the application of caffeine. Therefore, diffusional coupling between RyR channel clusters is bypassed and thus does not give rise to the level of fluctuations in our system.

Hence, it appears that the stochastic modeling cannot explain the distribution of the peak and plateau values, and that the gist for experimentally observed variations lies somewhere else. Therefore, in the following, we use the deterministic model with variable model parameters to simulate cell-to-cell variability.

5.2. Role of cell-to-cell variability

We examined the impact of cell-to-cell variability on calcium dynamics in airway smooth muscle cells using two procedures, (i) single reverse sensitivity analysis, in which we vary only a single

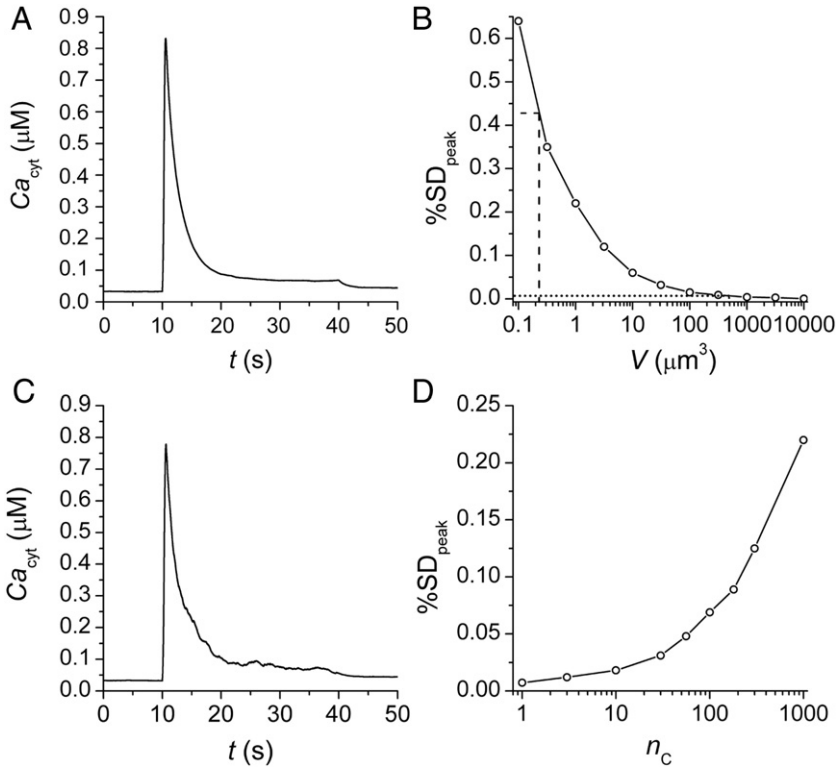

Fig. 2. Stochastic simulations of Ca^{2+} responses. (A) A typical trace of cytosolic calcium concentration obtained by the stochastic simulation for $V = 500\ \mu m^3$. (B) Standard deviation of peak values $\%SD_{peak}$ in dependence on the cellular volume used for the Gillespie's stochastic integration procedure. Dashed line denotes the experimentally obtained reference value of standard deviation, whereas the dotted line denotes the theoretically predicted standard deviation obtained if using genuine. (C) A typical trace of cytosolic calcium concentration obtained by the stochastic simulation, whereby channel clustering is considered — $n_C = 100$ and $V = 500\ \mu m^3$. (D) $\%SD_{peak}$ in dependence on the number of ryanodine channels in one cluster.

(one-by-one) parameter at the same time, and (ii) overall reverse sensitivity analysis, allowing simultaneous variations of all parameters (see Materials and methods).

Add (i): to carry out the single reverse sensitivity analysis, a Gaussian distribution of the tested parameter was considered, and we determined the SD inverse sensitivity coefficients $\%SD_{i,peak}$ and $\%SD_{i,plateau}$, which denote the SD of the parameter values required to obtain an SD of predicted peak and plateau values similar to the experimentally observed one [see Eqs. (17) and (18)], i.e. 41% and 43% for the peak and the plateau, respectively (see Experimental results).

Results showing values of $\%SD_{i,peak}$ (grey bars) and $\%SD_{i,plateau}$ (black bars) are presented in Fig. 3. Apparently, only six model parameters have a large enough impact, which enables them to provoke a dispersion of peak and plateau values, in the same extent as observed in the experiment. According to these results, the system seems to be highly robust with very few parameters significantly influencing the system and no hypersensitive parameters. The most sensitive parameters have values around 45% and 25% for peak and plateau, respectively. The plateau appears to be more sensitive than the peak to parameter variation. The parameters to which the plateau is especially sensitive are the total Ca^{2+} concentration (Ca_{tot}), the total concentration of buffering proteins (BPr_{tot}) (slow Ca^{2+}-binding proteins), and the on and off rate constants of Ca^{2+} binding to these proteins (k_{on}, k_{off}). The peak is less sensitive to parameter variations. As for the plateau, it is remarkably sensitive to Ca_{tot} and BPr_{tot}, but also to the concentration of signaling protein (SPr_{tot}) (fast Ca^{2+}-binding protein) and its dissociation constant (K_{SPr}). Furthermore, in contrast to the peak, the plateau value is not considerably sensitive to SPr_{tot} and K_{SPr} (red arrows in Fig. 3 indicate, that even a 75% variation of those parameters can not cause a 43% SD of the plateau values), whereas the peak is not significantly sensitive to variations in k_{on} and k_{off} (red arrows in Fig. 3).

Add (ii): the principle of the overall reverse sensitivity analysis is to determine what should be the range of distribution of all parameters that would induce a variation in the output variables corresponding to the observed cell-to-cell variability. According to Eq. (19), all model parameters are simultaneously varied in ranges which are determined by X. Results are presented in Fig. 4A. The

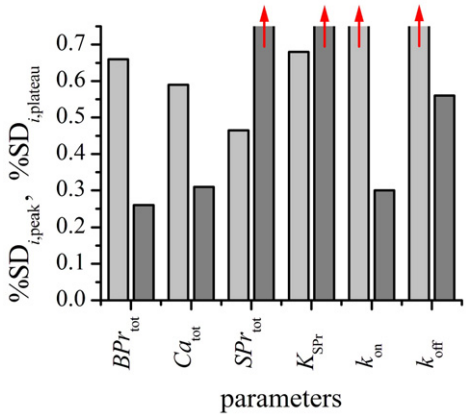

Fig. 3. The determination of the required variations of individual model parameters that cause the same dispersion Ca^{2+} responses as observed in experimental recordings. Values of $\%SD_{i,peak}$ (light grey columns) and $\%SD_{i,plateau}$ (grey columns). Red arrows indicate that the required SD_i of those parameters would exceed 75%.

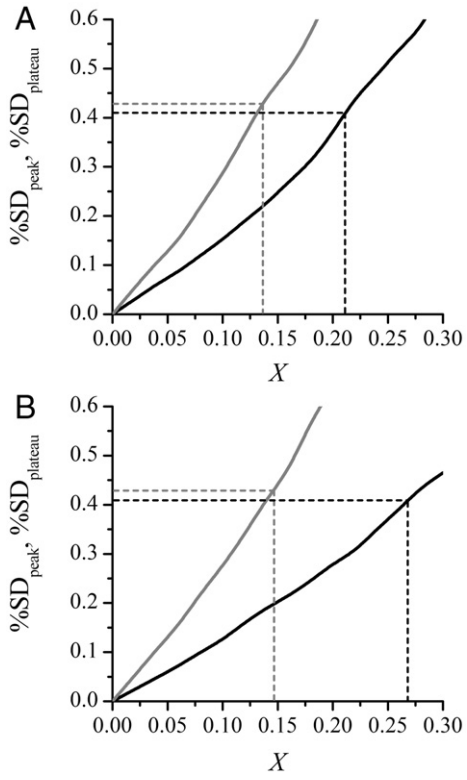

Fig. 4. Modeling of the cell-to-cell variability. (A) $\%SD_{peak}$ (black line) and $\%SD_{plateau}$ (grey line) in dependence on the degree of variation X of all parameters. (B) The same relation, whereby only the crucial 6 parameters determined in Fig. 3 were varied. In both panels, dotted lines denote experimentally observed standard deviations.

dotted lines indicate that for $X \approx 0.21$ the theoretical predictions best match the experimentally observed standard deviations of peak values ($\%SD_{peak} = 41\%$), whereas for $X \approx 0.13$ the results are in agreement with the SD of plateau values ($\%SD_{plateau} = 43\%$). Compared with the single reverse sensitivity analysis, one can observe, although somehow expected, that quite smaller simultaneous variations of model parameters lead to as considerable variations in the output variables as observed in experimental recordings.

In order to realize the relative importance of the crucial parameters, which were identified in Fig. 3, we additionally perform the overall sensitivity analysis, whereby only the crucial six parameters instead of all the parameters are simultaneously varied, as given by Eq. (19). Results, which are presented in Fig. 4B clearly indicate, that in this case not much larger variations of those parameters are required to achieve the same dispersion of the output variables. Especially for the plateau, this difference is almost negligible ($X \approx 0.15$), whereas for the peak the difference is somewhat larger ($X \approx 0.27$), but still not essential. According to these results we can conclude, that only a smaller subset of parameters is considerably involved into the formation of the calcium signal patterns. Furthermore, all those crucial parameters represent some intrinsic characteristics of the whole system and are not restricted to properties of its components and they also do not depend on the type of stimulation.

6. Discussion

Experimental study of caffeine-induced calcium responses in isolated airway myocytes, which are characterized by a transient peak followed by a progressive decay to a plateau phase, have shown rather large disperses in peak and plateau values. This may be surprising, if we consider the Ca^{2+} response as an encoding signal. The meaning and consequence of the high variation of encoding signal on decoding processes and final physiological response is beyond the scope of this study. But it should be first noticed that such variability is not specific to this study. SD values in the same range have been observed in our previous studies on the same cell type, either with caffeine [43,47], ACh [48,49], extracellular ATP [50] or high external KCl [51]. Comparisons of dispersion of experimental data in this paper with the compiled data selected from the literature makes this observation more general. Though we cannot exclude in absolute that it is an artifact due to experimental manipulations, this variability is not created by the use of a specific fluorescent dye. It is neither due to the process of cell isolation. Finally, this variability is observed whatever the agonist, in different cell types, either muscle or non-muscle cells, obtained from distinct organs and organisms, including cells from animals and plants. Hence, the interindividual variability in the single cell Ca^{2+} signaling analyzed in our study is not specific to its experimental conditions and reflects the interindividual variation generally observed. It appears then that the variability observed and analyzed in the present study is only one example of a general property of cells.

In order to provide a theoretical explanation for these observations, we have examined a mathematical model for calcium dynamics upon airway smooth muscle cell stimulation by caffeine. We have taken into account the intrinsic fluctuations, which are an unavoidable feature of all chemically reacting systems with relatively small number of reacting molecules, by simulating the system with a stochastic algorithm. For a volume corresponding to the estimated cytosolic volume, we conclude that the stochastic effects are not significantly enough. For smaller volumes, however, the stochastic effects make the output signal much closer, also visually closer, to those observed in the experiments. The Monte Carlo procedure used in our study is based on the principle that the volume considered is homogenous, which is not actually the case for a real cell. However, even though we do not take into account the spatial dimension of the cell, and with that the non-homogenous distribution of its compartments, which can indeed give rise to the extent of stochasticity, we do not substantially underestimate the level of intrinsic fluctuations. Namely, as indicated in recent studies [28,38], the main contribution to the stochasticity arises from diffusional coupling of a few channels, where small localized Ca^{2+} release events are occurring, which can lead to a global Ca^{2+} signal. Since in our experimental conditions whole-cell stimulation with quenching agonist concentrations is performed, diffusional coupling between channel clusters is by-passed. Nevertheless, we partially address this issue, by taking into account ryanodine channel clustering, similar as performed by Dupont et al. [27,42], whereat the cell is considered as a homogenous object. Although the level of stochasticity in this case is indeed increased, the stochastic effects are not strong enough to cause differences in peak and plateau value in such an extent as this is observed in the experiments. Stochastic events produce dispersion in values similar to the observed one only for very small, i.e., unrealistic volumes or unrealistic clustering of receptors. However, in both cases the predicted trace exhibits calcium variations upon time much greater than the observed one on entire cells. This does not mean that cell-to-cell variability is not due to random processes, but, if so, they are not directly reducible to intrinsic noise within the system, and they are not correctly modeled by a stochastic model. Our findings thus suggest that even if the deterministic limit is more or less reached in a particular biological system, cell variability still remains and is therefore very important to consider.

Since stochastic modeling could not account for the dispersion of the observed calcium signal, we used the deterministic model and introduce variation in parameter values to analyze its impact on cell-to-cell variability. The fact that the change in parameter value alters the output value is the base of sensitivity analysis. Its classical procedure, consisting in varying in a given range of each parameter

value, is usually difficult to link to any experimental measurement. The interest of the reverse sensitivity analysis proposed and applied in this paper is that it is based on the fact that cell-to-cell variability, experimentally quantified by SD, is used as an experimental and quantitative indicator for sensitivity analysis.

The procedure for reverse sensitivity analysis is based on the idea that the parameter value vary from cell-to-cell according to a distribution law. We have hypothesized that the distribution was Gaussian, but the principle remains the same if another distribution law is applied. The distribution-based single reverse sensitivity analysis allows to identify the core of sensitivity of the model and to give a quantitative estimate of what should be considered as realistically robust. Indeed, the model should be considered as hypersensitive if, applying reverse sensitivity analysis, the required dispersion of some critical parameter is smaller than what should be considered as realistic. Additionally, if experimental values were available for the parameters (which was not the case in our study), their actual distribution can be used in the reverse sensitivity analysis, and the predicted dispersion compared to the experimental one.

Single reverse sensitivity analysis, is based on one-by-one change in parameter value. This is a relevant approach if each parameter can be actually modulated independently, as it is the case in metabolic control analysis, but not so relevant in the modeling of signaling processes, even if it still provides some information about the importance of particular parameters. The more relevant approach should therefore consider the overall parameter variation, which was in our study realized by randomly distributing all of the parameters. Compared to the single one, overall reverse sensitivity analysis shows that the system is more sensitive to parameter variations if these parameters can vary simultaneously. Furthermore, by performing a parallel calculation, where only the crucial parameters were varied instead of the entire set, we reveal that the key parameters contribute mostly to the observed cell-to-cell variability. Thus, rather small, but simultaneous variations of the crucial parameters can cause considerable variations in the output variables, whereas the majority of parameters is not so essential in this point of view. We are aware that in our calculations only an estimation for the realistic parameter dispersion is acquired. But even if detailed information about the dispersion of individual parameters can in this manner not be achieved, it surely provides useful insights into cell robustness.

According to our study, the calcium signaling system of a single cell appears to be quite robust; the pattern of the calcium signal seems to be controlled by few parameters, all of them involved in general Ca^{2+} homeostasis, and not stimulation-specific: the total Ca^{2+} amount, concentration and binding properties of the Ca^{2+}-buffering proteins, to the concentration of signaling proteins and to its dissociation constant. Incidentally, most of them, like concentrations, are intrinsic characteristics of the system itself, not reducible to properties of its components. This might be of important physiological relevance providing a highly effective, reliable, and costly reasonable control of cellular signaling by spending the energy for fine tuning only a reasonable small amount of parameters while the system is robust to changes in the other parameters. It can be hypothesized that this highly effective control of the system by having a small subset of model parameters with small variation is probably evolutionarily developed by reaching an optimum in the number of such parameters. It is obvious that a biological system controlling all the parameters at the same time would be complicated, difficult and costly inefficient, whereas on the other hand, tuning only one parameter would be too risky and also difficult to carry out.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bpc.2010.02.006.

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