

Prevalence of stochasticity in experimentally observed responses of pancreatic acinar cells to acetylcholine

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Calcium ions play an important role in both intra- and intercellular signaling. In pancreatic acinar cells intracellular Ca^{2+} regulates exocytotic secretion and fluid secretion. In this paper we study the typical experimental traces of Ca^{2+} responses in pancreatic acinar cells obtained in response to the physiological agonist acetylcholine. To determine whether they are stochastic or deterministic in nature, we analyze the traces with methods of nonlinear time series analysis. In particular, by performing surrogate data tests and employing a determinism test for short time series, we show that the responses of pancreatic acinar cells to acetylcholine are stochastic with only faintly expressed deterministic features. Presented results thus corroborate the notion that mathematical models should take stochasticity explicitly into account when describing intra- and intercellular processes, and that indeed further efforts should be directed toward this subject. © 2009 American Institute of Physics. [DOI: 10.1063/1.3160017]

Calcium ions (Ca^{2+}) play an important and versatile role as second messengers in living cells. Ca^{2+} signaling controls several cellular functions in different cell types and is thus of key importance for normal functioning of living organisms. Activation of the cell via external stimuli triggers a series of biochemical reactions, which often leads to periodic elevations of cytosolic Ca^{2+} concentration. In this manner, information is transmitted to target proteins. A thorough understanding of the rather complex molecular and cellular mechanisms that govern Ca^{2+} oscillations requires mathematical modeling. During the past years several mathematical models have been developed, which are, especially the early ones, mainly deterministic. However, those models are approximate, treating molecules expressed in concentrations as continuous variables, neglecting fluctuations associated with the finite number of molecules composing the reactions as well as the fact that concentrations of molecular species can only vary by a discrete amount. Indeed, biochemical reactions occur as rapid successions of individual elementary events, whose exact timing is effectively random. Furthermore, recent experimental and theoretical investigations on cellular signaling indicate that stochasticity plays a vital role by the coordination of cellular processes. As a result, stochastic modeling is gaining more and more attention. Despite the fact that several discussions about the importance of stochastic versus deterministic approaches in the modeling of biochemical processes have been put forward in the past few years, the question about the real nature of Ca^{2+} signaling seems not yet to be completely resolved. Only a few experimental observations were devoted to the analysis of stochastic versus

deterministic origin of Ca^{2+} oscillations and all of them indicated a great extend of stochasticity in intracellular Ca^{2+} dynamics. In this paper we examine whether the experimentally measured Ca^{2+} oscillations in pancreatic acinar cells within the intact tissue are stochastic or deterministic in nature. For this purpose we utilize methods of nonlinear time series analysis. This is an important consideration also because irregularly appearing traces are often advertised as chaotic, while in fact their origin has little or nothing to do with deterministic nonlinearities responsible for the onset of deterministic chaos. Our results indicate a prevalence of stochasticity in intracellular Ca^{2+} dynamics in pancreatic acinar cells.

I. INTRODUCTION

In many excitable and nonexcitable eukaryotic cell types, including pancreatic acinar cells, Ca^{2+} has been recognized as an important second messenger in intracellular signaling.^{1,2} In response to extracellular agonists, such as certain hormones and neurotransmitters (e.g., acetylcholine), the intracellular Ca^{2+} concentration usually increases. The response to agonists is frequently characterized by repeated elevations of this concentration, which is known as Ca^{2+} oscillations.²⁻⁴ These oscillations are maintained, controlled, and shaped by a complex interplay of Ca^{2+} fluxes between the cytosol, intracellular Ca^{2+} stores, Ca^{2+} -binding proteins, and the external environment, often limited to just one pole of the cells under physiological conditions.⁵ Localized Ca^{2+} oscillations in pancreatic acinar cells occur via both the inositoltriphosphate (IP_3) and the ryanodine pathway.

Many theoretical studies have been conducted in order to explain the phenomenon of Ca^{2+} oscillations (for review see Refs. 6 and 7). The mechanisms of Ca^{2+} oscillations have

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been mainly modeled as deterministic processes.⁶ However, stochastic effects in biological cells often cannot and should not be neglected. In absolute terms, the number of membrane receptors, ion channels, and calcium ions in some organelles is very low. Thus, the stochastic effects can play an important role⁷ and the modeling of particular aspects of Ca^{2+} signaling in cells definitely requires stochastic treatment. Due to these facts, a range of stochastic models has been developed for the modeling of single Ca^{2+} channels,⁷⁻⁹ intracellular Ca^{2+} oscillations,¹⁰⁻¹³ coupled oscillators,^{11,14-17} and intercellular Ca^{2+} wave propagation.¹⁸⁻²⁰ In order to demonstrate the importance of stochastic versus deterministic modeling, some direct comparisons of stochastic and deterministic models have been performed as well.^{12,21-23}

Theoretical predictions about the role of stochasticity at the cellular level can give important hints for experimental investigations. There is still a lack of direct experimental evidence confirming either the stochastic or the deterministic nature of intracellular Ca^{2+} signals. However, only recently Dupont *et al.*²⁴ scrutinized the influence of stochasticity on Ca^{2+} oscillations in hepatocytes. Via a combined experimental and computational approach they revealed that the Ca^{2+} dynamics is strongly affected by inherent fluctuations. Another interesting contribution has been reported by Skupin *et al.*,²⁵ who succeeded in showing that intracellular Ca^{2+} signaling is clearly subjected to stochastic dynamics, thus implying that Ca^{2+} oscillations are a sequence of random spikes, which, however, can in some circumstances appear rather regular, indeed nearly deterministiclike. Their study is based on a thorough statistical analysis of measured time series in human embryonic kidney cells, in processed lipospirite cells, and in two types of glial cells (astrocytes and microglia). In our previous study we show that experimental Ca^{2+} traces in hepatocytes show an extremely high degree of stochasticity at the cellular level.²⁶ In the present paper we analyze the stochastic versus the deterministic nature of experimentally measured Ca^{2+} oscillations in pancreatic acinar cells. In contrast to previous studies, Ca^{2+} responses in isolated cells were measured within the intact tissue. The analysis is based on methods of nonlinear time series analysis.²⁷

It should be emphasized that the methods of nonlinear time series analysis have been successfully applied to experimentally obtained biomedical signals at the level of whole organs^{28,29} with prominent examples including the characterization of the dynamics of cardiac tissue,³⁰ networks of neural cells,³¹ or the human locomotion apparatus.^{32,33} The results of these analyses found several medical applications, e.g., a noninvasively detection of “silent” heart arrhythmias or imminent heart failure or the extraction of the fetal electrocardiogram from maternal recordings.³⁴ Furthermore, the analyses of electroencephalographic recordings can be used to diagnose epilepsy,³⁵⁻³⁷ whereas recordings obtained from the human locomotion apparatus can be used to determine neurodegenerative diseases like Parkinson’s disease, Huntington’s disease, or amyotrophic lateral sclerosis.³⁸⁻⁴⁰ Although these studies analyzing experimental traces obtained at the organ level have proved that nonlinear time series analysis methods have vast potential and applicability in

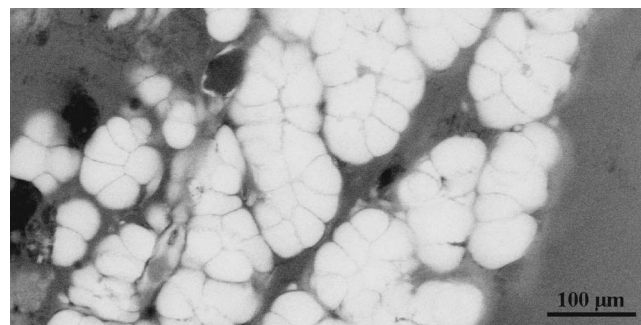


FIG. 1. Two-photon excitation image of the acinar cell structure within living whole pancreas slice. The intercellular spaces are visualized with the extracellular solution containing a fluid-phase tracer, sulphorhodamine B. The image intensity has been inverted. A detailed description of the image preparation can be found in Ref. 43.

various fields of medicine and biology, there is still a lack of studies analyzing the experimental traces at the tissue and cellular level. Moreover, irregular behavior may often be mistakenly advertised as chaos, where in fact external or inherent unpredictable disturbances render the behavior stochastic rather than deterministically chaotic. In order to properly address these issues and to make a further step toward understanding the functioning of biological organisms at the cellular level, we use methods of nonlinear time series analysis to determine the stochastic/deterministic nature of intracellular Ca^{2+} responses in single pancreatic acinar cells, still embedded in their normal cellular context.⁴¹

First, we present the experimental methods and measurements of cytosolic Ca^{2+} concentration in pancreatic acinar cells stimulated with acetylcholine. These experimentally obtained traces are then analyzed with methods of nonlinear time series analysis, and their stochastic/deterministic nature is determined. We show that the responses of pancreatic acinar cells to acetylcholine are mainly stochastic with only minute markers of determinism. These results are discussed and compared to previous model predictions and studies of oscillatory experimental traces obtained at the organ level.

II. EXPERIMENTAL METHODS

To measure the concentration of intracellular Ca^{2+} ($[\text{Ca}^{2+}]$) in pancreatic acinar cells within the intact tissue whole pancreas slices were prepared as described previously.⁴¹ A typical image of the acinar cell structure within living whole pancreas slice is shown in Fig. 1. To observe $[\text{Ca}^{2+}]$ changes in a large number of acinar cells, slices were bulk loaded with 6 μM Fura-PE3 AM (stock, 4 mM in DMSO with 5% pluronic acid F-127; Molecular Probes). After loading for 60 min on an orbital shaker, the slices were incubated for at least 15 min in indicator-free ES at 32 °C to achieve a sufficient degree of de-esterification. Monochromatic light (Polychrome IV, TILL Photonics) at 380 nm was short pass filtered (at 410 nm), reflected by a dichroic mirror (centered at 400 nm), and directed through a 60 \times water immersion objective (NA=1) (NA denotes numerical aperture). The emitted fluorescence was transmitted by the dichroic mirror and further filtered through a 470 nm barrier filter. Images were obtained using a cooled emCCD

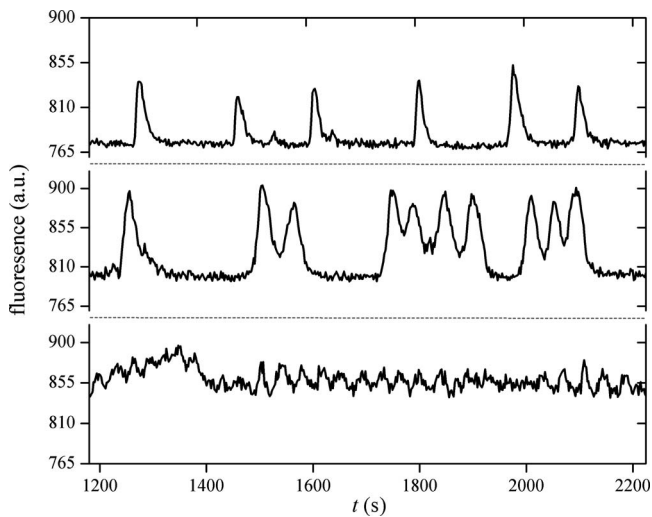


FIG. 2. Temporal traces of cellular responses evoked by $c_{\text{ACh}}=25$ nM, $c_{\text{ACh}}=50$ nM, and $c_{\text{ACh}}=250$ nM from top to bottom, respectively (see also the main text for details). The sampling rate was 0.5 s^{-1} .

camera (Ixon, Andor Technology) and native ANDOR software. $[\text{Ca}^{2+}]$ was calculated from the background-subtracted intensity images obtained at 380 nm excitation using the equation derived as described in Ref. 42. Images were recorded at the sampling rate of 0.5 s^{-1} . All necessary calculations were performed using a custom written MATLAB script, and image acquisition and hardware triggering parameters were calculated and controlled by a custom ANDORBASIC (Andor Technology) program.

Traces of the *in vivo* measurements of intracellular Ca^{2+} in pancreatic acinar cells are presented for three different concentrations of acetylcholine in Fig. 2. The sampling rate is 0.5 s^{-1} , whereas the Ca^{2+} concentration is presented by the fluorescence in arbitrary units. For the following analyses we have eliminated potential end-to-end mismatch from the experimental traces. In addition, all traces have been rescaled to the unit interval for simplicity. These alterations do not affect the results.

III. MATHEMATICAL METHODS AND RESULTS

By applying methods of nonlinear time series analysis on the experimental traces, we aim to determine whether their origin is deterministic or stochastic. First, we define x_i as the time series to be examined, where x is the rescaled value of the fluorescence presented in Fig. 2 and i is the discrete time index defined as the actual time t divided by sampling rate. We start by employing surrogate data methods,⁴⁴ which enable us to test different null hypotheses related to the presumably stochastic nature of the Ca^{2+} recordings. The three null hypotheses that we will test are the following: (A) x_i are independent (temporally uncorrelated) random numbers drawn from some fixed but unknown distribution, (B) x_i originate from a stationary linear stochastic process with Gaussian noise, and finally, (C) x_i originate from a stationary Gaussian linear process that has been distorted by a monotonic, instantaneous, time-independent nonlinear function.⁴⁵ Depending on the outcome of the surrogate data test a particular null hypothesis can be rejected or con-

firmed. Notably, several surrogates from the original series x_i have to be generated to achieve a desired significance level α by each test. Our goal is to achieve a significance level of $\alpha=0.99$ (99%) when confirming or rejecting a null hypothesis, which means that $[1/(1-\alpha)]-1$ surrogates need to be generated for a single-sided test.

As the characteristic marker of nonlinearity is able to discern stochasticity from determinism in a time series, we use the zeroth-order prediction error γ .²⁷ Specifically, we will use the notation γ_0 to indicate the results obtained on the original (rather than surrogate) traces. If $\gamma_0 < \gamma$ for all $[1/(1-\alpha)]-1$ surrogates and for all forward prediction steps n then a null hypothesis can be rejected with a significance level α . On the other hand, if $\gamma_0 > \gamma$ at any instance of the test the null hypothesis is confirmed. For further details we refer the reader to page 44 of Ref. 27 as well as to Ref. 26, where an identical analysis has been performed.

The simplest null hypothesis is, as mentioned above, that the data are independent random numbers drawn from some fixed but unknown distribution [hypothesis (A)]. Surrogates for hypothesis (A) are generated simply by randomly shuffling the data without repetition. This procedure yields time traces (surrogates) with exactly the same distribution but independent construction. However, since it is clear solely from visual observations that the recordings presented in Fig. 2 are not independent random numbers, we do not show results for this particular test. Of course it holds that γ_0 is always smaller than γ , irrespective of n . Formally, we can thus reject the null hypothesis that the studied data sets are composed of independent random numbers.

A more interesting null hypothesis is that the recordings originate from a stationary linear stochastic process with Gaussian noise [hypothesis (B)]. Such a process is uniquely determined by the mean, the variance, and the autocorrelation function. Appropriate surrogates therefore consist of correlated data points with the same autocorrelation function as the original recording, which can be realized by randomizing the phases of the Fourier transform of the original recording, and then perform the inverse Fourier transform to obtain the desired temporal traces. The blue vertical columns in Fig. 3 show γ in dependence on n . Indeed, the trend of γ in dependence on n for the surrogates is quite closely related to the trend of γ_0 , and for some n it can be observed that $\gamma_0 \geq \gamma$, particularly for $c_{\text{ACh}}=50$ nM ($n \geq 3, 4$) and $c_{\text{ACh}}=250$ nM (all n). We can refine this surrogate test further by accounting for the amplitude distortion imposed by the randomization of phases of the Fourier transform by using the so-called amplitude adjusted surrogates.^{27,45} Results of this test are shown by red vertical columns in Fig. 3, and indeed, except for the $c_{\text{ACh}}=25$ nM case, it is impossible to reject the null hypothesis that the studied data sets originate from a stationary linear stochastic process with Gaussian inputs. Note that for $c_{\text{ACh}}=50$ nM and $c_{\text{ACh}}=250$ nM γ_0 fall within the distribution of γ for all n . From this we conclude that stochastic influences prevail in experimentally observed single-cell responses within intact tissue, which is especially obvious in case of higher agonist concentrations. Higher stochasticity levels by higher agonist concentrations can be attributed to higher baseline Ca^{2+} concentrations and a larger influence of

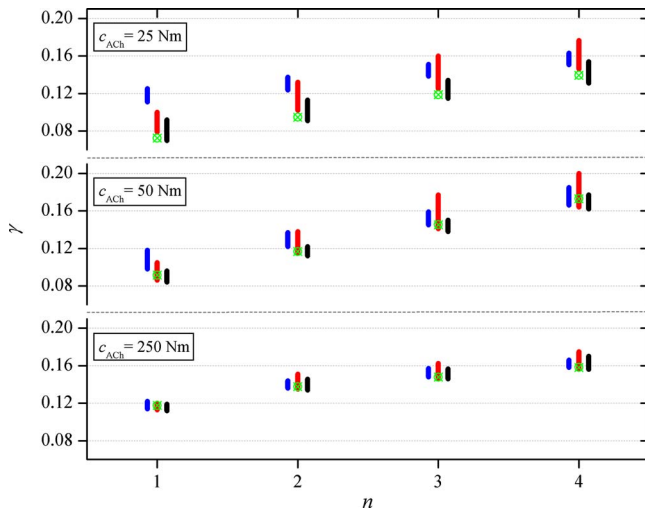


FIG. 3. (Color online) Surrogate data test for different null hypotheses. Colored vertical columns indicate the distribution of zeroth-order prediction errors γ for the three different surrogates and green circles denote prediction errors for the original recordings (γ_0) both in dependence on the number of prediction step n . More precisely, blue (gray when printed) and red (light gray when printed) vertical columns show the distribution of γ for the surrogates that originate from a stationary linear stochastic process with Gaussian noise. The first test [results presented by blue (gray when printed) vertical columns] is most permissive and accordingly the null hypothesis can be rejected for $c_{ACh}=25$ nM but no longer for all n if $c_{ACh}=50$ nM or $c_{ACh}=250$ nM. By applying a more rigorous surrogate generation routine for this null hypothesis [hypothesis (B)] [results presented by red (light gray when printed) vertical columns], the result indicated by blue (gray when printed) columns is further strengthened with the $c_{ACh}=25$ nM trace passing only marginally. Black vertical columns show the distribution of γ for the surrogates that originate from a stationary Gaussian linear process that has been distorted by a monotonic, instantaneous, time-independent nonlinear function. Clearly, all γ_0 are within the spans of γ , thus preventing the rejection of the null hypothesis [hypothesis (C)]. This leads to the conclusion that apart from an instantaneous nonlinear function acting on a noisy output, further markers of determinism in examined Ca^{2+} recordings are not present. All γ and γ_0 were calculated by embedding each time series into a three-dimensional phase space with delay $\tau=1$. Neighbors for prediction were sought among those points that were inside 10% of maximal distance to the reference.

channel inhibition, as can be inferred from Fig. 2.

The most common deviation from the null hypothesis (B) is that the data do not follow a Gaussian distribution. Accordingly, a more general null hypothesis is that the time series originated from a stationary Gaussian linear process that has been distorted by a monotonic, instantaneous, time-independent nonlinear function [hypothesis (C)]. Appropriate surrogates can be generated via an iterative procedure proposed by Schreiber and co-worker,²⁷ which uses an implementation similar to a Wiener filter to enforce the correct spectrum to the resulting surrogates. As for the other hypotheses, we have generated $[1/(1-\alpha)]-1$ such surrogates and calculated the zeroth-order prediction error γ in dependence on n . It is fascinating to discover that none of the experimental recordings are able to pass the test, as can be concluded from comparing γ (black vertical columns) with γ_0 (green symbols) in Fig. 3. Note that in all cases γ_0 is well within the distribution of γ irrespective of n . This further corroborates the fact that experimentally observed cellular responses are imbued with stochastic features, and that indeed, apart from an instantaneous nonlinear function acting on the underlying

noisy output, markers of determinism are grossly lacking.

Results from surrogate data testing can be additionally supported by the application of a determinism test for short time series, recently proposed by Binder *et al.*⁴⁶ This is useful since the method of surrogate data is not actually a determinism test, as it can only serve to reject certain null hypotheses. The determinism test exploits statistical properties of the growth of small separations between trajectories in the phase space, in particular, the expression $d(t) \approx d_0 e^{\lambda t}$ that describes their temporal evolution. While for a deterministic system, either regular or chaotic, this expression holds whereby λ is related to the largest Lyapunov exponent, a random system will have d independent of d_0 . This fact inspired Binder *et al.*⁴⁶ to propose a determinism test for short time series, which can be summarized as follows. For a series of n points generate all possible $n(n-1)/2$ distances d_0 between distinct points in the phase space, reconstructed from the time series with the embedding dimension m and delay τ . Next, evolve all initial distances forward in time for a fix number of time steps i and calculate the resulting distances d_i . Finally, plot the graph d_i versus d_0 , whereby different values of d_0 should be averaged over small bins to annihilate statistical fluctuations. If the binned d_i versus d_0 dependence for small d_0 can be fitted well by a line with a positive slope and zero intercept, the origin of the studied time series is likely to be deterministic, while independent d_i with respect to different d_0 and a positive intercept of the y axis are a sure sign of random origin. Notably, the determinism test proposed by Binder *et al.*⁴⁶ is robust and generally applicable, yet it has some difficulties with sinusoidal time series if average separations between the trajectories fluctuate substantially, and even more so if the sampling frequency is incommensurable with the main oscillation frequency of the series. However, the analyzed Ca^{2+} responses are clearly not sinusoidal, which lead to the conclusion that the determinism test is suitable for our purposes. Figure 4 features the results of the analysis for the three considered experimental recordings and different values of i . Irrespective of which time series is used and the value of i , all data points do not intercept the vertical axis at 0, thus supplementing nicely the results and conclusions derived from the surrogate tests, showing conclusively that stochasticity prevails in experimentally observed responses of pancreatic acinar cells to acetylcholine within the intact tissue.

IV. DISCUSSION

Our results show that the Ca^{2+} traces measured in pancreatic acinar cells in response to acetylcholine are mainly stochastic with only minute markers of determinism. Surrogate data testing has revealed that in case of lower, physiologically more relevant acetylcholine concentrations, the null hypothesis (C) could not be rejected. Thus we can conclude that the underlying processes that govern the intracellular Ca^{2+} oscillations at these stimulation levels are indeed stochastic in nature, although certain signs of nonlinear components that are involved in the signal generation seem to be present. By higher stimulation levels, even the null hypothesis (B) could not be rejected, thus indicating that the

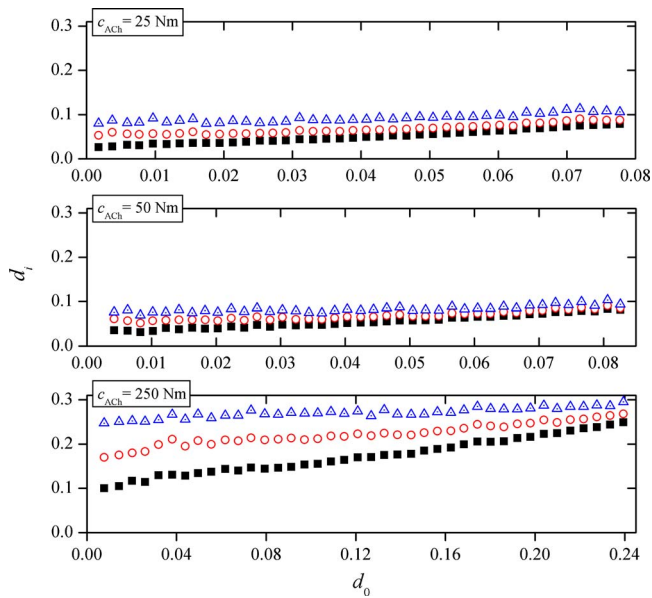


FIG. 4. (Color online) Results of the determinism test for $c_{\text{ACh}}=25$ nM, $c_{\text{ACh}}=50$ nM, and $c_{\text{ACh}}=250$ nM from top to bottom as denoted also in the individual panels. Each recording was analyzed by using the parameters $m=3$, $\tau=1$, and $i=1, 2, 3$ (squares, circles, and triangles). While all data points can be fitted fairly well with a straight line, none of them has an intercept at 0, which is according to Binder *et al.* (Ref. 46) is a clear sign for lack of determinism. In addition, it is evident that as i increases even marginally the dependence of d_i on d_0 becomes flat fast (d_i becomes independent of d_0), which further strengthens the conclusion that the analyzed traces have stochastic origin.

stochasticity is then so high that the recorded traces cannot be distinguished from a stochastic Gaussian linear process. We supplemented these findings with a determinism test for short time series, which has also confirmed lack of determinism in measured Ca^{2+} responses. In addition, the highest levels of stochasticity have been found at high acetylcholine concentrations, which is also in agreement with the results of the surrogate data method.

It should be noted that in our study we have used the full time series of Ca^{2+} oscillations for the analysis. Other recent studies^{24,47} deviate from our approach in that they take into account only the time intervals between successive Ca^{2+} peaks. Because of this difference a discrepancy between the reported results can emerge. In Ref. 47 the authors stated that stimulated oscillations have smaller entropy compared to spontaneous oscillations, which can be seen as a contradiction to our results presented in Fig. 3, where we conclude that higher stimulation levels lead to higher stochasticity in the recorded traces. However, considering full records of Ca^{2+} oscillations also takes into account the base levels of Ca^{2+} that are highly sensitive to internal and external perturbations. A similar effect can also be observed at high levels of overstimulated Ca^{2+} responses, where high-frequency oscillations appear at higher Ca^{2+} base levels, thereby substantially contributing to the overall levels of stochasticity.

Our results indicate that stochasticity is an important factor in the dynamics of intracellular Ca^{2+} oscillations. Indeed, the results suggest that mathematical models should take inherent fluctuations explicitly into account when describing cellular processes. The reported results are fully in agree-

ment with the previous analysis of experimental data and theoretical predictions.^{24–26} Combined with previous results, they represent an important experimentally based confirmation of previous theoretical declarations,^{7–23} which hypothesized that stochasticity must be taken into account when processes at the cellular level are modeled. Moreover, the unquestionable existence of inherent fluctuations confirms the feasibility of noise-induced phenomena at the single cell scale, such as stochastic or coherence resonance, which were frequently observed and studied theoretically.^{48,49}

Finally, we also compare our results, showing that cellular signals at cellular level are mainly stochastic, with the results of nonlinear time series analysis of biological signals measured at the level of the organ which indicate a high degree of determinism.^{30–33,50} This apparent discrepancy between the stochastic nature of cellular signals and deterministic nature of signals in tissues is in full agreement with the recent model predictions obtained for Ca^{2+} oscillators in diffusively coupled cells.¹⁶ The model predicts a transition from stochasticity to determinism in Ca^{2+} oscillations when going from responses of isolated cells to responses of a large number of coupled cells. It has been shown that the collective dynamics of coupled cells is, unlike that of isolated cells, deterministic for large-enough ensemble sizes. These model predictions are in best agreement with the nonlinear time series analysis of experimental results at the cellular level, where the extent of stochasticity is rather high,²⁶ and at the level of the organ, where it has been shown that the nature of measured signals is predominantly deterministic.^{50,51} In the future, it would certainly be of great interest to observe if ensembles of cells forming the tissue exhibit a more deterministic dynamics as individual cells. Namely, the tissue can provide conditions for apparently regular oscillations as described before;²⁵ however this depends on the level of communication among the cells as well as the network structure underlying the interactions. Apparently, such conditions are not met within the intact acini of pancreatic tissue slices.

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