

# How Does Intracellular $\text{Ca}^{2+}$ Oscillate: By Chance or by the Clock?

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**ABSTRACT**  $\text{Ca}^{2+}$  oscillations have been considered to obey deterministic dynamics for almost two decades. We show for four cell types that  $\text{Ca}^{2+}$  oscillations are instead a sequence of random spikes. The standard deviation of the interspike intervals (ISIs) of individual spike trains is similar to the average ISI; it increases approximately linearly with the average ISI; and consecutive ISIs are uncorrelated. Decreasing the effective diffusion coefficient of free  $\text{Ca}^{2+}$  using  $\text{Ca}^{2+}$  buffers increases the average ISI and the standard deviation in agreement with the idea that individual spikes are caused by random wave nucleation. Array-enhanced coherence resonance leads to regular  $\text{Ca}^{2+}$  oscillations with small standard deviation of ISIs.

## INTRODUCTION

How are random molecular events orchestrated into reliable cellular behavior like global intracellular  $\text{Ca}^{2+}$  oscillations? If many molecules are involved, it is generally assumed that the cell behaves like a continuously stirred reactor and the law of large numbers establishes predictable behavior (1). Most models of the dynamics of intracellular  $\text{Ca}^{2+}$  release adopt this idea (2–4), but it is not consistent with experimental analyses showing that global oscillations arise from  $\text{Ca}^{2+}$  waves initiated locally (5–9). Such a local mechanism is predicted to lead to stochastic oscillations because although each cell has many  $\text{IP}_3\text{Rs}$  and  $\text{Ca}^{2+}$  ions, the law of large numbers does not apply to the initiating event (3,10), which is restricted to very few  $\text{IP}_3\text{Rs}$  by steep  $\text{Ca}^{2+}$  concentration gradients (7,9,11). Indeed, the law of large numbers may not apply either to other signaling pathways that generate steep intracellular concentration gradients (12).

Oscillations of intracellular  $\text{Ca}^{2+}$  dynamics exhibit spike trains which appear rather regular by casual inspection. This led to formulation of models with an oscillatory deterministic regime (2–4), but cells might exhibit stochastic dynamics which produce very similar global signals. That type of dynamics, often described as a stochastic medium (10,13–16), was predicted theoretically for reaction-diffusion systems with discrete stochastic sources of the diffusing species. Intracellular  $\text{Ca}^{2+}$  dynamics may be perceived as a stochastic medium: the diffusing species is cytosolic  $\text{Ca}^{2+}$  and the discrete stochastic sources are ion channels. Such systems generate spatiotemporal structures by wave nucleation due to thermal noise. In general, that leads to random spike sequences. The randomness does not arise from small numbers

of molecules in the system, but rather from the fact that global events are initiated locally. Stochastic media exhibit almost regular oscillations in the regime of array enhanced coherence resonance (AEER) (10,13–16). Herein wave initiation is frequent enough to ensure that waves emerge as soon as the refractory period has passed. While there is experimental and theoretical evidence for the nucleation of global  $\text{Ca}^{2+}$  release events by local events (7,9,10), we investigate here whether spike sequences are random.

Transient changes in cytosolic  $\text{Ca}^{2+}$  concentration are used with great versatility to signal within cells (17). Under appropriate conditions and in many cell types, these transients form oscillations that encompass the entire cell (6,7,9,17). The interspike interval (ISI) of these oscillations varies from a few seconds to many minutes and their temporal profiles include spikes, sinusoidal oscillations, and bursts (2,3,7).

In many cells, including those examined in this study, oscillatory changes in free cytosolic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) result from the periodic release of  $\text{Ca}^{2+}$  from intracellular stores within the endoplasmic reticulum (ER). Release of  $\text{Ca}^{2+}$  occurs via intracellular  $\text{Ca}^{2+}$  channels, notably inositol 1,4,5-trisphosphate receptors ( $\text{IP}_3\text{Rs}$ ), and  $\text{Ca}^{2+}$  is then resequenced into the ER by  $\text{Ca}^{2+}$  pumps (SR/ER  $\text{Ca}^{2+}$ -ATPases).  $\text{IP}_3\text{Rs}$  are regulated by the  $\text{Ca}^{2+}$  they conduct, such that fast activation by cytosolic  $\text{Ca}^{2+}$  and slower inhibition lead to a bell-shaped  $\text{Ca}^{2+}$ -dependence of the stationary open probability of the  $\text{IP}_3\text{R}$  (18–20). Within the membrane of the ER, clusters of  $\text{IP}_3\text{Rs}$  are separated by perhaps 1–7  $\mu\text{m}$  (8,9,21), and clustering is also dynamically regulated (22,23). These clusters of channels generate global concentration spikes via a hierarchy of  $\text{Ca}^{2+}$  release events (5–7) that depend upon  $\text{Ca}^{2+}$  diffusing between  $\text{IP}_3\text{Rs}$  to ignite the activity of successive clusters (3,17). The smallest  $\text{Ca}^{2+}$  release events, blips (5), probably reflect random openings of single  $\text{IP}_3\text{Rs}$ . Larger events, puffs, lasting tens of milliseconds and restricted to a volume of  $<0.5$  fl, reflect the

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almost simultaneous opening of a few  $\text{IP}_3\text{Rs}$  within a cluster (5,8,9,21). Many coordinated puffs form oscillations and waves (3,24,25).  $\text{Ca}^{2+}$  oscillations therefore depend upon both the spatial organization of  $\text{IP}_3\text{Rs}$  and their regulation by  $\text{Ca}^{2+}$  (19,20,26).

The links between  $\text{IP}_3\text{R}$  behavior and  $\text{Ca}^{2+}$  oscillations are poorly understood. Is oscillatory behavior a feature of individual  $\text{IP}_3\text{Rs}$  and their local interaction with  $\text{Ca}^{2+}$  and  $\text{IP}_3$ , with channels opening and closing in oscillatory fashion? Or do oscillations arise from properties that depend upon a higher level of organization involving many  $\text{IP}_3\text{Rs}$ ? Is AECR the new quality added by the more complex organization that would allow oscillations to emerge even from nonoscillatory elements?

With oscillatory dynamics arising from local interactions between individual  $\text{IP}_3\text{Rs}$  and their ligands, each  $\text{IP}_3\text{R}$  cluster is also an oscillator because channels within a cluster are synchronized by  $\text{Ca}^{2+}$  diffusion (11). Characteristics of the global oscillation, like period and amplitude, would be determined by local parameters such as channel state dynamics. Spatial coupling of channel clusters by  $\text{Ca}^{2+}$  diffusion would serve only to synchronize the oscillations. The global oscillation is deterministic: it has regular ISI and the timing of each spike is predictable. But recent theoretical studies suggest that  $\text{IP}_3\text{R}$  clusters are not deterministic oscillators (10,27). A deterministic model generates oscillations only if  $\text{IP}_3\text{Rs}$  are exposed to  $\text{Ca}^{2+}$  concentrations similar to those that cause their half-maximal regulation ( $\sim 0.1\text{--}10\ \mu\text{M}$ ) (18–20,26). Local  $\text{Ca}^{2+}$  concentrations near open  $\text{IP}_3\text{Rs}$  are much higher, and therefore do not allow for deterministic oscillations (11,27–29).

Another possibility is that stochastic fluctuations render  $\text{IP}_3\text{R}$  clusters oscillatory. Stochastic behavior of  $\text{IP}_3\text{R}$  clusters is observed experimentally as  $\text{Ca}^{2+}$  puffs (6,9). Simulations show that random binding and dissociation of  $\text{Ca}^{2+}$  and  $\text{IP}_3$  to  $\text{IP}_3\text{Rs}$  are sufficient sources of stochastic fluctuations to explain the random generation of  $\text{Ca}^{2+}$  puffs (10,30,31). However, these fluctuations alone cannot generate the observed timescale of global oscillations because puff durations and frequencies are 3–100 times faster than the ISI in cells (6). Processes occurring within a single  $\text{IP}_3\text{R}$  cluster do not, therefore, transform random molecular behavior into oscillations. Local processes are not oscillatory, and so the idea that channel clusters are oscillators is untenable. A higher level of spatial organization is required to generate oscillations. The next spatial level is the cell, with its array of  $\text{IP}_3\text{R}$  clusters each capable of generating  $\text{Ca}^{2+}$  puffs.

That suggests that intracellular  $\text{Ca}^{2+}$  dynamics behave as a stochastic medium. Experimental and theoretical studies show that  $\text{Ca}^{2+}$  puffs can initiate global  $\text{Ca}^{2+}$  spikes (3,6,7,9,10). But theoretical analysis suggests that cells would not then oscillate deterministically; instead, they would produce random sequences of global  $\text{Ca}^{2+}$  release events (3,10,27,32). In particular, factors that modify spatial coupling would affect both frequency and the ability to oscillate (3,10).

## METHODS

### Measurements of $[\text{Ca}^{2+}]_i$ in glia

Glial cell cultures were prepared from brains of newborn NMRI mice (33) using whole brains for microglia, but only cortex for astrocyte cultures. Cells were loaded with fluo4-AM (5  $\mu\text{M}$ ) for 30 min at 20°C in glial medium (148.9 mM NaCl, 5.4 mM KCl, 1 mM  $\text{MgCl}_2$ , 10 mM  $\text{CaCl}_2$ , 5 mM HEPES, and 10 mM D-glucose, at pH 7.4). Cultures were fixed within the microscope chamber by a U-shaped Pt wire and superfused with glial medium at 20°C. Cells were illuminated (495 nm) from a monochromator (TILL Photonics, München, Germany) and fluorescent images (515–545 nm) collected every 3 s with a 12-bit camera (SensiCam, Cooke, Romulus, MI) on an upright microscope and visualized with ImagingCellsEasily software (Max Dulbrück Center, Berlin, Germany).

### Measurements of $[\text{Ca}^{2+}]_i$ in human embryonic kidney (HEK) cells

HEK293 cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 supplemented with fetal bovine serum (10 %) and L-glutamine (2 mM), in a humidified atmosphere (95% air, 5%  $\text{CO}_2$ , 37°C). Medium was replaced every third day, and cells were passaged when they reached 80% confluence. For single cell imaging, cells were plated onto 22-mm round glass coverslips coated with 0.01% poly-L-lysine and used after two days. They were loaded with 2  $\mu\text{M}$  Fura-2-AM in HEK medium (135 mM NaCl, 5.9 mM KCl, 1.2 mM  $\text{MgCl}_2$ , 1.5 mM  $\text{CaCl}_2$ , 11.6 mM HEPES, 11.5 mM D-glucose, and pH 7.3) supplemented with 1 mg/ml bovine serum albumin and 0.02% Pluronic F-127. Cells were loaded for 45 min at 20°C and then washed for 45 min in HEK medium to allow deesterification of the Fura-2-AM. Measurements of  $[\text{Ca}^{2+}]_i$  in single cells were performed as previously described (34), with fluorescence collected at intervals of 5 s. Fluorescence ratios were calculated after correction for autofluorescence.

### Measurements of $[\text{Ca}^{2+}]_i$ in processed lipoaspirate (PLA) cells

The study conforms to the Declaration of Helsinki and all cell donors gave their informed written consent to use part of their fat tissue for the generation of processed lipoaspirate (PLA) cells.

PLA cells were isolated from fat tissue (35) and cultured in 25  $\text{cm}^2$  flasks in 5%  $\text{CO}_2$ , humidified air at 37°C in Ham's F-10 medium (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (Sigma, St. Louis, MO), 2 mM glutamine, 0.1 mM  $\beta$ -mercaptoethanol, 1–100 nonessential amino acids (Biochrom), 100 units/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin (Invitrogen, Carlsbad, CA). Subconfluent cell cultures were dissociated with a 0.2% trypsin-0.05% EDTA solution and subcultured onto 12 mm coverslips. For  $\text{Ca}^{2+}$  measurements, subconfluent cells on coverslips were incubated for 30 min with 10  $\mu\text{M}$  fluo3-AM (Invitrogen) and then washed once in culture medium. Fluo-3 fluorescence was recorded in single cells by confocal laser-scanning microscopy (laser scanning microscope model No. 510 Meta; Zeiss, Jena, Germany) using a 25 $\times$  objective with a numerical aperture of 0.8 (Plan Neofluor; Zeiss). Excitation was at 488 nm and emission was recorded using a 515 nm longpass filter.

## DATA ANALYSIS

All fluorescence signals, whether derived from single wavelength or ratiometric indicators, are expressed relative to the baseline fluorescence ( $\Delta F = \text{signal}/\text{basal}$ ). Because we analyze only interspike intervals (ISIs), there is no need to calibrate  $\Delta F$  to  $[\text{Ca}^{2+}]_i$ . For each single-cell time series of fluorescence, the time points of the maxima above a minimal level were determined resulting in a series of spike times,

which was used to determine the ISIs. The calculations of statistical characteristics require a stationary ISI series. However, most experimental records of HEK cells have a slight trend and eventually stop oscillating. We reduced that trend by leaving out parts of the ISI series in the beginning and end showing very obvious trends and removed linear trend from the remaining series before we calculated the average period  $T_{av}$ , the standard deviation  $\sigma$ , and correlations. This is illustrated in Supplementary Material Fig. S7.

Correlation coefficients  $\rho_k$  between an ISI and its  $k^{\text{th}}$  successor for the  $i^{\text{th}}$  ISI-series were calculated according to

$$\rho_k^{(i)} = \left\langle \frac{\text{Cov}(ISI_i^{(i)}, ISI_{i+k}^{(i)})}{\sigma_i^{(i)} \sigma_{i+k}^{(i)}} \right\rangle_i. \quad (1)$$

With the  $\rho_k^{(i)}$ , we formed the population average across  $n$  cells by  $\rho_k = (1/n) \sum_{i=1}^n \rho_k^{(i)}$ .

### $T_{av}$ - $\sigma$ relation for time-dependent global nucleation rate

We show that a global nucleation probability increasing by a relaxation to an asymptotic value provides  $T_{av}$ - $\sigma$  relations compatible with our findings. We choose a time-dependent nucleation rate  $\Lambda$  like

$$\Lambda(t) = \lambda(1 - e^{-\xi t}), \quad (2)$$

with the regeneration rate  $\xi$ . The value  $\xi$  could describe, e.g., the recovery from inhibition (36). The probability of observing a global wave at time  $t$  is given by

$$p(t) = \lambda(1 - e^{-\xi t}) \exp \left[ - \int_0^t \lambda(1 - e^{-\xi t'}) dt' \right]. \quad (3)$$

With this expression we can calculate the first two moments as

$$T_{av} = \int_0^\infty t \lambda(1 - e^{-\xi t}) \exp \left[ - \int_0^t \lambda(1 - e^{-\xi t'}) dt' \right] dt, \quad (4)$$

$$\langle T^2 \rangle = \int_0^\infty t^2 \lambda(1 - e^{-\xi t}) \exp \left[ - \int_0^t \lambda(1 - e^{-\xi t'}) dt' \right] dt. \quad (5)$$

That can be integrated by parts,

$$T_{av} = \int_0^\infty t e^{-f(t)} f'(t) dt = -t e^{-f(t)} \Big|_0^\infty + \int_0^\infty e^{-f(t)} dt, \quad (6)$$

where the first term vanishes, leading to

$$T_{av} = \int_0^\infty e^{-\lambda \left( t + \frac{1}{\xi} e^{-\xi t} - \frac{1}{\xi} \right)} dt \quad (7)$$

for  $T_{av}$ .  $\langle T^2 \rangle$  is given by

$$\begin{aligned} \langle T^2 \rangle &= \int_0^\infty t^2 e^{-f(t)} f'(t) dt = 2 \int_0^\infty t e^{-f(t)} dt \\ &= 2 \int_0^\infty t e^{-\lambda \left( t + \frac{1}{\xi} e^{-\xi t} - \frac{1}{\xi} \right)} dt. \end{aligned} \quad (8)$$

Integration yields

$$T_{av} = \frac{e^{\frac{\lambda}{\xi}} \left( \frac{\lambda}{\xi} \right)^{1-\frac{\lambda}{\xi}}}{\lambda} \left[ \Gamma \left( \frac{\lambda}{\xi} \right) - \Gamma \left( \frac{\lambda}{\xi}, \frac{\lambda}{\xi} \right) \right], \quad (9)$$

$$\langle T^2 \rangle = \frac{2e^{\frac{\lambda}{\xi}}}{\lambda^2} \mathcal{F} \left[ \left( \frac{\lambda}{\xi}, \frac{\lambda}{\xi} \right), \left( 1 + \frac{\lambda}{\xi}, 1 + \frac{\lambda}{\xi} \right), -\frac{\lambda}{\xi} \right], \quad (10)$$

where  $\Gamma(x)$  denotes the Euler  $\Gamma$ -function,  $\Gamma(x, y)$  the incomplete  $\gamma$ -function, and  $F[\mathbf{x}]$  is the generalized hypergeometric function  ${}_2F_2(\mathbf{x})$ . With the relation for the standard deviation

$$\sigma = \sqrt{\langle T^2 \rangle - T_{av}^2}, \quad (11)$$

we obtain the  $T_{av}$ - $\sigma$  relations shown in Fig. 1.

We did not consider a deterministic part  $T_d$  of the ISI in the above calculations, which represents an absolute refractory part of the ISI. Such a deterministic part simply moves the curves in Fig. 1 to larger values of  $T_{av}$ . The complete distribution including  $T_d$  is

$$p(t) = \begin{cases} 0, & t < T_d \\ \lambda(1 - e^{-\xi(t-T_d)}) \exp \left[ - \int_{T_d}^t \lambda(1 - e^{-\xi(t'-T_d)}) dt' \right], & t \geq T_d \end{cases} \quad (12)$$

The analysis of experimental data showed that  $T_{av}$ ,  $\sigma$ , and  $T_d$  vary between individual cells of the same cell type.

## RESULTS AND DISCUSSION

To establish whether intracellular  $\text{Ca}^{2+}$  oscillations are deterministic or stochastic, we performed comprehensive measurements of ISI series in different cell types. In each case,  $\text{IP}_3\text{Rs}$  evoke the  $\text{Ca}^{2+}$  oscillations (37–39) (Fig. S8). Fig. 2 shows representative time series of global oscillations in astrocytes, microglia, PLA stem cells from human adipose

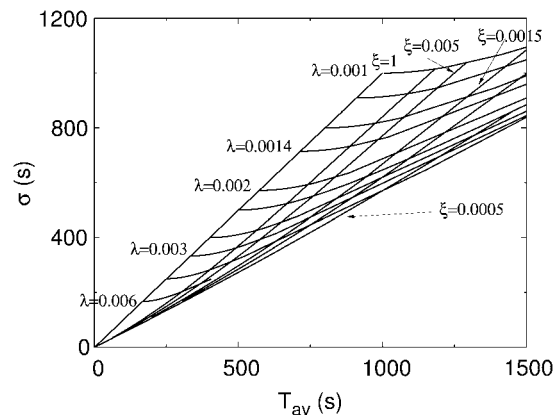


FIGURE 1  $T_{av}$ - $\sigma$  relation resulting from Eqs. 7 and 8. The lines  $\xi = \text{constant}$  arise by varying  $\lambda$ . They start at (0,0) with  $\lambda = \infty$  and converge to  $T_{av} = \sigma$  with increasing  $\xi$ . The lines  $\lambda = \text{constant}$  start on  $T_{av} = \sigma = \lambda^{-1}$  with  $\xi \gg \lambda$  very large. Note that the  $T_{av}$ - $\sigma$  relation becomes linear for  $\lambda/\xi \rightarrow 0$  or  $T_{av} \rightarrow \infty$  and  $\sigma \rightarrow \infty$  and that the slope decreases from one for  $\xi \rightarrow 0$ . The values  $\xi$  and  $\lambda$  are measured in  $\text{s}^{-1}$ .

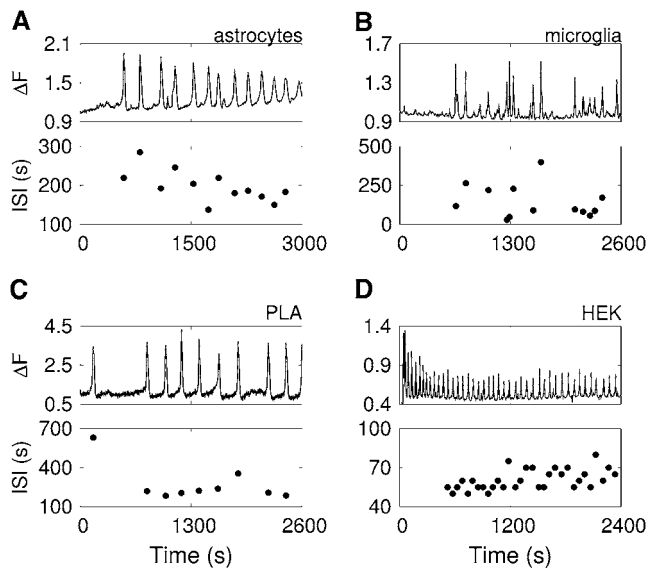


FIGURE 2  $\text{IP}_3$ -mediated  $\text{Ca}^{2+}$  spikes in diverse cells. Representative examples of oscillation time series for astrocytes (A), microglia (B), PLA cells (C), and HEK cells stimulated with  $30 \mu\text{M}$  carbamyl choline (CCh) (D). The oscillations in panels A–C are spontaneous. Changes in the fluorescence of the  $\text{Ca}^{2+}$  indicator relative to its basal level ( $\Delta F$ ) report the increase in free cytosolic  $[\text{Ca}^{2+}]$ . Lower panels show the ISI after each spike, with ISI defined as the interval between consecutive fluorescence maxima.

tissue, and human embryonic kidney (HEK) cells. These examples show that ISIs are not regular. The length of the ISI changes randomly. Records like these were used to determine the serial correlation coefficients  $\rho_k$ , mean values  $T_{\text{av}}$ , and standard deviations  $\sigma$  of ISI. For all cell types and with all degrees of stimulation,  $\sigma$  increases with  $T_{\text{av}}$  (Fig. 3).

Standard deviations are of the same order of magnitude as averages for most points, and for large  $T_{\text{av}}$ ,  $\sigma$  and  $T_{\text{av}}$  are similar. Hence, the uncertainty in predicting the occurrence of a spike is of the same order as the mean ISI: i.e.,  $\text{Ca}^{2+}$  spikes are random events. If spikes represented the active phase in a deterministic oscillation, the standard deviation of ISI would instead be in the range of the global interpuff interval, i.e., between a few hundred milliseconds for short  $T_{\text{av}}$  and a few seconds for long  $T_{\text{av}}$ . The uncertainty in spike timing shows in the correlation coefficients  $\rho_k$  of the ISI time series between the  $i^{\text{th}}$  and  $(i + k)^{\text{th}}$  ISI (Fig. 4). Consecutive intervals are not correlated, confirming the randomness of the spike-generating mechanism.

We conclude that global oscillations result from a sequence of randomly occurring global  $\text{Ca}^{2+}$  spikes. The data show also that  $\sigma$  almost vanishes at the smallest values of  $T_{\text{av}}$ , indicating that almost regular oscillations with ISIs close to  $T_d$  do exist. The results are similar for spontaneous oscillations in astrocytes, microglia, and PLA cells, and for oscillations evoked by different levels of stimulation in HEK cells, suggesting that  $\text{IP}_3$ -evoked  $\text{Ca}^{2+}$  oscillations are sequences of stochastic spikes in many cell types.

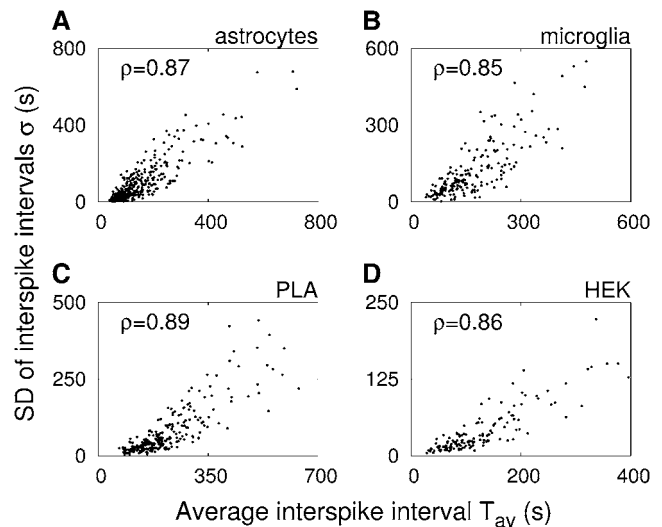


FIGURE 3  $\text{Ca}^{2+}$  spikes occur randomly. Dependence of the standard deviation  $\sigma$  of ISI on the average ISI  $T_{\text{av}}$  for 366 astrocytes (A), 224 microglia (B), 270 PLA cells (C), and 137 HEK cells stimulated with  $30 \mu\text{M}$  CCh (D).  $\sigma$  and  $T_{\text{av}}$  were obtained from time series of single cells by temporal averaging. The correlation coefficient  $\rho$  shows that  $\sigma$  and  $T_{\text{av}}$  are highly correlated in all four cases.

Our conclusion is compatible with the idea that each  $\text{Ca}^{2+}$  spike reflects the passage of a  $\text{Ca}^{2+}$  wave across the cell driven by successive activation of  $\text{IP}_3\text{R}$  clusters by  $\text{Ca}^{2+}$  diffusing between them (6,7,9). This mechanism can generate the spectrum of observed shapes of oscillations (3,10). Our results show that waves initiate randomly. The time of initiation is not set by a deterministic process, such as recovery from  $\text{Ca}^{2+}$ -inhibition or a progressive sensitization of  $\text{IP}_3\text{Rs}$  by  $\text{Ca}^{2+}$ .

Stochastic models of repetitive waves show that if  $\sigma$  is of the same order as  $T_{\text{av}}$ , it is dominated by the probability ( $P_{\text{trig}}$ ) of triggering a wave after the cell has recovered from the previous one (10). The smaller the value of  $P_{\text{trig}}$ , the longer it takes on average for the next wave to occur, and the larger is the value of  $\sigma$ . For such repetitive triggering of waves,  $\sigma$  increases linearly with  $T_{\text{av}}$ , and  $\sigma = 1/P_{\text{trig}}$  holds for large values of  $T_{\text{av}}$ . Even if  $P_{\text{trig}}$  relaxes exponentially from 0 immediately after a spike to an asymptote, the linear relation between  $\sigma$  and  $T_{\text{av}}$  for large  $T_{\text{av}}$  still holds and it has a slope  $< 1$  (see Fig. 1 and Fig. 3 D). Such a relaxation of  $P_{\text{trig}}$  appears to apply also to wave initiation reported in Marchant and Parker (9). Almost regular oscillations arise when  $P_{\text{trig}}$  is very large, because then as soon as the cell has recovered from one spike, the next one is triggered. We refer to the length of the ISI of these regular oscillations as the deterministic part  $T_d$  of the ISI. It might be set by a variety of processes depending on cell type, for example: store refilling,  $\text{IP}_3\text{R}$  inhibition, or  $\text{Ca}^{2+}$ -feedback to the  $\text{IP}_3$  concentration. Each of these processes may also cause a time-dependence of  $P_{\text{trig}}$  such as that described in a simplified way by the relaxation with time constant  $\xi$  in Eq. 2.

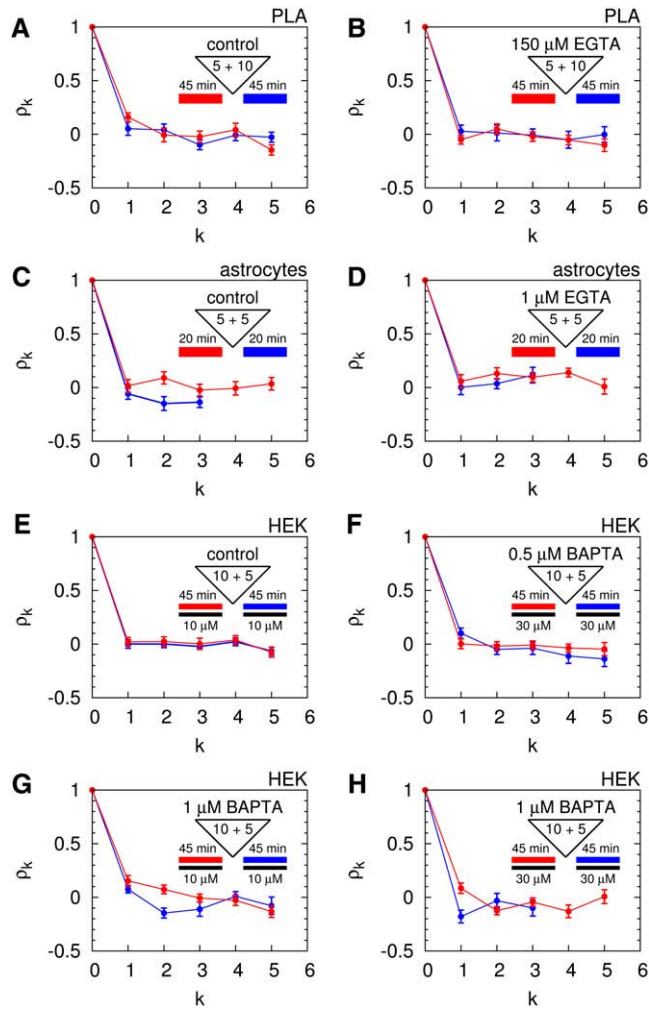


FIGURE 4 Successive ISI are not correlated. Correlation coefficient  $\rho_k$  between the  $i^{\text{th}}$  and  $(i+k)^{\text{th}}$  ISI averaged across  $n$  time series obtained in the experiments shown in Fig. 6 before (red) and after (blue) loading with additional  $\text{Ca}^{2+}$  buffer. The protocols are shown in the insets (see also caption of Fig. 6). The number of time series measured for the first and the second periods were, respectively: (A)  $n_1 = 27$ ,  $n_2 = 20$ ; (B)  $n_1 = 35$ ,  $n_2 = 18$ ; (C)  $n_1 = 36$ ,  $n_2 = 35$ ; (D)  $n_1 = 33$ ,  $n_2 = 23$ ; (E)  $n_1 = 35$ ,  $n_2 = 35$ ; (F)  $n_1 = 35$ ,  $n_2 = 28$ ; (G)  $n_1 = 31$ ,  $n_2 = 22$ ; and (H)  $n_1 = 29$ ,  $n_2 = 18$ . Error bars show standard error for averaging over  $n$  time series.

The time-dependence of the global nucleation rate differs between the different cell types as revealed by the slopes of the relationship between  $\sigma$  and  $T_{\text{av}}$ . Fig. 5 shows values of the parameters of the time-dependent global nucleation rate (Eq. 2) for the different cell types. The spontaneous oscillations in astrocytes and microglia have a slope of the  $\sigma$ - $T_{\text{av}}$ -relation close to 1 and an asymptotic nucleation rate  $\lambda$  much smaller than the relaxation rate  $\xi$ . Despite the relatively fast recovery from the previous spike, these oscillations are neither fast nor regular, since the asymptotic nucleation rate  $\lambda$  of these cells is small. The stimulated oscillations in HEK cells show the inverse relation between  $\lambda$  and  $\xi$ . That is correlated with the information content of stimulated and spontaneous oscillations which increases with increasing  $\lambda/\xi$  (40).

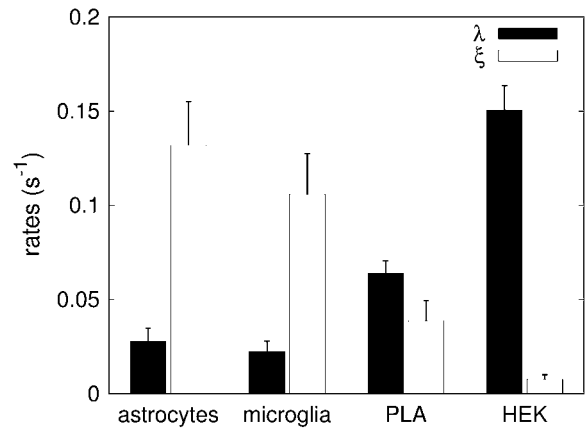


FIGURE 5 Values of the parameters  $\lambda$  and  $\xi$  of the time-dependent global nucleation rate Eq. 2 for the different cell types. The values were obtained by fitting  $\sigma$  and  $T_{\text{av}}$  of individual time series to Eqs. 10 and 11 to obtain  $\lambda$  and  $\xi$ , which were then averaged across all cells of the same type. We have approximated  $T_d$  by the smallest value of  $T_{\text{av}}$  observed for the corresponding cell type in the fitting procedure. Error bars show standard error.

Recovery from the previous spike is relatively slow in HEK cells. That corresponds to the initial decline in spike amplitudes in Fig. 2 during which the cell reaches a state corresponding to incomplete recovery from a spike during each ISI. One possibility is that insufficient refilling of the ER causes the decline. We might then suggest that immediately after stimulation,  $\text{Ca}^{2+}$  reuptake during the ISI fails to keep pace with release during a spike and successive  $\text{Ca}^{2+}$  transients decrease in amplitude. But during the stationary phase of oscillations, the two fluxes come into balance such that  $\text{Ca}^{2+}$  uptake by the stores during the ISI matches the amount released during the preceding spike. The time-dependent nucleation rate  $\Lambda(t)$  reaches only 60% of  $\lambda$  during an average ISI due to the small value of  $\xi$ . However, since the asymptotic nucleation rate  $\lambda$  is rather large, the oscillations in HEK cells have smaller  $\sigma$  than astrocyte oscillations. The values for  $\lambda$  and  $\xi$  of PLA cells lie between those for astrocytes and HEK cells.

We can further examine the stochastic component of wave triggering by manipulating the probability of wave initiation, for example by increasing the  $\text{Ca}^{2+}$ -buffering capacity of the cytosol (10,11). Decreasing  $P_{\text{trig}}$  increases the average ISI, but if wave triggering is random, the standard deviation  $\sigma$  should also increase.  $P_{\text{trig}}$  is actually the probability of two sequential events: that a puff occurs ( $P_{\text{puff}}$ ) and then that the puff ignites a wave ( $P_{\text{wave}}$ ). The latter turns the puff frequency into the smaller wave frequency ( $P_{\text{trig}} = P_{\text{puff}}P_{\text{wave}}$ ) and the standard deviation of interpuff intervals into the standard deviation of interspike intervals.  $P_{\text{wave}}$  is expected to decrease when diffusion of free  $\text{Ca}^{2+}$  between clusters (the spatial coupling) is reduced by buffers (11). The precise effect depends on the dynamic regime of the cell. With slow irregular waves, a slight decrease in coupling is predicted to increase  $T_{\text{av}}$  and  $\sigma$ , while larger decreases are predicted to abolish the

self-amplifying mechanism required for oscillations leaving only moderate puff activity (10,11). Oscillations are predicted to be very sensitive to addition of  $\text{Ca}^{2+}$  buffers with fast binding and dissociation rates, and with  $K_d^{\text{Ca}}$  similar to resting cytosolic  $\text{Ca}^{2+}$  concentration because such buffers effectively reduce diffusion of free  $\text{Ca}^{2+}$  at distances similar to the likely spacings of  $\text{IP}_3\text{R}$  clusters (11). Because the buffers we use do not change the resting cytosolic free  $\text{Ca}^{2+}$  concentration, the  $\text{IP}_3$  concentration, or the interactions between  $\text{IP}_3\text{R}$ s within clusters (41), we do not expect them to affect  $P_{\text{puff}}$ .

We measured the effect of  $\text{Ca}^{2+}$ -buffering on oscillations in astrocytes, HEK cells, and PLA cells. Oscillations were first recorded to establish  $T_{\text{av}}$  and  $\sigma$ . The buffering capacity of the cells was then increased by incubating the cells with BAPTA-AM or EGTA-AM, before continuing measurements of  $[\text{Ca}^{2+}]_i$ . BAPTA loading caused most of the HEK cells to stop oscillating, and for the cells that resumed oscillations ( $\sim 50\%$  for cells loaded with 0.5 or 1 M BAPTA-AM),  $T_{\text{av}}$  and  $\sigma$  were both increased in a concentration-dependent way (Fig. 6, D–F). EGTA loading of PLA cells and astrocytes also caused oscillations to terminate in  $\sim 80\%$

of cells, and increased both  $T_{\text{av}}$  and  $\sigma$  in the cells that resumed oscillations (Fig. 6, A–C, G–I).

The results demonstrate both the exquisite sensitivity of  $\text{Ca}^{2+}$  oscillations to  $\text{Ca}^{2+}$  buffering, whether provided by fast (BAPTA) or slow (EGTA)  $\text{Ca}^{2+}$  buffers, and the increase in both  $T_{\text{av}}$  and  $\sigma$  predicted for a stochastic mechanism dependent on repetitive  $\text{Ca}^{2+}$  waves (10).

We have shown that cells use a stochastic mechanism to generate global  $\text{Ca}^{2+}$  spikes. With  $\sim 10^4$   $\text{IP}_3\text{R}$ s in each HEK cell (unpublished) and far more  $\text{Ca}^{2+}$  ions, why does random behavior play such an important part in  $\text{Ca}^{2+}$  spiking? The reason is that, as for any stochastic medium, global events are initiated by local processes. A puff changes the local free  $\text{Ca}^{2+}$  concentration by 100–1000-fold (11) and the gradients are so steep that only neighboring  $\text{IP}_3\text{R}$  clusters experience the change. Each puff, occurring within only a small part of the cell, is a potential wave initiation site, and the few  $\text{IP}_3\text{R}$  clusters that participate in the initiation of each wave are too few for the process to become deterministic. These properties identify intracellular  $\text{Ca}^{2+}$  dynamics as a random medium. The existence of so-called hot-spots (5) does not change these

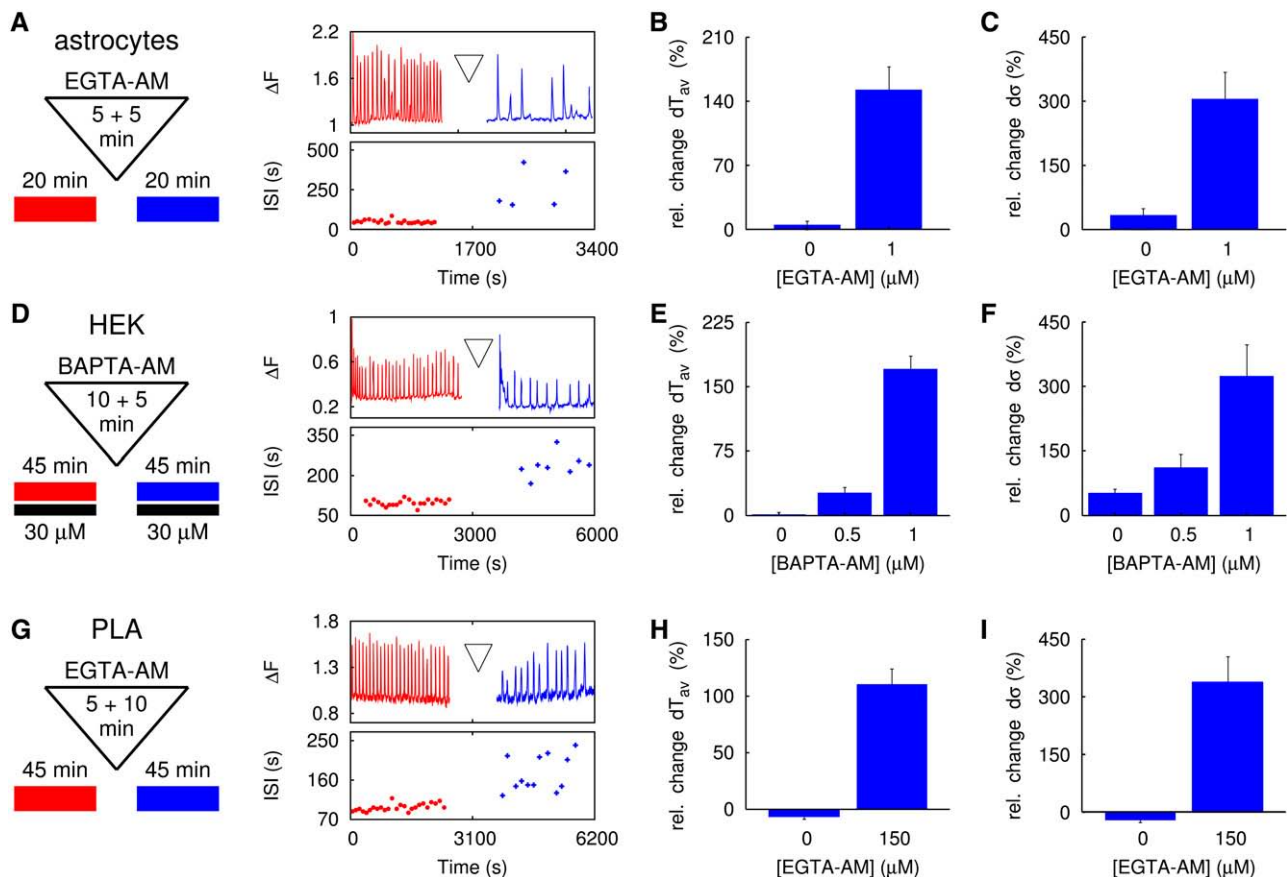


FIGURE 6  $\text{Ca}^{2+}$  buffers render spike times even less predictable. Results show  $\text{Ca}^{2+}$  spikes in astrocytes (A–C), HEK cells stimulated with 30  $\mu\text{M}$  CCh (D–F), and PLA cells (G–I) before (red) and after (blue) loading with additional  $\text{Ca}^{2+}$  buffer. The protocols and typical results are shown in panels A, D, and G; the durations of the loading and subsequent hydrolysis times are shown in the triangles. Changes to concentrations of EGTA-AM or BAPTA-AM are shown in the histograms. For the 50% of cells that resumed  $\text{Ca}^{2+}$  spiking, changes  $dT_{\text{av}}$  and  $d\sigma$  in  $T_{\text{av}}$  and  $\sigma$  are shown relative to the values obtained before incubation with the  $\text{Ca}^{2+}$  buffer. Error bars show standard error from different cells and experiments.

conclusions; it simply means that  $P_{\text{puff}}$  is not spatially homogeneous.

Intracellular  $\text{Ca}^{2+}$  spikes occur randomly. But a regular regime exists if  $P_{\text{trig}}$  is high due to the existence of a deterministic part of the ISI. The mean and the standard deviation of the ISI distribution depend on spatial coupling. The regular  $\text{Ca}^{2+}$  spikes are thus an example of array enhanced coherence resonance, an intrinsically stochastic phenomenon (10,13,15,16,42). This is consistent with hierarchical recruitment of  $\text{Ca}^{2+}$  release events (6,7). Hence, oscillations are an emergent property of arrays of  $\text{IP}_3\text{R}$  clusters and not a property of single  $\text{IP}_3\text{Rs}$ .  $\text{Ca}^{2+}$  oscillations are the first example of the constructive use of noise in cell signaling and the first experimental verification of AECR in a natural system.

These conclusions are based on the data presented here, previous measurements on the initiation of spikes and waves (6,7,9,43) and on theoretical studies investigating the role of noise and spatial coupling for global signals (10,16,42,44,45). It is accepted that elemental events like  $\text{Ca}^{2+}$  puffs and sparks are random (3,30,31,46–53), but this has not previously been shown for global events. Indeed, and despite the observation that over a large area of the cytosol ( $8\ \mu\text{m} \times 8\ \mu\text{m}$ ) of a *Xenopus* oocyte  $\sigma$  increases with  $T_{\text{av}}$  (9), it has been assumed that global signals are deterministic (2).

The probability of initiating a wave ( $P_{\text{trig}}$ ) is determined by local properties, such as channel state dynamics, numbers of channels in a cluster, release currents, and by diffusion of free  $\text{Ca}^{2+}$  between clusters.  $P_{\text{trig}}$  sets  $\sigma$  of the ISI distribution. Information on local properties, like the open probability in vivo, can thus, in principle, be obtained from the fluctuations of the global  $\text{Ca}^{2+}$  signal.

We have shown that in many different cell types  $\text{IP}_3$ -evoked  $\text{Ca}^{2+}$  spikes are caused by random wave nucleation with a regular regime arising from AECR. The randomness of spike trains does not prevent  $\text{Ca}^{2+}$  oscillations from transmitting information. As long as the ISI distribution of stimulated spike trains is sufficiently different from the ISI distribution of spontaneous spiking (40), cells can distinguish the message from the noise.

## SUPPLEMENTARY MATERIAL

To view all of the supplemental files associated with this article, visit [www.biophysj.org](http://www.biophysj.org).

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