Mechanism of long-range Ca^{2+} signalling in the nucleus of isolated rat hepatocytes

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Ca^{2+} regulates a wide range of cell proteins, in both the cytosol and nucleus. It enters the nucleus from stores along the nuclear envelope, but how it then spreads through the nuclear interior is unknown. Here we used high-speed confocal line-scanning microscopy to examine the propagation of Ca^{2+} waves across nuclei in isolated rat hepatocytes. Nuclear Ca^{2+} waves began at the nucleus/cytosol border as expected, then spread across the nucleus at less than half the speed of cytosolic Ca^{2+} waves. High concentrations of caffeine slowed Ca^{2+} waves in the cytosol but not in the nucleus. We developed a mathematical model based on diffusion to analyse these data, and the model was able to

describe the nuclear but not cytosolic Ca^{2+} waves that were experimentally observed. These findings suggest that Ca^{2+} waves cross the nucleus by simple diffusion, which is distinct from the reaction-diffusion mechanism by which Ca^{2+} waves propagate across the cytosol. Since the range of messenger action for Ca^{2+} in the cytosol is much smaller than the distance across the nucleus, this also suggests that the unique environment and geometry of the nuclear interior may permit this simple mechanism of Ca^{2+} wave propagation to control Ca^{2+} -mediated processes in a relatively large region despite Ca^{2+} release pools that are spatially limited.

INTRODUCTION

Ca^{2+} is an important second messenger in both the cytosol and nucleus [1,2]. In the cytosol, the range of messenger action for Ca^{2+} is approx. $0.1\ \mu\text{m}$ when Ca^{2+} is transiently released from a single site [3], and the range of Ca^{2+} action remains far less than $1\ \mu\text{m}$ even if a cluster of Ca^{2+} channels open simultaneously [4]. Cytosolic Ca^{2+} (Ca_i^{2+}) signals therefore must depend on reaction-diffusion mechanisms [1,5] and on other messengers such as $\text{Ins}(1,4,5)\text{P}_3$ [3] in order to spread across the entire cell. Unlike in the cytosol, Ca^{2+} release in the nucleus occurs only along its boundary, at the nuclear envelope [6,7]. Increases in either cytosolic or nuclear $\text{Ins}(1,4,5)\text{P}_3$ [8] and perhaps cyclic ADP-ribose (cADPR) [6] induce discharge of Ca^{2+} from the nuclear envelope into the nucleoplasm. Since no Ca^{2+} uptake or release mechanisms have been identified within the nuclear interior, simple diffusion may be responsible for the spread of nuclear Ca^{2+} (Ca_n^{2+}) waves inward from the nuclear envelope. However, nuclei may be many micrometres in diameter, so this mechanism of Ca_n^{2+} wave propagation would require that, unlike in the cytosol [3], Ca^{2+} can act as a global messenger in the nucleus. Here we investigated the kinetics of Ca_n^{2+} wave propagation to determine whether such waves can be described by simple diffusion inward from the nuclear envelope.

MATERIALS AND METHODS

Animals and materials

Male Sprague–Dawley rats (180–250 g; Camm Research Laboratory Animals, Wayne, NJ, U.S.A.) were maintained on Purina rodent chow under a constant light cycle and used for all experiments. [Arg^8]Vasopressin and caffeine were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.) and fluo 3 acetoxymethyl ester was from Molecular Probes (Pitchford, OR,

U.S.A.). All other chemicals were of the highest quality commercially available.

Isolation of rat hepatocytes

Rat hepatocytes and hepatocyte couplets were isolated in the Hepatocyte Isolation Core of the Yale Liver Center as described previously [9–11]. Briefly, rat livers were perfused with Hanks A then Hanks B medium containing 0.05% collagenase (Boehringer-Mannheim Biochemicals, Indianapolis, IN, U.S.A.) and 0.8 unit of trypsin inhibitor (Sigma)/unit of tryptic activity. Livers were then excised, minced, passed through serial nylon mesh filters, and the resultant cells washed. These cells were suspended at a concentration of 6×10^6 cells/ml in Liebovitz L-15 medium (Gibco, Grand Island, NY, U.S.A.) containing 50 units of penicillin and $50\ \mu\text{g}$ of streptomycin per ml, and plated on to glass coverslips or coverslip fragments. Cells were incubated at 37°C and used 2–6 h after plating. Cell viability by Trypan Blue exclusion was measured 2 h after plating and exceeded 90%.

Experimental measurements of nuclear and cytosolic Ca^{2+}

Coverslips containing the cells were loaded with fluo 3 acetoxymethyl ester ($6\ \mu\text{M}$), transferred to a chamber on the stage of a Zeiss Axiovert microscope, perfused at 37°C with a Hepes-buffered solution and observed using a Bio-Rad MRC-600 confocal imaging system. Ca^{2+} signals were detected using the line-scanning mode of the confocal microscope [9,10,12]. With this approach, fluorescence is determined at each point along a single line across the image, rather than at each point across the entire image. Confocal line-scanning microscopy was used rather than other types of cell imaging for several reasons. First, hepatocytes in short-term culture are spherical with diameters of up to $25\ \mu\text{m}$, so confocal rather than epifluorescence imaging is necessary for adequate spatial resolution of

Abbreviations used: Ca_i^{2+} , cytosolic Ca^{2+} ; Ca_n^{2+} , nuclear Ca^{2+} ; cADPR, cyclic ADP-ribose.

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fluorescence signals within subcellular structures such as the nucleus [9,13]. With the confocal imaging system used here, a spatial resolution of $0.26\ \mu\text{m}$ was obtained in the plane of focus, and the depth of focus was $0.7\ \mu\text{m}$ [9]. Second, Ca^{2+} waves cross hepatocytes at speeds of up to $100\ \mu\text{m/s}$ [9,10]. One to several seconds are required to obtain conventional two-dimensional confocal images, but line scans can be obtained as quickly as every 4–6 ms [9,10,13]. Scans were obtained at a rate of 12 ms/pixel (83.3 Hz) here. Certain confocal microscopes use an acousto-optical deflector to obtain two-dimensional images at video rates (30 Hz), but pilot studies suggested that this approach did not provide sufficient spatial resolution to detect Ca^{2+} waves within the nucleus of hepatocytes. Line scanning can lead to overestimates of Ca^{2+} wave speeds, especially if the wave begins perpendicular to the scan line, but these overestimates are within 15% of the actual speed on average [10]. Finally, use of fluo 3 is potentially limited by the fact that the dye is both excited and detected at single wavelengths, so that photobleaching may introduce measurement errors. However, line scanning reduces fluo 3 fluorescence in hepatocytes by only approx. 0.01% per scan, which corresponds to less than 4% during the 512 scans acquired per experiment [9].

Velocities of intracellular Ca^{2+} waves were determined from the rate at which initial increases in fluorescence progressed along the scan line [12]. Confocal machine settings were adjusted to measure fluo 3 fluorescence in the mid-region of the dynamic range, so that fluorescence intensity was approximately linearly related to cytosolic or nuclear Ca^{2+} . This approach allowed the diffusion coefficient D to be estimated, since estimates depend on accurate measurements with respect to time and distance, but only require Ca^{2+} concentrations to be determined to within a scale factor. In addition, in cases where fluo 3 fluorescence was non-uniform at baseline, the fluorescence over time at each point across the cell was normalized by the initial fluorescence at that point.

Mathematical modelling and data analysis

Model simulations, parameter estimations, sensitivity coefficients and graphical representations of the data were performed using a computer program written for this purpose. The model equation was solved numerically using the Crank–Nicholson method [14]. Mesh increments in both time and space were chosen by comparing Crank–Nicholson calculations with the analytical solution for a test model with sinusoidally varying boundary conditions constrained to agreement within 0.0001% throughout the entire time–space domain. This test model was also used with simulated data sets to verify that the program could accurately estimate both D and the experimental standard deviation. In working with the actual data, the time-dependent boundary conditions were represented by the least-squares fit of one of several arbitrarily chosen curve-fitting functions, each of which gave nearly identical results. To estimate D , two-dimensional data sets were used that consisted of up to 28 points in the x direction (at $0.26\ \mu\text{m}$ intervals for Ca_n^{2+} and $1\ \mu\text{m}$ intervals for Ca_i^{2+}) and up to 512 points in the t direction (at 12 ms intervals). D was determined by using a Levenberg–Marquardt method [14] to minimize the sum of the squared residual differences between the model and these data. Then 95% confidence intervals were calculated by the Bootstrap method using repeated Monte Carlo simulations with synthetic data sets [14]. Finally, two-dimensional residual maps and plots of calculated versus experimental Ca_n^{2+} were generated and inspected for goodness-of-fit and for pronounced errors.

RESULTS AND DISCUSSION

Subcellular patterns of Ca^{2+} signals were detected in isolated rat hepatocytes using confocal line-scanning microscopy [9,10,12]. Increases in Ca^{2+} began as Ca^{2+} waves both in the cytosol, as described previously [9–11,15], and in the nucleus (Figure 1). Ca_n^{2+} waves began at the nucleus/cytosol interface in each of 37 hepatocytes, which is consistent with previous observations that Ca^{2+} within the nucleus is derived from $\text{Ins}(1,4,5)\text{P}_3$ -sensitive and cADPR-sensitive stores along the nuclear envelope [6]. Several types of Ca_n^{2+} wave patterns were observed, though. Increases in Ca_n^{2+} either crossed the nucleus as a single wave, or else spread inward from the boundaries (Figure 1). Furthermore increases in free Ca^{2+} were equally likely to begin on the cytosolic or nucleoplasmic side of the nuclear membrane, although Ca_n^{2+} and Ca_i^{2+} began nearly simultaneously on average; Ca_n^{2+} increases preceded increases in Ca_i^{2+} that were detected in the region of cytosol adjacent to the nucleus by $6.6 \pm 136.7\ \text{ms}$ (mean $\pm$ S.E.M.; range, 336 ms before to 324 ms after the increase in Ca_i^{2+}). Although hormone-induced increases in free Ca^{2+} begin in the cytosol rather than in the nucleus [17], these findings demonstrate that Ca_n^{2+} increases are nearly simultaneous with and can even precede Ca_i^{2+} increases along specific regions of the nucleus/cytosol interface. Moreover, these findings demonstrate that Ca_n^{2+} signals begin as waves that spread inward from the nuclear envelope.

To examine the mechanism of Ca_n^{2+} wave propagation, Ca^{2+} wave speed was measured in hepatocytes stimulated with vasopressin (10 nM), either with or without pretreatment with

Figure 1 Patterns of Ca_n^{2+} signals detected by confocal line-scanning microscopy

Two types of Ca_n^{2+} wave patterns were observed in response to stimulation with vasopressin (10 nM). Ca_n^{2+} either travelled across the nucleus as a single wave (left) or as separate waves inward from the boundaries (right). Representative line scans are shown, in which distance (across the nucleus) is along the horizontal axis and time is along the vertical axis. Spatial resolution here and in Figure 2 is $0.26\ \mu\text{m}/\text{pixel}$, and temporal resolution here and in subsequent Figures is 12 ms/pixel. Total elapsed time (from top to bottom) is 6 s, total distance across the nucleus on the left is $6.5\ \mu\text{m}$, and total distance across the nucleus on the right is $5.2\ \mu\text{m}$. Measurements were taken of Ca^{2+} signalling across entire hepatocytes, but only Ca^{2+} signalling in the nucleus is shown here for clarity. The grey scale used for images here and in subsequent Figures ranges from black to grey to white as Ca_n^{2+} increases; Ca_n^{2+} typically increases from 100 to 200 nM at baseline to 400 to 600 nM at peak [16]. Thus the Ca_n^{2+} wave in the line scan on the left begins along the left edge of that nucleus (after a delay of approx. 3.4 s), then spreads across the width of the nucleus to the right edge over about the next 0.3 s. In contrast, the Ca_n^{2+} increase in the line scan on the right begins as two separate waves, moving inward from the left and right edges (after a delay of about 1.3 s) and meeting in the middle of the nucleus within about the next 0.3 s.

Figure 2 Propagation of Ca_n²⁺ waves described by simple diffusion

Top, typical line scan demonstrating Ca_n²⁺ over time as experimentally determined (left), predicted appearance of the line scan based on diffusion inward from the nuclear envelope (centre), and pattern of residuals that demonstrates the difference (i.e. error) between the actual data and the model simulation (right). The predicted appearance was based on Fick's second law of diffusion, using the value of D_n (14.2 μm²/s) that was determined by non-linear regression from the experimental data shown on the left. The experimentally observed and predicted line scans (left and centre respectively) are represented according to the grey scale used in Figure 1. The residuals map (right) is also represented by a grey scale, in which underestimates of the data are dark grey to black, and overestimates are light grey to white. Note that the errors in the model are small and that no systematic pattern is evident. Total elapsed time (from top to bottom) is 6 s, and total distance across the nucleus is 4.42 μm. Middle, graphical representation of the experimental versus predicted values for Ca_n²⁺ at four

caffeine (20 mM). This concentration of caffeine inhibits both Ins(1,4,5)P₃ receptor-mediated and ryanodine receptor-mediated increases in Ca_i²⁺, each of which may contribute to Ca_i²⁺ signalling in hepatocytes [11,18]. Inhibition of Ins(1,4,5)P₃ receptor-mediated Ca²⁺ release occurs by inhibition of both Ins(1,4,5)P₃ formation [19] and Ins(1,4,5)P₃ receptor activation [20], whereas the apparent inhibition of Ca²⁺ release from ryanodine receptors actually results from increasing the open probability of these Ca²⁺ channels [21,22], which then leads to the premature discharge of the associated Ca²⁺ stores [11]. Moreover, this concentration of caffeine has been shown to slow the speed of Ca_i²⁺ waves in hepatocytes [11]. Since Ca_n²⁺ waves may begin by release of Ca²⁺ from channels along the nuclear face of the nuclear envelope [6], it would be useful to know the extent to which caffeine permeates the nuclear interior. The caffeine concentration in the nucleus was not measured here, but it is probably similar to the caffeine concentration of the medium and the cytosol, since cytosolic caffeine concentration equilibrates with the extracellular space within 10 s [19], and since molecules under 500 Da freely exchange between the nucleus and cytosol [23]. Here we confirmed that caffeine slowed the speed of vasopressin-induced Ca_i²⁺ waves, from 49 ± 5 to 26 ± 3 μm/s ($P = 0.001$; $n = 14$ each). In contrast, caffeine had no effect on the speed of Ca_n²⁺ waves, which was 26 ± 2 μm/s without and 24 ± 3 μm/s with caffeine ($P = 0.26$; $n = 12$ and 17 respectively). Thus in cells stimulated with vasopressin alone, Ca_i²⁺ wave speeds were twice Ca_n²⁺ wave speeds ($P = 0.001$ by paired t test), whereas cytosolic and nuclear Ca²⁺ wave speeds were no different in cells treated with caffeine before stimulation with vasopressin ($P = 0.10$ by paired t test). These findings demonstrate that Ca²⁺ waves travel faster in the cytosol than in the nucleus, and that cytosolic but not nuclear Ca²⁺ wave speed depends on a caffeine-sensitive Ca²⁺-release mechanism.

Since Ca²⁺ is discharged into the nucleus from release channels along the nuclear envelope [6,7], and since our current findings plus previous observations [6,16,17] together suggest that there are not additional Ca²⁺ uptake or release mechanisms within the nuclear interior, we hypothesized that Ca²⁺ waves must cross the nucleus solely by diffusion. To test this hypothesis, we modelled Ca_n²⁺ using Fick's second law of diffusion:

$$\partial \text{Ca}_n^{2+}(x, t) / \partial t = D_n \partial^2 \text{Ca}_n^{2+}(x, t) / \partial x^2$$

where D_n is the diffusion constant for Ca_n²⁺. Since release of Ca²⁺ into the nucleus is independently controlled by Ins(1,4,5)P₃ [8] and possibly cADPR [6], the Ca²⁺ signals measured over time at the nucleus/cytosol interface were taken as the boundary conditions for this equation. We estimated the value of D_n for each of eight representative experiments by minimizing the least-squared error between the experimentally observed Ca_n²⁺ signalling patterns and the values predicted by simulating this model [14]. The model described each data set closely (Figure 2); each estimate of D_n was well determined, with 95% confidence limits that were within 14% of the estimates on average, and residual errors were small and without systematic patterns. These

different locations in the nucleus. Tracing 1 is taken from the left edge of the nuclear interior, tracing 2 is from one-third of the distance across the nucleus (1.47 μm from the left edge), tracing 3 is from two-thirds of the distance across the nucleus (2.95 μm from the left edge), and tracing 4 is from the right edge of the nuclear interior (4.42 μm from the left edge). Tracings are offset for clarity. Bottom, sensitivity analysis of the diffusion-based model. The sensitivity coefficient, $\partial \text{Ca}_n^{2+} / \partial D_n$, was evaluated near the nuclear boundary (tracing 1) and near the centre of the nucleus (tracing 2). Note that the coefficient is nearly 0 when Ca_n²⁺ is at steady-state (before $t = 3$ and after $t = 4.5$ s) and is greater near the centre of the nucleus than near its boundary.

findings suggest that propagation of Ca^{2+} waves into the nuclear interior can be described by diffusion of the Ca^{2+} released from the nuclear envelope.

A sensitivity analysis of the model was performed to determine the extent to which changes in D_n would result in changes in the predicted value for Ca_n^{2+} . This analysis helps define the experimental conditions under which reliable estimates for D_n can be obtained, since changes in the data will only lead to changes in the estimate for D_n if the sensitivity coefficient $\delta\text{Ca}_n^{2+}/\delta D_n$ is sufficiently elevated [24]. Here, the sensitivity coefficient was only elevated during the 1–2 s that Ca_n^{2+} was actively changing in the nucleus, and the coefficient was much greater near the centre of the nucleus than near the nuclear envelope (Figure 2, bottom). This sensitivity analysis demonstrates the importance of using confocal line-scanning microscopy to obtain the data for estimates of D_n in nuclei within intact cells, since these estimates critically depend on measurements obtained during the brief time interval and in the precise subnuclear region in which $\delta\text{Ca}_n^{2+}/\delta D_n$ is elevated.

Estimates for D_n from the eight data sets fell into two groups. One of the two groups contained four of the eight estimates for D_n , which were very similar ($18.2 \pm 3.9 \mu\text{m}^2/\text{s}$; range, 14.2–22.2 $\mu\text{m}^2/\text{s}$). These values are in close agreement with estimates for the diffusion coefficient D for Ca^{2+} in cytosol that have been reported in two other studies [3,25]. In one of the two studies, a combination of patch clamp and fluorescence imaging techniques was used to identify the effects of endogenous (mobile and immobile) Ca^{2+} buffers plus the exogenous buffer fura 2 on the apparent D [25]. From these measurements, the actual diffusion coefficient for Ca_i^{2+} was calculated to be 10–40 $\mu\text{m}^2/\text{s}$ [25]. In a separate study, D was estimated instead by examining the spatial distribution over time of $^{45}\text{Ca}^{2+}$ injected into tubes of cytosol extracted from *Xenopus* oocytes [3]. In that study, estimates for D ranged from 13 to 38 $\mu\text{m}^2/\text{s}$ when Ca^{2+} was 10–100 nM [3]. By estimating that D_n lies in this range, the present study therefore suggests that the diffusion coefficient for Ca^{2+} is very similar in nucleus and cytosol. Moreover, this work demonstrates a technique by which D_n can be estimated in an intact functioning cell responding to a physiological stimulus.

Values for D_n estimated from the second group of data sets were much higher than those estimated from the first group, described above. The estimated values for D_n for this second group ($n = 4$) were $133.5 \pm 42.2 \mu\text{m}^2/\text{s}$ (range 96.7–180.9 $\mu\text{m}^2/\text{s}$; $P < 0.001$ relative to the first group). To understand the basis for this finding, we examined the relationship between D_n and the relative increase in Ca_n^{2+} [measured as (peak nuclear fluorescence)/(baseline nuclear fluorescence)]. We found that the relative increase in Ca_n^{2+} in response to vasopressin stimulation was higher among those nuclei with higher values for D_n ($P = 0.011$). This observation suggests that Ca_n^{2+} rose higher in those nuclei, or that fluo 3 loading was greater in them, or both. Indeed, it has been shown that D increases with increasing free Ca^{2+} [3], which probably reflects that Ca^{2+} buffers in cytosol become saturated as Ca_i^{2+} increases, in turn allowing Ca_i^{2+} to diffuse more quickly [3]. It also has been shown that higher concentrations of fura 2 will result in a higher apparent value for D [25]. Together, these findings therefore suggest that the higher values we obtained for D_n in this second group of nuclei represent overestimates of the actual diffusion constant. These findings also support the idea that measurements of D for Ca^{2+} will be most accurate if increases in free Ca^{2+} are not excessive and if dye loading is minimized [25,26].

Since mathematical models can sometimes describe experimental data even if the models inaccurately reflect the physical events involved [27], we examined whether this same diffusion-

Figure 3 Demonstration that Ca_i^{2+} waves do not propagate by simple diffusion

Shown are a typical line scan demonstrating Ca_i^{2+} over time (left), the predicted appearance of the line scan (centre), and the pattern of residuals that demonstrates the error between the actual data and the model simulation (right). The predicted appearance was based on the same diffusion-based model used to describe Ca_n^{2+} waves, and D here (263.4 $\mu\text{m}^2/\text{s}$) was also determined by non-linear regression from the experimental data shown on the left. The experimentally observed and predicted line scans (left and centre respectively) and the residuals map (right) are represented by grey scales, as in Figures 1 and 2. Total elapsed time (from top to bottom) is 6 s, although here the spatial resolution is 1 $\mu\text{m}/\text{pixel}$, so that total distance across the cell is 22 μm . In contrast with the case for the nucleus, here the mathematical model systematically overestimates Ca_i^{2+} near the left boundary of the cell (where the Ca_i^{2+} wave originates) and underestimates Ca_i^{2+} near the right boundary. This in turn reflects that the predicted (i.e. diffusional) wave dissipates as it travels from left to right across the cytosol, whereas the actual Ca_i^{2+} wave regenerates.

based model could describe Ca_i^{2+} waves in these hepatocytes. Ca_i^{2+} waves in hepatocytes [5,11] and other cell types [1,5] result from a reaction-diffusion mechanism whereby Ca^{2+} is sequentially released from Ca^{2+} stores that are distributed across the cytosol. We fitted this model to the data from each of three representative experiments in which Ca_i^{2+} waves were measured (Figure 3). In contrast with Ca_n^{2+} waves, values of D for Ca_i^{2+} waves were all greater than 300 $\mu\text{m}^2/\text{s}$, which is much higher than previous estimates [3,25,28–30], and were poorly determined. Moreover, large residual errors with systematic error patterns were observed (Figure 3). These findings confirm that cytosolic Ca_i^{2+} waves cannot be adequately modelled by simple diffusion and suggest that the good ability of this model to describe nuclear Ca_n^{2+} waves probably reflects the fact that diffusion is the responsible mechanism.

In cytosol, the effective range of action for $\text{Ins}(1,4,5)\text{P}_3$ is over 20 μm , whereas for Ca^{2+} the range is under 1 μm [3]. Endogenous Ca^{2+} buffers and organelles that sequester Ca_i^{2+} are largely responsible for limiting the range over which Ca_i^{2+} released from a single site will spread [3], although closely spaced Ca^{2+} -release sites across the cell permit Ca_i^{2+} waves to propagate over many micrometres nonetheless [5]. In rat hepatocytes, the nucleus can be up to 8–10 μm in diameter, but Ca^{2+} storage and release sites for this organelle are limited to the nuclear envelope. Since Ca^{2+} regulates processes deep within the nucleus, including DNA synthesis and repair, transcription and apoptosis [31], Ca^{2+} fluxes at the nuclear envelope must therefore be able to regulate Ca_n^{2+} at relatively distant sites. Our work suggests that the geometry and perhaps the internal milieu of the nucleus provide a uniquely protective environment in which Ca_n^{2+} diffuses rapidly over several micrometres without significant dissipation of its con-

centration gradient. This in turn permits nearly uniform signalling patterns throughout the nucleoplasm.

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