Biophysical Journal, Volume 99

Supporting Material

RhoA regulates calcium independent periodic contractions of the cell cortex

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Materials and Methods

Cell culture and reagents

Swiss 3T3 and CHO cells were grown in medium containing 10% FBS (Biowhittaker Lonza, Basel, Switzerland) and 4 mM L-glutamine (Gibco, Invitrogen, Carlsbad, CA.) in 4.5 g/L D-glucose DMEM (Gibco) for 2 days to confluency and trypsinized with 1x trypsin-EDTA (Gibco) for 5 min. The supernatant was then spun down at <1000 RPM and the cells were resuspended in 5 mL of media. 1 mL of this suspension was then added to 35 mm Mat Tek (Mat Tek corporation, Ashland, MA.) glass bottom dishes, along with an additional 1.5 mL of media. Reagents were added at the time of cell plating unless otherwise noted; these included thapsigargin 1.3 μM in DMSO (Invitrogen, Carlsbad, CA.), colcemid 1μM in DMSO (Sigma-Aldrich, St. Louis, MO.), colchicine (10 μM in dH_2O, Sigma), LPA 1-8 μg/mL in DMSO (Sigma), calyculin 25 nM (Sigma), Y-27632 10μM in DMSO (Invitrogen), H-1152 10μM in DMSO (EMD4 Biosciences, Gibbstown, NJ.) and orange calcein-am 20 μg/mL in DMSO (Invitrogen). Serum free experiments were performed on cells grown in serum and then plated in serum free DMEM as described above. Calcium free experiments were performed on cells plated in serum free media; the media was removed 1 h after plating and cells were washed and then incubated in DMEM media containing no serum and no calcium. For siRNA experiments, Swiss 3T3 cells were grown for 2 days prior to transfection. The human RhoA ON-TARGET plus SMART-pool siRNA set and a scrambled siRNA set (Dharmacon, Lafayette, CO.) were transfected using an Amaxa Cell Line Nucleofector Kit R (Lonza) and electroporated using an Amaxa electroporator (Lonza). We also tested the effects of electroporation on oscillations. Cells were then re-plated for the experiments 24 h after electroporation according to the above protocol.

Phase contrast imaging

Cells were transferred to a Nikon Diaphot 300 microscope (Melville, NY.) with a 37˚C open perfusion microincubator (model PDMI-2, Harvard Apparatus Inc., Holliston, MA.) and a 5% CO_2 solution was perfused into the chamber. Phase contrast images were taken at 10 s intervals at 20x magnification for a period of 0.5-2 h after cell plating using a Hamamatsu (Bridgewater, NJ.) dual mode cooled CCD camera C4880 and recorded using Metamorph 8.0 software (Molecular Devices, Downington PA.).

DIC and orange calcein-am fluorescence imaging.

After the 25 min incubation time, cells were transferred to an Olympus (Center Valley, P.A.) IX-81 microscope. The temperature was maintained at 37˚C by a Warner Instruments (Hamden, C.T.) incubator and a 5% CO_2 solution was perfused into the chamber. Images were obtained with a SensiCam QE CCD camera (Cooke Corporation, Romulus, M.I.) at 10s intervals and recorded and processed with Metamorph 8.0 software. DIC images were taken at 60x magnification. Orange calcein-am fluorescence imaging was interspersed sequentially with phase contrast at 20x magnification.

Quantification of cell oscillations

The area of analysis was defined for each cell individually, as cells varied in size and shape. The area of analysis included only single cells not in contact with other cells (see Fig. S1 *A* and Fig. S2). All cells in the population meeting this criterion were included in the population analysis. We developed a Matlab (Mathworks, Natick, MA.) program to automate the analysis of isolated cells. For each frame, the center of intensity (X_{CI}, Y_{CI}) was calculated as follows:

$$\left(X_{\mathrm{CI}}, Y_{\mathrm{CI}}\right) = \sum_{j=1}^{r} \sum_{i=1}^{c} I_{ij} \left(x_{ij}, y_{ij}\right) / \sum_{j=1}^{r} \sum_{i=1}^{c} I_{ij} \qquad \text{(S1)}$$

where the sums are over the *r* rows and *c* columns of the selected area and I_{ij} and (x_{ij},y_{ij}) are the intensity and position of the *(i, j)*th pixel, respectively. A time series for the center of intensity consisting of 256 frames (corresponding to 50 min) was used to compute the power spectrum of the Fourier transform for all experiments except those in which the media was changed or a reagent was added after the onset of oscillations; in such cases, 128 frames corresponding to 25 min were used for each experiment. This observation window was chosen as the ideal time frame based on the fact that the cells maintained a fairly consistent morphology during this time, were well attached, and did not move out of the frame. We excluded period values above 400 s from the power spectrum, as these correspond to irrelevant longer time scale trends in the signal. We then normalized the remainder of the signal values so that the discrete values added to 1. Power spectra from both the x and y axes (Fig. S1 *A*) were compared for each cell, and the axis exhibiting the largest spectral amplitude meeting our criteria was selected to characterize the oscillation period for that cell. Only cells that oscillated with periods within the range of 40-120 s were considered in the analysis. Cells with periods below 40 s were excluded because our frequency of image acquisition (10 s between frames) was not sufficient to properly sample those oscillations. Cells with periods above 120 s were excluded as outliers because their periods were greater than three standard deviations above the mean period.

To simplify our study of the oscillation period, we limited our analysis to cells with a single frequency. A cell is considered to be oscillating with a single frequency if the following criteria are met: i) the largest peak amplitude value must be greater than three standard deviations above the average amplitude of the spectrum (as seen in Fig. S1 *D* and *E*); ii) when more than one peak is above threshold and the associated periods differ by more than 10s, the maximum peak must have a spectral amplitude at least twice as large as that of the secondary peak (as seen in Fig. S1 *E*). If the difference between two peaks is less than 10s, we use the largest peak as representative of the cell's period. Fig. S1 *C* and *D* represent non-oscillating cells based on these criteria, whereas Fig. S1 *E* represents an oscillating cell. Power spectra from both the x and y axes (Fig. S1 *A*) were compared for each cell, and the spectrum from the axis exhibiting the largest amplitude meeting our criteria was selected to characterize the oscillation period for that cell. (See also Supplementary Movies S1-S3)

The amplitude of the signal was determined from averaging the difference between each consecutive maximum and minimum (extrema) of the center of intensity signal along a given axis. The amplitude is thus defined as:

$$\frac{\sum_{i=1}^{N}\left|x_{i+1}-x_i\right|}{N-1} \qquad \text{(S2)}$$

where N=number of extrema, i=index of extrema, and x_{i+1}- x_i is the particular amplitude. We also categorize all cells that have average amplitudes less than that which was observed by the same analysis of the background light transmission of a phase contrast image (equivalent to 0.05 μm) as non-oscillating.

We confirmed that the use of phase contrast was a valid measurement for changes in the distribution of cell volume during the oscillatory cycle by co-imaging the fluorescent volume indicator, calcein-AM, with phase contrast. The center of mass for both signals was calculated as described above. To compare signals, we normalized the amplitudes of both signals to show that the first moment of the volume signal and phase contrast intensity of the oscillating cell are essentially in phase and have the same period (Fig. S1 *B*).

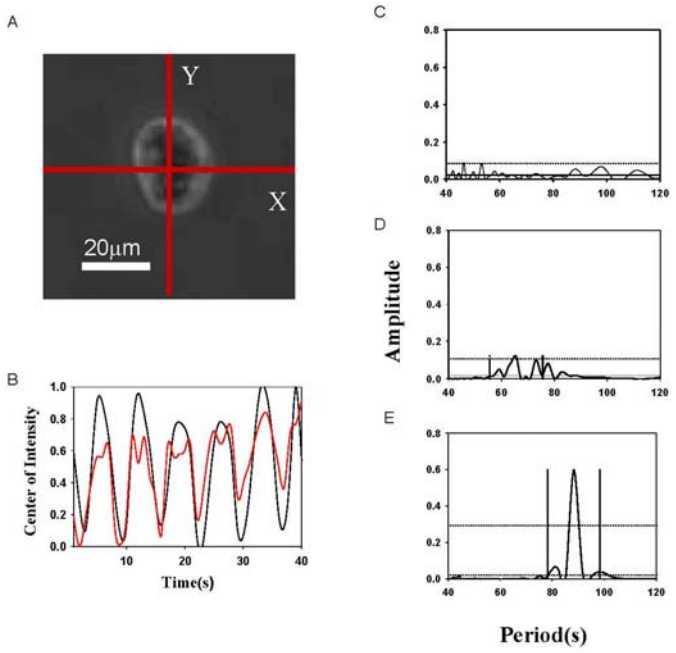
Immunofluorescence and f-actin and nuclear staining

After fixation with 3.7% formaldehyde for 15 min at 37°C, cells were permeabilized with 0.1% Triton-X100 for 20 min. Cells were then washed with PBS and stained with AlexaFluor-594-phalloidin (Molecular Probe) for 20 min or with 500 nM DAPI (4',6-diamidino-2-phenylindole, Molecular Probe) in PBS at room temperature for 5 min.

Rabbit phospho-myosin light chain 2 (Ser19) antibody (anti-pMLC; Cell Signaling) was used for indirect immunostaining of phosphorylated myosin light chain. For indirect immunostaining, cells were plated on glass bottom dishes (Mattek-35mm) with a 10 μg/ml fibrinogen precoating. 10 μM colchicine was added during the plating. Cells were imaged using phase contrast microscopy every 10 s to ensure that they exhibited morphological oscillations. After 30 min of defined oscillations, cells were fixed with 4% formaldehyde for 15 min, washed and incubated for 1 h in blocking buffer (PBS+5% goat serum+0.4% Triton), then washed with PBS and incubated overnight in 4ºC with rabbit anti-pMLC. Cells were then incubated for 2 h at room temperature with fluorescently labeled secondary antibodies (Alexa Fluor488 goat anti-rabbit, 1:2000, Invitrogen). Images were acquired using an Olympus FluoView1000 confocal scanning microscope Olympus FluoView1000 with a 60x water immersion objective

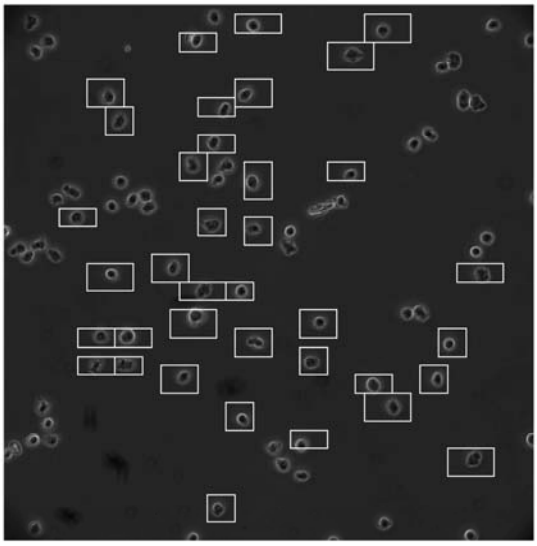
Images were exported as Tiff files and analyzed using ImageJ and ImageSurfer (UNC, (www.imagesurfer.org)) software. Our Matlab code, described above, was used for analysis of nucleus movement and determination of oscillation period.

Supplementary Figures and Movies



Supplementary Figure 1: Method of Analysis. In our analysis, each oscillating cell is analyzed for displacement of the center of phase contrast intensity as a function of time. *(A)* Phase contrast (20x) image of a spreading Swiss 3T3 cell treated with colchicine (bar =20 μm). The area of analysis is designated by hand. The *x* and *y* axes are analyzed individually to find the center of intensity as described in Materials and Methods (Supporting Material). *(B)* The temporal variation of the position of the first moment of intensity along a single axis from both calcein-orange (red) and phase contrast (black) images of an oscillating cell are shown. In contrast to the phase contrast signal, (the fluorescent signal is subject to probe bleaching. Both the phase contrast and the fluorescence signal are in phase and give similar periods but the amplitude of the fluorescent signal is smaller by a factor of 10 as compared to the phase contrast signal. Fig. S1 *C-E* give examples of Fast Fourier Transform power spectra calculated from the excursions of the first moment of intensity along a single axis for three different cells imaged with phase contrast. Several criteria were employed so that only cells with highly periodic signals were used for analysis. In each figure, the two horizontal dotted lines are the mean amplitude and the "mean + the 3 std dev" amplitude value. The vertical dotted lines show the 10 s interval surrounding the maximum amplitude value. Supplementary videos of each cell are available (Movies S1-S3). In Fig. S1 *C*, the cell has no predominant power spectrum peak and is therefore considered non-oscillating. In Fig. S1 *D*, the cell has frequency peaks above the threshold of "mean + 3 std dev" that we

established, however, these frequencies are more than 10 s apart and the satellite peaks are more than ½ the dominant peak. Therefore we do not include this cell as oscillating. In Fig. S1 *E*, the cell has a dominant peak that meets the aforementioned criteria, and we therefore consider this cell as "oscillating."



Supplementary Figure 2: 20x phase contrast image of spreading confluent Swiss 3T3 cells treated with colcemid. The white boxes are selected by hand in Matlab for analysis of the center of intensity within the area over time. Only cells not in contact with other cells were chosen for the analysis.

Supplementary Movies:

S1 Non-oscillating cell: Swiss 3T3 cells were imaged at 20x in phase contrast. The FFT spectrum of this cell is shown in the Methods text (Fig. S1 *C*). The movie was recorded 1 h after plating with a timelapse interval of 10s.

S2 Multi-frequency contracting cell: The FFT spectrum of this cell is shown in the Methods text (Fig. S1 *D*). This cell is not considered oscillating because it contracts with more than one frequency.

S3 Oscillating cell: The FFT spectrum of this cell is shown in the Methods text (Fig. S1 *E*). This cell is considered "oscillating" because it contracts with a single frequency.

S4 DIC images of oscillating cell: Movie corresponding to the 60x DIC image used in the kymograph in Figure 1. Swiss 3T3 cell was plated in 10 μM colchicine. The movie was recorded 1 h after plating with a timelapse interval of 10 s.

S5 Nuclear oscillations: Timelapse of DIC co-imaged with nuclear fluorescence images (See Fig. 6 *D*).

S6 3D reconstruction of a high fluorescence isovalue of f-actin, active myosin and nucleus: See Fig. 7 *F,G*.