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Spectral components of cytosolic $[Ca^{2+}]$ spiking in neurons

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We show here, by means of evolutionary spectral analysis and synthesis of cytosolic Ca^{2+} ($[Ca^{2+}]_c$) spiking observed at the single cell level using digital imaging fluorescence microscopy of fura-2-loaded mouse cerebellar granule cells in culture, that $[Ca^{2+}]_c$ spiking can be resolved into evolutionary spectra of a characteristic set of frequencies. Non-delayed small spikes on top of sustained $[Ca^{2+}]_c$ were synthesized by a main component frequency, 0.13 2 ± 0.01 2 Hz, showing its maximal amplitude in phase with the start of depolarization (2 5 mM KCl) combined with caffeine (1 0 mM) application. Delayed complex responses of large $[Ca^{2+}]_c$ spiking observed in cells from a different set of cultures were synthesized by a set of frequencies within the range 0.018–0.11 7 Hz. Differential frequency patterns are suggested as characteristics of the $[Ca^{2+}]_c$ spiking responses of neurons under different conditions.

Key words Caffeine; $[Ca^{2+}]_c$ spiking; Cerebellar granule cells; Spectral components

Introduction

Since Cobbold and collaborators reported that oocytes[1] and hepatocytes[2] can give rise to oscillatory signals in cytosolic free $[Ca^{2+}]$ ($[Ca^{2+}]_c$), oscillation in $[Ca^{2+}]_c$ has been reported in excitable pituitary cells[3] and in many other non-neuronal cells.[4,5] Glutamate-specific sustained $[Ca^{2+}]_c$ oscillations and propagating waves observed in cultured astrocytes suggest a role for glial long-range Ca^{2+} signalling within the brain.[6] In the CNS, nerve fibre stimulation triggered $[Ca^{2+}]_c$ oscillations in periaxonal glial cells in the optic nerve.[7] Oscillatory $[Ca^{2+}]_c$ signalling is less well documented among neurons; however, examples such as caffeine-induced rhythmic hyperpolarizations and depolarizations,[8] as well as $[Ca^{2+}]_c$ oscillations[9] in sympathetic neurons or spontaneous fluctuations of $[Ca^{2+}]_c$ in cultured rat hyppocampal neurons[10] indicate the occurrence of oscillatory $[Ca^{2+}]_c$ signalling within the neurons as well. Our findings that mild depolarization applied to cultured mouse cerebellar granule cells induced significant $[Ca^{2+}]_c$ fluctuations,[11] together with the novel mechanism for Ca^{2+} channel modulation in these cells (coupling of ryanodine receptors (RyRs) to Ca^{2+} entry through L-type Ca^{2+} channels by type-1 metabotropic glutamate receptors produced a cyclical facilitation of the L-type Ca^{2+} channel)[12] hinted at oscillatory $[Ca^{2+}]_c$ signalling in these cells. In this study we investigated whether a non-stationary stochastic process such as caffeine-induced $[Ca^{2+}]_c$ spiking in cultured mouse cerebellar granule cells could be quantitatively depicted. As the classical fast Fourier transformation formalism assumes stationarity, the method of evolutionary spectral analysis seemed to be more appropriate. Based on the theory of evolutionary spectra,[13] we used the technique of complex demodulation[13] for its continuous time resolution and favourable statistical properties.[14]

Materials and Methods

Pregnant mice were obtained from the animal quarters at the Panum Institute (Copenhagen, Denmark). Petri dishes (3 5 mm) were purchased from NUNC A/S (Denmark), No. 1 glass coverslips from Mentzel (Germany) and fetal calf serum from Sera-Lab Ltd (Sussex, UK). 4,5,6,7-Tetrahydro-isoxazolo-[5,4-c]-pyridin-3-ol (THIP) was kindly provided by P. Krogsgaard Larsen (Royal Danish School of Pharmacy). Polylysine (mol. wt > 300 000), trypsin inhibitor, DNAse, amino acids , *p*-aminobenzoic acid, cytosine arabinoside, HEPES and caffeine were obtained from Sigma Chemical Co. (St Louis, MO. USA), insulin from Novo Nordisk (Denmark) and penicillin from Leo (Demnark). Caffeine was

dissolved in HEPES-buffered salt solution pH 7.4 (HBS) containing 135 mM NaCl, 5 mM KCl, 0.6 mM $MgSO_4$, 10 mM glucose, 10 mM HEPES prepared with either 1.8 mM $CaCl_2$ or without Ca^{2+} ion added, or with 25 mM KCl. HBS buffer containing 25 mM KCl was prepared by addition of KCl and equimolar reduction of NaCl concentration. Stock solution of fura-2 acetoxy methylester (fura-2/AM, Molecular Probes, Eugene, OR, USA) was freshly prepared in dimethylsulphoxide (DMSO).

Cerebellar granule neurons obtained from dissociated cerebella of 7-day-old mice were cultured for 7 days.[11] THIP (150 μM) was added to the cultures after 2–4 days *in vitro*.[11] The cell suspension was plated into Petri dishes housing glass coverslips.[15] On the day of the experiment, the cell cultures were incubated in the presence of 3 μM fura-2/AM for 20 min in the dark at 37°C then washed with fresh medium and incubated for 30–60 min before a change to HBS and transfer to the microscope stage (loading and washing). The glass coverslip with the loaded cells, appropriately fixed in a superfusion chamber,[15] was placed on the stage of the microscope in a thermostatted (37°C) black box. HBS buffer, maintained at 37°C, was superfused at 2 ml/min using a peristaltic pump. Detecting the fura-2 epifluorescence emission at 510–535 nm, $[Ca^{2+}]_c$ measurements were performed with the technique of double excitation (340 nm and 380 nm) ratio imaging combined with an inverted Zeiss Axioconvert 100 TV microscope and an intensified CCD-camera (C-4700, Hamamatsu Ltd, Japan) as described elsewhere.[15] Images were acquired automatically the digitized image pairs, stored in a database and processed on a pixel-to-pixel basis to calculate $[Ca^{2+}]_c$ values for a selected region of neurons.[15] Calibration was performed using Calcium Calibration Buffer Kit II (Molecular Probes Eugene, OR, USA).

Values of $[Ca^{2+}]_c$ from each of the neurones in the group selected, automatically arranged separately in a spreadsheet, were used for the spectral analysis and synthesis of the observed $[Ca^{2+}]_c$ spiking. Evolutionary spectra for the component frequencies of $[Ca^{2+}]_c$ spiking in the soma were obtained in two steps. First the component frequencies were determined by autoregression (AR) spectra.[16] Using the above AR frequencies as parameters the evolutionary spectra of the $[Ca^{2+}]_c$ signal were obtained in modulation–demodulation sequences (complex demodulation technique).[13] As a local harmonic analysis describing the local power–frequency distribution at each instant of time, complex demodulation can give rise to the time-dependent behaviour of a component frequency present in the stochastic signal by shifting its frequency down to zero (modulation), with a suitably designed low-pass filtering of the modulated signal all the component frequencies but the term in question were removed. Thereafter, the modulated and filtered signal was shifted back from zero to its original frequency (demodulation). $[Ca^{2+}]_c$ spiking in the soma was synthesized by calculating intensity-weighted linear combination of the evolutionary spectra of the component frequencies followed by rectification and by adding the slowly varying trend component. Complex demodulation and other processings were carried out by self-devised programs developed in the Matlab for Windows 4.2c software environment. AR spectrum was estimated by our Matlab adaptation of the memcof procedure of the software library Numerical Recipes.[17]

Results

First values for the resting $[Ca^{2+}]_c$ of cells ($[Ca^{2+}]_{c,r}$) from the particular set of cultures were determined. In cultures characterized by $[Ca^{2+}]_{c,r} = 50$ nM the HBS buffer containing 1.8 mM $CaCl_2$ was applied in the loading, washing and experimental protocols (high-calcium condition). By contrast, in cultures with $[Ca^{2+}]_{C,r} = 200$ nM, the HBS buffer prepared without added Ca^{2+} ion ($[Ca^{2+}]_{buffer} \sim 1$ μM) was applied throughout the above procedures (low-calcium condition). Thus, by taking the advantage of variations in $[Ca^{2+}]_{c,r}$ cultured mouse cerebellar granule neurons with low $[Ca^{2+}]_c$ were conditioned in high-calcium conditions, and *vice versa*.

When exposed to 10 mM caffeine in combination with depolarization (25 mM KCl), cells in the high-calcium condition showed non-delayed small spikes on top of sustained $[Ca^{2+}]_c$ (Fig. 1A). Cells in the low-calcium condition displayed complex responses of large $[Ca^{2+}]_c$ spiking which appeared in the washout period after the application of 10 mM caffeine in HBS buffer containing 1.8 mM Ca^{2+} (Fig. 2A). Thereafter, spectral analysis and synthesis of $[Ca^{2+}]_c$ spiking under low- and high-calcium conditions was performed. AR analysis for the component frequencies of $[Ca^{2+}]_c$ spiking in a group of cultured mouse cerebellar granule cells kept under high-calcium conditions gave frequencies of 0.132 ± 0.012 Hz, 0.409 ± 0.023 Hz, 0.853 ± 0.042 Hz, 1.21 ± 0.05 Hz, 1.56 ± 0.04 Hz, 2.00 ± 0.18 Hz and 2.56 ± 0.29 Hz with relative AR intensities of 1.00, 0.09, 0.01, 0.02, 0.01, 0.01 and 0.01, respectively. Evolutionary spectra for the slowest, main component (0.132 ± 0.012 Hz, $n = 3$) derived by complex demodulation showed that its amplitude was maximal at the start of the application of 10 mM caffeine in HBS buffer containing 25 mM KCl (Fig. 1B). Synthesis of the observed $[Ca^{2+}]_c$ spiking from this single evolutionary frequency component is presented in Fig. 1C. By contrast, AR spectral analysis for the component

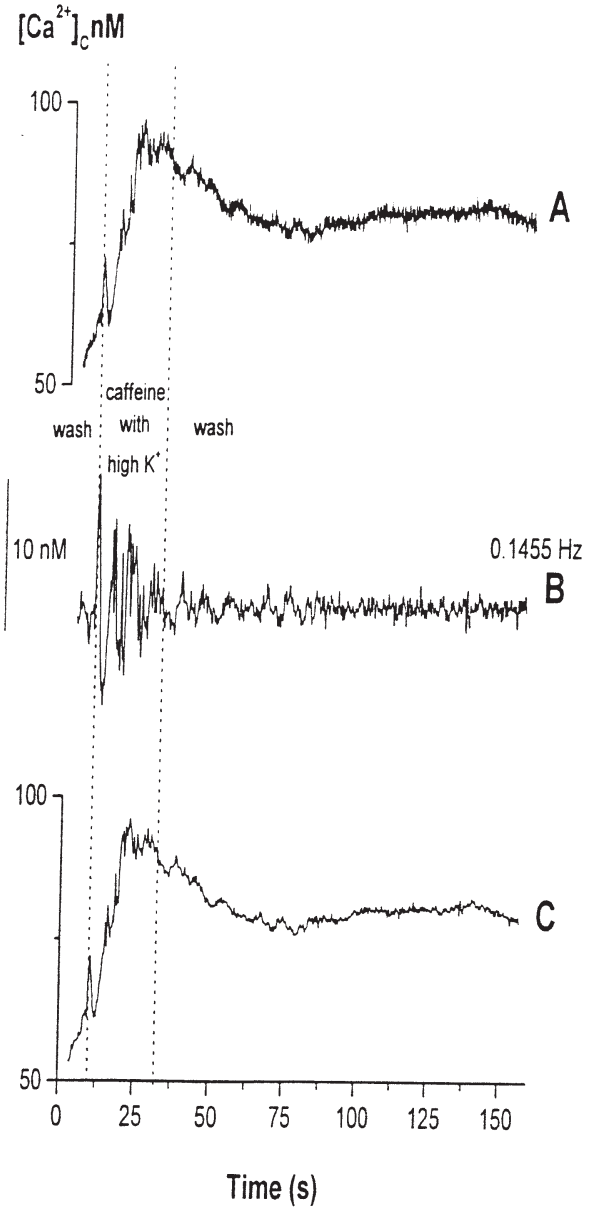

FIG. 1. Spectral analysis and synthesis of $[Ca^{2+}]_c$ spiking observed in the soma of three of eight mouse cerebellar granule cells in culture conditioned and washed with buffer containing 1.8 mM Ca^{2+} ions. Images were acquired every 80 ms for 3 min. Vertical bars indicate the time during which the cells were exposed to depolarization (25 mM KCl) combined with caffeine (10 mM). (**A**) Observed $[Ca^{2+}]_c$ spiking; (**B**) evolutionary spectrum for the main component frequency. As a type of stochasticity, the noise superimposed on the 0.1455 Hz harmonic component was inherent in $[Ca^{2+}]_c$ spiking during this condition. (**C**) $[Ca^{2+}]_c$ spiking synthesized from evolutionary spectrum (see Materials and Methods). Note the maximal amplitude of the 0.1455 Hz component in phase with the start of the application. Cell spiking in each soma showed the same pattern with minor differences in the signal to noise ratio and spike intensities.

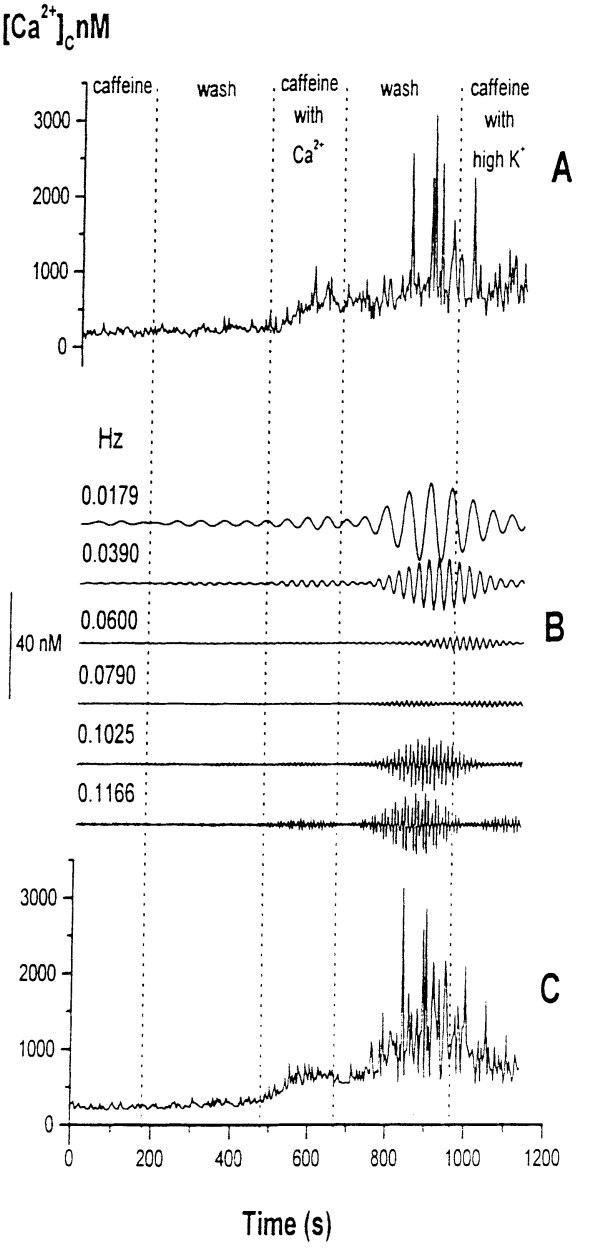

FIG. 2. Spectral analysis and synthesis of $[Ca^{2+}]_c$ spiking in the soma of mouse cerebellar granule cells in culture for nine of 25 cells conditioned and washed in buffer with no Ca^{2+} ion added. Images were acquired every 3.6 s for 19 min. Vertical bars indicate the time during which the cells were exposed to either caffeine (10 mM) or caffeine (10 mM) and Ca^{2+} ion (1.8 mM) or depolarization (25 mM KCl) combined with caffeine (10 mM). (**A**) Observed $[Ca^{2+}]_c$ spiking; (**B**) evolutionary spectra for the component frequencies; (**C**) $[Ca^{2+}]_c$ spiking synthesized from evolutionary spectra (see Materials and Methods). Note the delayed progressive appearances of $[Ca^{2+}]_c$ spikes in the 5 min washout period after a 3 min application of caffeine with Ca^{2+} ion. Cell spiking in each soma showed the same pattern with differences in the signal to noise ratio and spike intensities. The above cell showed the highest signal to noise ratio and definite high intensity spiking.

frequencies of $[Ca^{2+}]_c$ spiking observed in a group of cells in the low-calcium condition gave several slow components of high-intensity in the frequency range 0.018–0.117 Hz. Evolutionary spectra for the component frequencies displayed their maximal amplitudes in the washout period after the application of 10 mM caffeine in HBS buffer containing 1.8 mM $CaCl_2$ (Fig. 2B). Synthesis of the observed $[Ca^{2+}]_c$ spiking from this set of evolutionary frequency components is shown in Fig. 2C. When components seen in the

low-calcium condition and the mean 0.132 ± 0.012 Hz component seen in the high-calcium condition are compared (one-sided test for the difference of the mean *vs* population mean), the following *p*-values were obtained: 0.0179 Hz, $p = 0.0018$; 0.0390 Hz, $p = 0.0028$; 0.0600 Hz, $p = 0.0046$; 0.0790 Hz, $p = 0.0083$; 0.1025 Hz, $p = 0.0255$; 0.1166 Hz, $p = 0.0781$.

Discussion

Cultured mouse cerebellar granule neurons with low (50 nM) and high (200 nM) $[Ca^{2+}]_{c,r}$ were subjected to high- and low-calcium conditions, respectively, to test whether this external challenge, opposite to $[Ca^{2+}]_{c,r}$ would enhance the probability of oscillatory calcium signalling for these neurons. Caffeine-induced $[Ca^{2+}]_c$ spiking in these cells, when transmembrane Ca^{2+} ion influx was facilitated either by depolarization under the high-calcium condition or by a 2000-fold increase of extracellular $[Ca^{2+}]$ ion under the low-calcium condition. These findings were consistent with the recent proposal[12] for a tight functional coupling between ryanodine receptors and L-type calcium channels in these neurons, a mechanism which has also been suggested to provide the capacity of bidirectional signalling.[12]

Caffeine-induced $[Ca^{2+}]_c$ spiking in cultured mouse granule neurons seemed to be quite different under the different conditions: non-delayed small spikes on top of sustained $[Ca^{2+}]_c$ observed under the high-calcium condition were distinguishable from the delayed complex responses of large $[Ca^{2+}]_c$ spiking observed under the low-calcium condition. The possibility that a stochastic process such as $[Ca^{2+}]_c$ spiking could be deterministic[18] led us to examine the spectral components for these different responses of $[Ca^{2+}]_c$ spiking. The non-delayed small $[Ca^{2+}]_c$ spikes could be quantitatively depicted by a single frequency component of 0.132 ± 0.12 Hz, although several other higher frequency and low relative AR intensity noise components also contributed to the response. With a nonlinear activation of RyRs interplaying in the signal one can assume that these noise components are in fact manifestations of the nonlinear dynamics underlying the phenomena. Cyclical facilitation of the L-type channel activity by RyRs has been reported[12] in a similar frequency range with these neurones. The delayed complex responses of large $[Ca^{2+}]_c$ spiking were best fitted with the set of evolutionary spectral components within a somewhat separated range of slower frequencies. We suggest that differential patterns of component frequencies are characteristic of the $[Ca^{2+}]_c$ spiking response of a granule neurone under different conditions and represent simple or complex oscillatory $[Ca^{2+}]_c$ signalling.

Non-linearly coupled oscillators, such as L-type Ca^{2+} channels controlled by RyRs,[12] were assumed to be sources of the frequency-encoded $[Ca^{2+}]_c$ spiking responses of these neurones. Appreciation of differential spectral components of $[Ca^{2+}]_c$ spiking from different neurons may be relevant to bidirectional signalling in the CNS.

Conclusion

Caffeine-induced $[Ca^{2+}]$ spiking of cultured mouse cerebellar granule cells can be described quantitatively by characteristic sets of component frequencies. The resulting frequency fingerprints reveal differential $[Ca^{2+}]_c$ spiking responses of neurones to changes occurred under physiological, adaptive or pathological conditions.

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