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Rescaled Range Analysis applied to the study on intracellular Ca^{2+}

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Abstract: The present study revealed the stimulatory effects of NMDA on intracellular Ca^{2+} concentration in rat dorsal root ganglion (DRG) neurons. Fura-3/AM, an intracellular calcium fluorescent indicator was used to monitor the fluctuation of $[Ca^{2+}]_I$. Using the Confocal microscopy technique and the rescaled range method, Intracellular Ca^{2+} concentration variation was studied. The time-related variation of fluorescence intensity was observed. The Rescaled Range analysis showed that there were persistent correlations. The Hurst coefficients were calculated for four kinds of different external solutions which are 0.559±0.0542(n=13) for 0.5 ml of 2×10^{-4}M NMDA and 3.3mM Ca^{2+} bath solution; 0.8488±0.0805 (n=10) for 0.5 ml of 2×10^{-4} M NMDA and 6.6 mM Ca^{2+} bath solution; 0.8924±0.0679 (n=11) for 0.5 ml of 2×10^{-4} M NMDA and 10mM Ca^{2+} bath solution; 0.9192±0.037 (n=8) for 0.5 ml of 2×10^{-4} M NMDA and 0 mM Ca^{2+} bath solution adding high Ca^{2+} (10mM). These results indicate that persistent correlation for the long intervals can arise from the fact that the natural system is bounded. It also suggests that the mean and the variance of the intracellular Ca^{2+} concentration may not be good measures to estimate the intracellular Ca^{2+} kinetics and to determine the effectiveness of NMDA.

Key words: NMDA, dorsal root ganglion, Confocal microscopy, Fura3/AM, Rescaled range analysis

Introduction

It is generally agreed that NMDA plays a role of extracellular chemical messenger, either as a neurotransmitter or as a co-transmitter acting on peripheral neurons including primary sensory, sympathetic and parasympathetic neurons and on CNS neurons in various brain areas. In recent years, some biologists have used patch-clamp to record ion-channel currents, for example, the currents of NMDA activated channels in mouse CNS neurons in magnesium-free solutions (Lester *et al.,* 1990; Ascher *et al.,* 1988). The inhibition of single NMDA-activated channel by zinc ions on cultured rat neurons (Legendre and Westbrool, 1990) and the single channel's properties of NMDA receptors have been reported (Gao Tian-Ming, 1995). A quantitative description of NMDA receptor-channel kinetic behavior was also reported (Jahr *et al.*, 1990). Distinct NMDA-activated channels in different types of neurons dissociated from rat dorsal root ganglions play an important role in the extracellular calcium ion permeability (Gibb *et al.,* 1992; Laketic-Ljubojevic *et al.,* 1999; Llinas *et al.,* 1992). Imaging techniques had been proved very powerfully in highly localizing gradients of intracellular free calcium concentrations $[Ca^{2+}]_I$ and some published imaging approaches can provide the time resolution and spatial distribution state (Sharon *et al.*, 2000; Funatsu *et al.*, 1995; Oheim and Loerke, 1998; Cleeman and Morad, 1997; Ohei *et al.,* 1997; Piston *et al.,* 1994; Monck *et al.,* 1994 and Macklin *et al.,* 1996). Classic measures of the arithmetic mean and variance are the most widely used statistical measures. It is not always fully appreciated that the usefulness of these measures depends on the properties of the data satisfying certain assumptions. If the data do not match these assumptions, the results of the analysis may not be meaningful. Moreover, the results of the analysis will not be able to lead us toward an understanding of the physical mechanism that generated the data.

A few techniques have been developed for analyzing fractal time series, among which is the rescaled range analysis (Hurst, 1951). The method is comparatively simple and rather faithful (Bassingthwaighte and Raymond, 1994). Hurst himself has found that for many natural phenomena H is, in average, about 0.73, that is, the phenomena exhibited correlation rather than random time series. Many biological processes were successfully examined for their fractal nature by different statistical measures, but the Hurst method was used only in a few cases. Recently in cellular biology, the rescaled range analysis has been used to analyze records in time produced by the mechanical motions of cells growing in tissue culture (Giaever and Keese, 1989) and the R/S analysis was applied to the study of patch clamp records of human T-lymphocytes (Churilla *et al.*, 1996). (Nogueira *et al.*, 1995) shown that for the Ca^{2+}-activated K^+ channels of Leyding cells, the Hurst exponent was equal to 0.75, that is the successive openings and closings assumed to be a process with memory, similarly (Wamberto *et al.*,

2000); (Kochetkov *et al*., 1999) was reported rescaled range method applications.

We used the Hurst method to examine the data obtained on NMDA stimulatory effects on intracellular Ca^{2+} in rat dorsal root ganglion (DRG) neurons. The analysis was aimed at revealing time correlation.

Materials and Methods

Isolation of dorsal root ganglion neurons: Two-Three-weeks-old Sprague-Dawley rats, irrespective of sex, were decapitated and the thoracic and lumbar segments of vertebrate column were dissected and longitudinally divided into two halves along the median lines on both dorsal and ventral sides. The DRGs together with dorsal and ventral roots and attached spinal nerves were taken out from the inner side of each half of the dissected vertebrate and transferred into Dulbecco's Modified Eagle's Medium (DMEM, Sigma) at pH=7.4. After the removal of attached nerves and surrounding connective tissues, the DRGs were minced with iridectomy scissors and incubated with enzymes including trypsin (type III, Sigma) 0.5 mg ml^{-1}, collagenase (type IA, sigma) 1.0 mg ml^{-1} and DNase (type IV, sigma) 0.1 mg ml^{-1} in 5ml DMEM at 35^{0}C in a shaking bath for 40 min. To stop the enzymatic digestion 1.25 mg ml^{-1} soybean trypsin inhibitor (type II-S1, Sigma) was added. The isolated neurons were transferred into a 35-mm culture dish and kept still for at least 30 min. All experiments were performed at room temperature (20-30^{0}C) (Hong-Zhen and Zhi-wang, 1996 and 1997; Hu *et al*., 1997).

External solution, chemicals and drugs: The external solution contained (in mM) N_aCI 150, KCI 5, C_aCI_2 3.3, M_gCI_2 1, HEPES 10, D-glucose 10, its osmolarity was adjusted to 340 m Osm with sucrose and pH was adjusted to 7.4 with N_aOH. Chemicals and Drugs used in the experiments were: NMDA, Fura-3/AM (purchased from Molecular Probe). Other ordinary drugs are purchased from local pharmaceutical companies.

Fura-3 loading: The cells in the holes of the testing plate were washed 3-5 times with external solution after being incubated in 2-4 μM fura-3/AM for 50 min at 25^{o}C. The external solution contains (mM): N_aCI 150, KCI 5, $C_aCI_2$3.3, M_gCI_2 1, HEPES 10, D-glucose 10, PH=7.4.

Intracellular Ca^{2+} measurements: The setup of fluorescence measurement was as follows: Confocal microscopy system MRC1024 (Bio-Rad, USA) and multiphoto excitation fluorescence microscopy A_r^+ Laser (American Laser Corporation), Excitation light at 488 nm or 514 nm was provided by a monophoton system (US) which was controlled by computer and an upright microscopy TE2000 (Nikon, Japanese). The fluorescence signals were collected by a photomultiplier and converted, then digitized and inputted to the computer. The fluorescence intensity was used in the experiments to reflect the fluctuation of $[Ca^{2+}]_I$.

Fluorescence data acquisition and data analysis: We sampled fluorescence with a spatial frequency of 70 nm (at 100x) or 140 nm (at 50x). Images were transferred directly to MB memory of P55/200 MHZ Pentium computer via a high-speed serial link, and taken at an acquisition rate of 10 Hz with an integration time of 60 ms for each image. We scanned consecutive images, and observed the fluorescence intensity variation time-related. We analyzed the effects of NMDA on neurons. At the same time, we computed Hurst exponential of fluorescence intensity with time variation on a sun blade 100 computer using MATLAB software.

Theory of the R/S analysis: The rescaled range analysis or R/S Hurst analysis is used to study records in time or a series of observations in different time. Hurst spent a lifetime studying the Nile and the problems related to water storage. He invented a new statistical method, the rescaled range analysis (R/S analysis) (Hurst, 1951). The problem is to determine the design of an ideal reservoir based upon the given record of observed discharges from the lake. Here the method of Hurst will be introduced and considered as a time dependent function$|\hat{I}_{i\xi}$, we divide it into N(T) adjacent segments, each of T points. To perform the rescaled range analysis requires that we compute a quantity called R/S for each T. For eliminating possible trend influence, the mean of the nth segment of length T is first computed:

$$<|\hat{I}>_{n,T}= \quad \frac{1}{T} \sum_{i=(n-1)T+1}^{nT} \xi \; i$$

The standard deviation $S_{n,T}$ of the nth segment of length T is defined as

$$S_{n,T} =[(\frac{1}{T})]^{½} \sum_{i=(n-1)T+1}^{nT} (\xi_i - < \xi >_{n,T})^2$$

For each point I in the time series, we compute

$$X_{n,T}(I)= \sum_{k=(n-1)T+1}^{i} (\xi(k) - < \xi >_{n,T})$$

$R_{n,T}$=max($X_{n,T}$(I))-min($X_{n,T}$(I))

Computed the rescaled range $(R/S)_{n,T}$ of that segment.

$$(R/S)_{n,T} = \frac{R_{n,T}}{S_{n,T}}$$

and averaged the rescaled ranges computed from the segments,

$$(R/S)_T = (\frac{1}{N(T)}) \sum_{n=1}^{N(T)} (R/S)_{n,T}$$

where N(T)=N_t/T.

We calculated the rescaled ranges for different duration T, and the logarithm of $(R/S)_T$ is plotted versus the logarithm of T. The slope of this plot is H, the Hurst coefficient. When 0<H<0.5, the self-similar correlations at all time scales are antipersistent, that is, increases at any one time are more likely to be followed by decreases over all later time scales. When H=0.5, the self-similar correlations are uncorrelated. When 0.5<H<1, the self-similar correlations at all time scales are persistent; that is, increases at any one time are more likely to be followed by increases over all later time scales.

Results

In this study, Confocal microscopy was used to investigate spatiotemporal regime of intracellular Ca^{2+} dynamics in rat dorsal root ganglion cells both at rest and at stimulation. Experiments were carried out on 42 freshly-isolated DRG neurons, the diameter of which were in the range of 15~60μm. The frequency distribution of the diameters is shown in the bar chart in Fig.1. The result was consistent with simulation result (Hu *et al.*, 1997).

Stimulatory effects of NMDA on the different cells imaging shown: Of all the cells which responded to the NMDA stimulus in different external solutions, Ca^{2+} concentration was showed FI change. Imaging techniques had also shown intracellular $[Ca^{2+}]_I$ concentration variation of different diameter cells at different time. At the same time, it could show its spatial distribution (image not shown). Here was shown FI variation after experiment beginning 40 second adding 0.5 ml of 2 $\times 10^{-4}$M NMDA and 3.3 mM Ca^{2+} bath solution (Fig. 2), (other situation not shown).

Stimulatory effects of NMDA on different external solution: Fig. 3 shows traces of FI recorded at different Ca^{2+} concentration external solutions and after experiment beginning 40 s adding 0.5 ml 2 x 10^{-4} (M) NMDA. After experiment beginning 40s adding NMDA, FI increase phase arisen, as FI increase maximum value, generally decrease trend arisen, then variation trend is kept up and down. Above situation happen condition was at external solution Ca^{2+} concentration 3.3, 6.6 and 10 M,

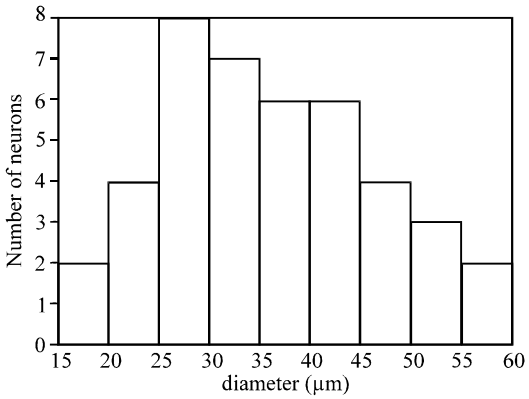

Fig. 1: The histogram of diameter distribution of neurons (n=42)

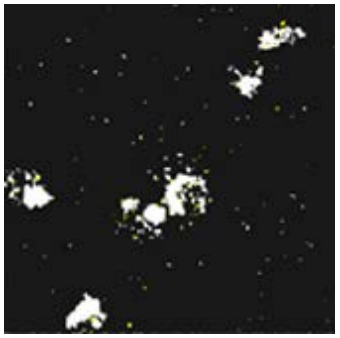

Fig. 2: Shows intracellular F1 after experiment beginning 40 sec adding 0.5 ml of 2x10^{-4} M NMDA and 3.3 mM Ca^{2+} hath solution

Table 1: Experimental values of H

Cell external solution concentration (mM)	H (mean±S.D.)	N
3.3mM	0.559±0.0542	13
6.6mM	0.8488±0.0805	10
10mM	0.8924±0.0679	11
0-C_a^{2+} adding high C_a^{2+} external solution(10mM)	0.9192±0.037	8

respectively. In particular, The application of 0.5 ml 2x10^{-4} M NMDA In 0-Ca^{2+} bath solution, evoked the decrease in $[Ca^{2+}]_I$. 5 minute after adding 500μl high Ca^{2+} solution (10 mM), results indicated that the NMDA-triggered $[Ca^{2+}]$I elevation may be attributed to extracellular Ca^{2+} entry. 8 min after adding 500μl high Ca^{2+} solution (10 mM), intracellular Ca^{2+} concentration continued to increase, which further indicated that the normal function of the NMDA channel opening may be dependent on some intracellular biochemical processes and may have correlation with anamnesis. Fig. 3a,b,c,d shown the cells intracellular Ca^{2+} concentration at different concentration respectively..

Instability of mean and variance: The classical analysis assumes that the sample means and variances will

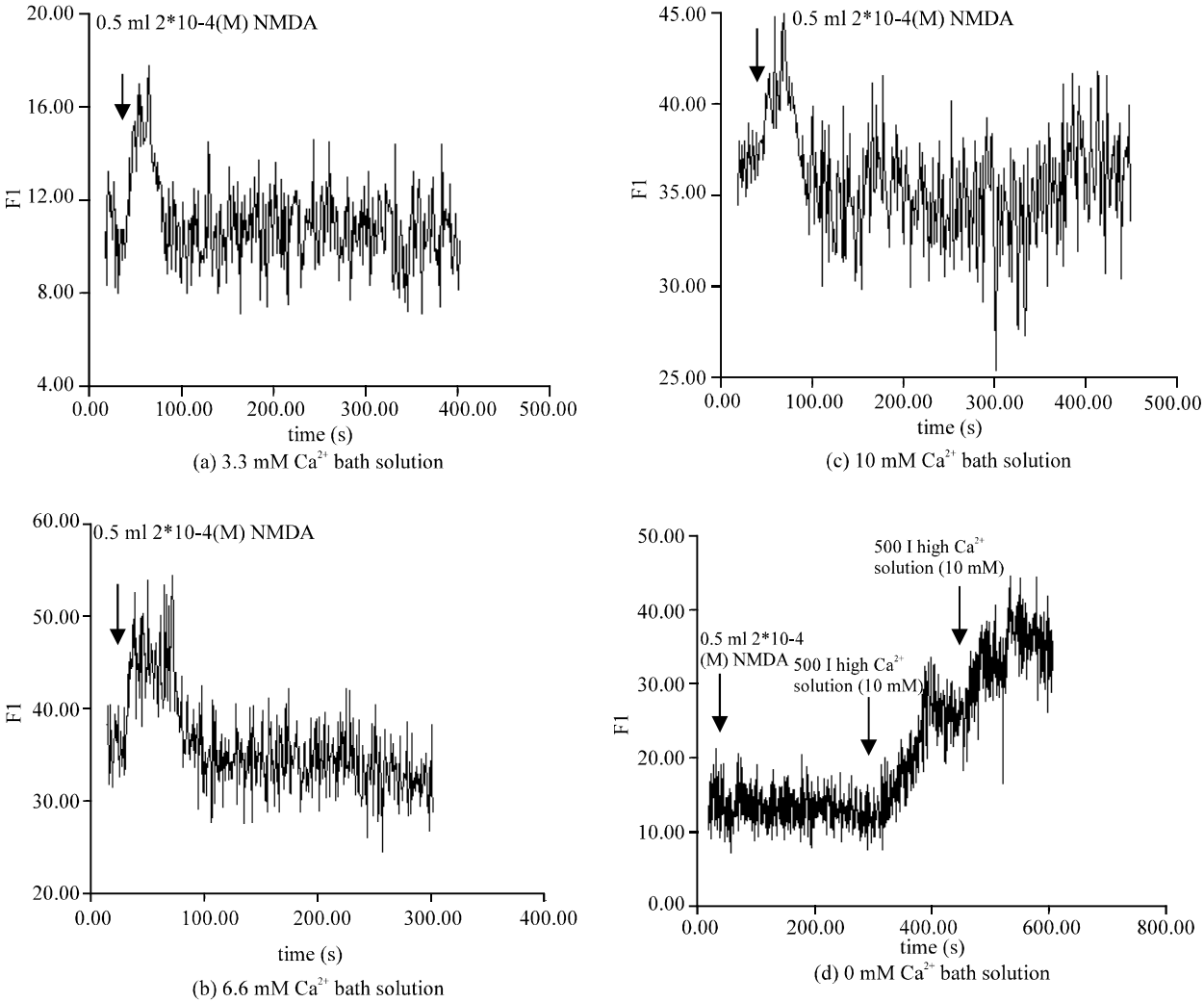

Fig. 3a,b,c,d: Shown four kinds different cells at different external solution FI variation respectively (a) 3.3mM Ca^{2+} bath solution, (b) 6.6mM Ca^{2+} bath solution, © 10mM Ca^{2+} bath solution, (d) 0mM Ca^{2+} bath solution

approach finite limiting values that we identify with the population mean and variance. Also we analysis the mean and variance of FI with time variation over different amounts of data. Starting with two intervals time and finishing with all the intervals time in a given set. The results are shown in Fig.4a, b, c, d, which indicates that means and variances do not always approach stability. Thus the means and variances of the FI that occur over some finite time interval does not describe the data and kinetics of intracellular Ca^{2+} properties appropriately, and could lead to an invalid assessment of kinetics. This is an important finding for analyzing intracellular C_a^{2+} concentration. Fig.4a, b, c, d showed the sample means and variances of the intracellular C_a^{2+} concentration that were plotted as a function of data length respectively in four of kinds different external solution diameter range in 15-30 μm cells (other not shown). The changes in those values indicate that the sample means and variances were not stable and did not necessarily converge to limiting values.

Concentration dependence of the hurst rescaled range (R/S): The Hurst exponent is the slope of the plot of the log (dispersion) vs log (number of values analysis). Fig. 5a, b shown two different diameters of cells double-logarithmic plots of the rescaled range R/S vs, the size of the samples (N_t) for a typical experimental record of FI at 3.3 mM C_a^{2+} bath solution. Values of H calculated for different external solution and applied through the confocal microscopy are shown in Table 1. As external

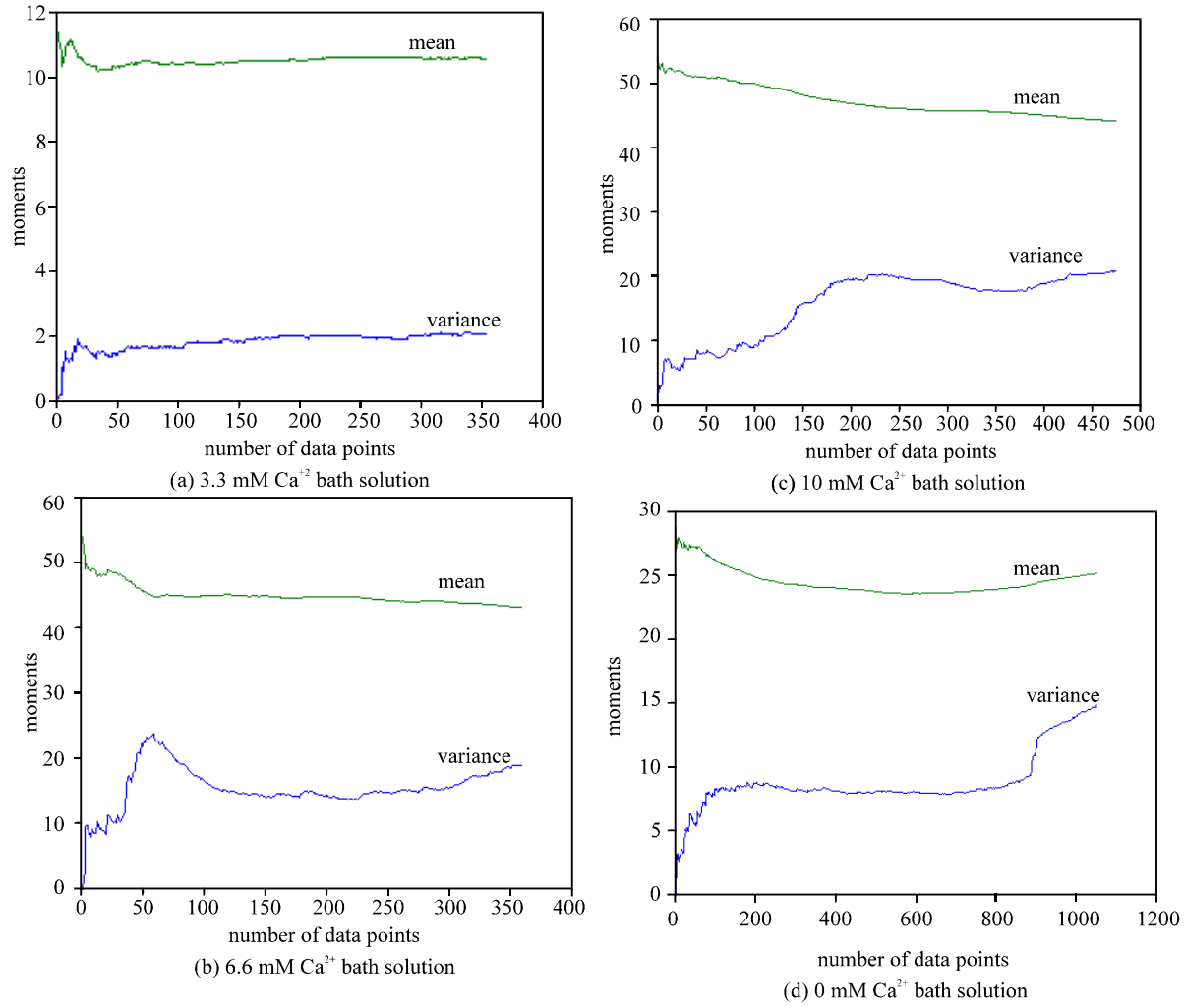

Fig. 4a,b,c,d: Shown the sample means and variances of four kinds of different external solution diameter range in 15-30μm cells (other not shown) cells the intracellular C_a^{2+} concentration that were plotted as a function of data length. The changes in those values indicate that the sample means and variances did not stable and did not necessarily converge to limiting values.
(a) 3.3 mM Ca^{2+} bath solution H=0.5048 (b) 3.3 mM Ca^{2+} bath solution H=0.6132

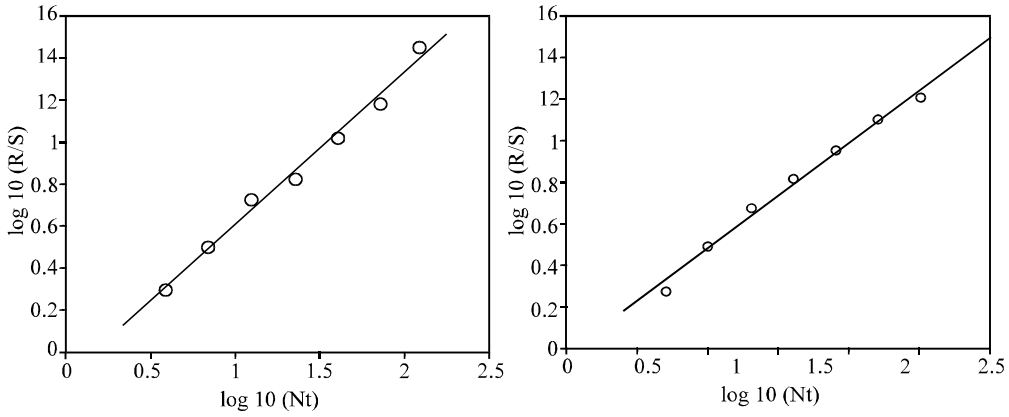

Fig. 5a,b: Shown H coefficient of two different cells at 3.3 mM Ca^{2+} bath solution (H=0.5048,H=0.6132), respectively

solution concentration was changed, also H was changed, thus Hurst rescaled range has Concentration dependence. It is interesting that the application of Ca^{2+} channel stimulant, 0.5 ml of 2×10^{-4} M NMDA In 0 Ca^{2+} bath solution, evoked the decrease in $[Ca^{2+}]_I$. the results indicated NMDA-triggered NMDA receptor channel open, intracellular Ca^{2+} influx. 5min after adding 500 μl high Ca^{2+} solution (10 mM), the results indicated that $[Ca^{2+}]I$ elevation may be attributed to extracellular Ca^{2+} entry. 8min after adding 500 μl high Ca^{2+} solution (10 mM), intracellular Ca^{2+} concentration continued to increase (Fig. 3), computation result indicated that H coefficient of 0mM Ca^{2+} bath solution added high Ca^{2+} solution approximated with H coefficient in 10mM Ca^{2+} bath solution.

Discussion

For investigating the stimulatory effects of NMDA on the intracellular FI variation, It was required that the experiments were carried out on DRG neurons with confocal microscopy. It is practical because there are different diameter sized DRG cells in response to NMDA according to the data obtained from our previous work.

We measured the cell size with confocal microscopy. It is evident from Fig. 2 that intracellular Ca^{2+} concentration varied. Specially, Fig. 2 shown intracellular FI after experiment beginning 40 sec adding 0.5 ml of 2×10^{-4} M NMDA and 3.3 mM Ca^{2+} bath solution. Fig. 3a,b,c shown adding 0.5 ml of 2×10^{-4} M NMDA four kinds different cells intracellular Ca^{2+} concentration variation time-related at different external solution respectively. NMDA-receptor channel opened, Intracellular C_a^{2+} concentration increase, generally decrease trend arisen, then variation trend is kept up and down. but It is of interest that Fig. 3 (d) showed after adding 0.5 ml of 2×10^{-4} M NMDA, NMDA receptor open, the first decay phase of $[Ca^{2+}]_I$ curve was owed to intracellular Ca^{2+} flowing out, adding high C_a^{2+} solution 500μl, the second climbing phase was owed to extracelluar Ca^{2+} influx, continue adding high Ca^{2+} solution 500 μl, the third climbing phase was also owed to extracelluar Ca^{2+} influx and denoted that the NMDA had an anomalous function. We had ever observed this anomalous phenomenon of NMDA modulation in another work (unpublished observation). Denoting NMDA triggered NMDA-receptor channel opening in rat dorsal root ganglion (DRG) neurons cells. We considered their nonlinear properties and non-linear kinetics model [27].

The basic question involved in the study of time series resulting from a sequence of measurements of FI that fluctuates in time. FI of one-dimension histogram and two-dimension histogram (previous work) was described its non-normalizable distributions. But data from such distributions cannot be meaningfully characterized by only mean and one variance. In fact, we showed that the mean and the variance of the number of FI per unit time at different external solutions did not always reach stability, limiting values was increasingly varied. These results suggest that the mean and the variance cannot describe intracellular Ca^{2+} concentration kinetic properties. The rescaled range analysis was used to study the FI variance. The calculated Hurst exponents were found to be in the range $0.5084 \le H \le 0.946$. This high value of H for different external solutions indicates a significant persistent correlation which may arise from nonlinear interactions between different time correlation and is concentration dependent. Another interesting point raised by our results is that the Hurst coefficient changes with extracellular solution concentrations.

What is the physiological significance of the results obtained from the present investigation? It is now open to discussion. In this work we use the cell body of DRG neuron as a simple and accessible sample for studying the characteristics of intracellular Ca^{2+}. On the one hand, NMDA can trigger NMDA-receptor channel open, cause intracellular Ca^{2+} concentration variation, on the other hand, we used the rescaled range analysis to study FI. The results indicated persistent of correlation intracellular Ca^{2+} in different time, H coefficient was characterized the statistical properties in these data properly. The experiment results also indicated that the NMDA had anomalous function, which suggests that NMDA may cooperate with other chemicals in activating peripheral nociceptive endings of sensory neurons, especially during tissue damage or inflammation.

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References

Ascher, P., P. Breggestovski and L. Nowak, 1988. N-methyl-D-aspartate-activated channels of mouse central neurons in magnesium-free solution. J. Physiol., 399: 207-226.

Bassingthwaighte, J.B. and G.M. Raymond, 1994. Evaluating rescaled range analysis for time series, Ann. Biomed. Eng., 22: 432-444.

Cleeman, L. and M. Morad, 1997. Visualization of calcium sparks in hert cells with total internal reflection fluorescence microscopy. Biophys J., 72: A45.

Churilla, A.M., W.A. Gottschalk, L.S. Liebovitch, L.Y. Selector, A.L. Todorov and S. Yeandle, 1996. Membrane potential fluctuations of human T-lymphocytes have fractal characteristics of fractional Brownian motion. Ann. Biomed. Eng., 24: 99-108.

Funatsu, T., Y. Harada, M. Tokunaga, K. Saito and T. Yanagida, 1995. Imaging of single fluorescent molecules and individual ATP turnovers by single myosin molecules in aqueous solution. Nature, 374: 555-559.

Gao Tian-Ming, Zou Fei and Chen Pei-Xi, 1995. Single channel properties of NMDA receptors in cortical neurons. Acta Physiologica Sinica, 47: 133-141.

Gibb, A.J. and D. Colquhoun, 1992. Activation of N-methyl-D-aspartate receptors by L-glutamate in cells dissociated from adult rat hippocampus. J. Physiol., 456: 143-179.

Giaever, I. and C.R. Keese, 1989. Fractal motion of mammalian cells, Physica D, 38: 128-133.

Hong-Zhen, H. and L. Zhi-Wang, 1996. Substance P potentiates ATP-activated currents in rat primary sensory neurons. Brain Res., 739: 163-168.

Hong-Zhen, H. and L. Zhi-Wang, 1997. Modulation by adenosine of GABA-activated current in rat dorsal root ganglion neurons. J. Physiol., 501: 67-75.

Hu, H.Z., Z.W. Li and J.Q. Si, 1997. Evidence for the existence of substance P autoreceptor in the membrane of rat dorsal root ganglion neurons. Neuroscience, 77: 535-541.

Hurst, H.E., 1951. Long-term storage-capacity of reservoirs. Trans. Am. Soc. Civil Eng., 116: 770-808.

Jahr, C.E. and C.F.A. Stevens, 1990. A quantitative description of NMDA receptor-channel kinetic behavior. J. Neurosci., 10: 1830-1837.

Kochetkov, K.V., V.N. Kazchenko, O.V. Aslanidi, N.K. Chemeris and A.B. Gapeyev, 1999. Non-Markovian gating of Ca^{2+}-activated K^{+} channels in cultured kidney cells vero. Rescaled range analysis. J. Biophysics, 25: 211-222.

Laketic-Ljubojevic, I., L.J.M. Maathuis, D. Sanders and T.M. Skerry, 1999. Functional characterization of NMDA acid-gated channels in bone cells.Bone, 25: 631-637.

Lester, R.A.J. and S.G. Cull-Candy, 1990. NMDA channel behavior on agonist affinity. J. Neurosci., 12: 635-643.

Legendre, P. and G.L. Westbrook, 1990. The inhibition of single N-methyl-D-aspartate-activated channels by zinc ions on cultured rat neuron. J. Physiol., 429: 429-449.

Llinas, R., M. Sugimori and R.B. Silver, 1992. Microdomains of high calcium concentration in a presynaptic terminal. Science, 256: 677-679.

Macklin, J.J., J.K. Trautman, T.D. Harris and L.E. Brus, 1996. Imaging and time-resolved specyroscopy of single molecules at an interface. Sci., 272: 255-258.

Monck, J.R., I.M. Robinson, A.L. Escobar, J.L. Vergara and J.M. Fernandez, 1994. Pulsed laser imaging of rapid Ca^{2+} gradients in excitable cells. Biophys J., 67: 505-514.

Nogueira, R.A., W.A. Varanda and L.S. Liebovitch, 1995. Hurst analysisin the study of ion channel kinetics,Brasilian J. Med. Biol. Res., 28: 491-496.

Ohei, M., R.H. Chow and S. Stuhmer, 1997. Total internal reflection fluorescence microscopy (TIRFM) resolves vesicle dynamics between distinct submembranous pools prior to fusion. Biophys. J., 72: A215.

Oheim, M. and D. Loerke, 1998. Walter stuhmer robert H. Chow: The last few milliseconds in the life of a secretory granule. Eur. Biophys. J., 27: 83-98.

Piston, D.W., M.S. Kirby, H. Cheng, W.J. Lederer and W.W. Webb, 1994. Two-photon-excitation fluorescence imaging of three-dimensional calcium-ion activity. Appl Opt., 33: 662-669.

Sharon, T. wong, A. Barbara, Atkinson, C. Lynne, Weaver, 2000. Confocal mocroscopic analysis reveals sprouting of primary afferent fibres in rat dorsal horn after spinal cord injury. Neuroscience Letters, 296: 65-68.

Wamberto, A. Varandn, S. Larry and Liebovitch, 2000. Jose N>Figueiroa and Romildo A.Nogueira. Hurst analysis applied to the study of single calcium-activated potassium channel kinetics. J. Theor. Biol., 206: 001-011.