Basal Protein Kinase Activity Maintains Spontaneous Synchronous Intracellular Ca^{2+} Oscillation Frequency in Mouse Primary Cultured Cortical Neuronal Networks

Fumie YASUMOTO [1]

E-mail : yasumoto@edu.u-toyama.ac.jp

Abstract

1. Spontaneous synchronous intracellular Ca^{2+} oscillations are essential for central nervous system development.
2. The broad-spectrum protein kinase inhibitor staurosporine reduced the frequency of spontaneous synchronous Ca^{2+} oscillations among primary cultured cortical neurons.
3. Basal protein kinase activity may contribute to neural circuit development and function through modulation of Ca^{2+} oscillation frequency.

Keywords : neuronal network; protein kinase; intracellular Ca^{2+} oscillation

Introduction

Neurons within the central nervous system (CNS) exhibit spontaneous oscillations in intracellular Ca^{2+} concentration that form complex spatiotemporal patterns across networks, such as calcium waves and synchronized bursts. These oscillations driven by neural burst firing are believed to transmit signals both within and among neurons through a frequency code. Such signaling is required for neuronal development, differentiation, and the establishment of functional networks (Garaschuk et al., 1998; Gray et al., 1989).

Primary cultured mouse neurons also exhibit synchronous burst firing activity driven by glutamatergic synapses in the absence of stimulation, and this activity is associated with periodic changes in the concentration of intracellular Ca^{2+} (synchronous oscillations) (Yasumoto et al., 2004a), suggesting that these cultures are an accessible model to study the contributions of Ca^{2+} oscillations to neural development. During development of the cerebral cortex, excitatory glutamatergic neurons mature through several sequential stages, including cell proliferation, cell fate specification, migration, dendrite and axon formation, and synaptogenesis (Price et al. 2006; Rakic 2006). Similarly, mouse cultured cortical neurons form networks through sequential developmental stages according to the number of days in vitro (DIV), and these stages are associated with gradually increasing spontaneous synchronous Ca^{2+} oscillation frequency (Yasumoto et al., 2004a and b).

In previous studies, I examined the role of protein kinase A (PKA) in this form of signaling and demonstrated that PKA inactivation reduces spike frequency (Yasumoto et al., 2004b). However, numerous additional kinases regulate Ca^{2+} signaling and thereby more complex synaptoplastic processes such as long-term potentiation (Linden et al., 1986; Linden et al., 1987; Malenka et al., 1986; Hu et al., 1987; Malinow et al., 1989) and long-term depression (Linden et al., 1991), including protein kinase C (PKC) (Saito et al., 2002).

The aim of the present study was to examine the contributions of basal kinase activity to

[1] Faculty of Education, Academic Assembly, University of Toyama, Toyama, Japan

synchronous Ca^{2+} oscillations within and among neurons in networks. Numerous studies have shown that staurosporine inhibits a broad -spectrum of protein kinases, and so has been used to assess kinase functions in various cell types (Yadav et al., 2015; Zhao et al., 2015; Nardo et al., 2014). For example, inhibition of protein kinase activity by staurosporine was reported to block neurite formation (Shea et al., 1991). However, another group reported that staurosporine induced neurite development in both N18TG2 mouse neuroblastoma cells and primary cultures of mouse cerebellar neurons. So specific effects may depend on neuronal type and/or stage of development. The current report demonstrates that staurosporine reduces the frequency of synchronized Ca^{2+} spikes in cultured mouse cortical neuronal networks.

Materials and Methods

Animals

Pregnant ICR mice were purchased from SLC (Shizuoka, Japan) and housed under controlled conditions (temperature, 24°C ± 1°C; humidity, 55% ± 5%) with ad libitum access to commercial CMF (Oriental Yeast, Tokyo, Japan) and tap water until cesarean operation. Experiments were performed in accordance with the Guidelines for Animal Experimentation, the University of Tokyo, and were approved by the Institutional Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo.

Primary culture of neurons from mouse cerebral cortex

Primary cultures of cortical neurons were prepared according to a previously reported method (Yasumoto et al., 2004a and b). Briefly, fetuses were removed at gestational day (E)17 and cerebral cortices isolated. Cortical tissues were then cut into pieces smaller than 1 mm^3 in isolation medium consisting of equal-volume Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, St. Louis, MO) and Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS). Sections were digested in 11.2 units/ml dispase, dispersed into a single cell suspension in DMEM supplemented with 10% horse serum, and plated at 1.1×10^4 cells/mm^2 on glass coverslips coated with polyethylenimine (PI) (Sigma). Cultured cerebral cortical cells were maintained in the same medium under a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Intracellular Ca^{2+} recording

Intracellular Ca^{2+} concentration measurements were initiated after 7 or 8 DIV according to a previously reported procedure (Ogura et al., 1987). Briefly, cells were loaded with the fluorescent calcium indicator Fura-2-AM (5 μM, Dojin, Kumamoto, Japan) in balanced salt solution (BSS). Fields of 10 × 10-μm were randomly chosen and the ratio of emission intensity from 340 and 360 nm excitation, which corresponds to the intracellular Ca^{2+} concentration, was recorded from individual cells using an Argus-50 imaging system (Hamamatsu Photonics, Hamamatsu, Japan). While the culture contained both neurons and glia, the plan of focus was maintained above the plate to selectively capture emission from the rounder neurons rather than flatter glial cells.

Chemicals

Staurosporine (Sigma) was diluted in BSS to a working concentrations (100 nM) and quickly administered by a pipette positioned just above the culture medium level. To quantify the changes in Ca^{2+} spike frequency in response to staurosporine, Ca^{2+} spikes were counted for 2.5 min prior to (pre) and after (post) staurosporine application and the ratio (post/pre) was calculated (so a ratio of 1 indicates no change). The vehicle alone had no effect on the frequency of Ca^{2+} spike.

Statistical analysis

Numerical values are presented as mean ± S.E.M., and the difference in spike frequency pre- vs. post-treatment was evaluated using the paired Student's t test. A $p < 0.05$ (two-tailed) was considered statistically significant for all tests.

Results

Intracellular Ca^{2+} concentrations changed spontaneously within individual cultured cortical neurons and synchronously among groups of neurons in the absence of external stimulation (synchronous oscillation) at 7–8 DIV (Fig. 1). To investigate the effects of basal protein kinase activity on these synchronized Ca^{2+} oscillations, staurosporine was bath-applied and spike frequency compared to baseline. Staurosporine induced a significant decrease in the frequency of synchronized Ca^{2+} spikes (number of separate cultures (Nc) = 3, number of total wells examined (Nw) = 3, number of responsive cells = 21/21) (Figs. 1 and 2), while vehicle had no effect on frequency (Nc = 5, Nw = 5, 35/35 cells all no effect) (Fig. 2).

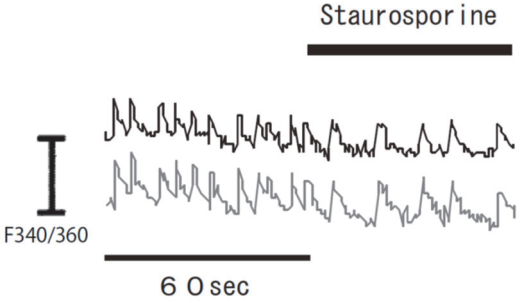

Fig. 1. Modulation of spontaneous Ca^{2+} oscillation frequency among mouse cultured cortical neurons by the broad-spectrum kinase inhibitor staurosporine. Two representative cortical neurons at 7 DIV are presented showing synchronous oscillations of intracellular Ca^{2+} concentration. Traces show representative recordings of synchronized Ca^{2+} spikes in two neurons randomly selected from the imaging field at baseline and after bath application of staurosporine (100 nM). Staurosporine induced a significant decrease in the frequency of synchronized Ca^{2+} spikes. Scale bar represents 60 seconds.

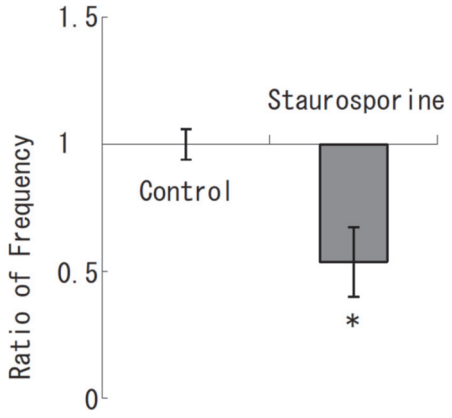

Fig. 2. Changes in synchronous Ca^{2+} oscillation frequency induced by staurosporine. Data are presented as the mean ± S.E.M. (control: 35/35 cells and staurosporine: 21 cells). Asterisks indicate significance versus control ($p < 0.05$).

Discussion

In this study, broad-spectrum kinase inhibition was demonstrated to reduce the frequency of spontaneous synchronous calcium oscillations among primary cultured cortical neurons (Figs. 1 and 2). A previous report indicated that these synchronous oscillations were accompanied by rhythmic membrane depolarizations and action potentials driven by glutamatergic synapses, a circuit behavior also observed during CNS development (Yasumoto et al., 2004b). Thus, basal cellular kinase activity may modulate neuronal development and circuit formation by maintaining the frequency of synchronized Ca^{2+} spikes.

The frequency of synchronous Ca^{2+} spikes increases with neuronal network development in primary culture, suggesting that such oscillations must be maintained for synaptic development and plasticity underlying circuit formation. Therefore, in addition to the induced activities of specific kinases, total basal protein kinase activity appears to be an important factor supporting neuronal network formation. However, several previous studies reported that staurosporine

induced neuronal apoptosis (Koh et al., 1995; Sodja et al., 2014, Ramiro-Cortés et al., 2011), suggesting that a basal level of kinase activity is also critical for neuronal survival (possibly by transducing trophic signals). Apoptosis assays, such as Annexin V staining or cleaved caspase activity measures, were not performed in this study; however, no dramatic morphological changes indicative of apoptosis were observed after recording intracellular Ca^{2+} concentrations (data not shown). Nonetheless, the long-term effects of broad-spectrum protein kinase inhibition on survival was not examined. Measurement of Ca^{2+} oscillations under conditions permitting longer-term monitoring are required to address these issues.

The results of this study suggest that a certain level of basal protein kinase activity is required to maintain the frequency of synchronized Ca^{2+} spikes. Further studies are required to examine if these changes in intracellular calcium dynamics influence neuronal differentiation, survival, and circuit formation.

Acknowledgement

This study was supported by the Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research (Grant Number 05J11569). The author would like to thank Enago (www.enago.jp) for the English language review.

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（2020 年 10 月 15 日受付）
（2020 年 12 月 8 日受理）