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Full-length article

MEK inhibitor PD98059 acutely inhibits synchronized spontaneous Ca^{2+} oscillations in cultured hippocampal networks

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Key words

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Abstract

Aim: To investigate the changes in synchronized spontaneous Ca^{2+} oscillations induced by mitogen-activated protein kinase kinase (MEK) inhibitor PD98059 at different concentrations in cultured hippocampal network. **Methods:** Hippocampal neurons in culture for 1–2 weeks were used for this study. Spontaneous synaptic activities of these hippocampal neurons were examined by Ca^{2+} imaging using calcium-sensitive dye. MEK inhibitor PD98059 (10, 30, and 60 μmol/L) and SB202474 (10 and 60 μmol/L), a negative control for mitogen-activated protein kinase (MAPK) cascade study, were applied to the cells under the microscope while imaging was taking place. **Results:** PD98059 at a lower concentration of 10 μmol/L had little effect on the Ca^{2+} oscillation. At the higher concentration of 30 μmol/L, 5 min after application of PD98059, the spike frequency was decreased to 25.38%±7.40% (mean±SEM, n=16, P<0.01 *vs* medium control) of that of the control period. At an even higher concentration of 60 μmol/L, 5 min after application of PD98059, the spike frequency was decreased to 14.53%±5.34% (mean±SEM, n=16, P<0.01 *vs* medium control) of that of the control period. The spike amplitude underwent a corresponding decrease. However, the negative control SB202474 at concentrations of 10 and 60 μmol/L had little inhibition effect on the Ca^{2+} oscillation. **Conclusion:** These results indicate that PD98059 inhibits synchronized spontaneous Ca^{2+} oscillation through inhibition of MEK, which hints that the MAPK cascade is required to maintain synchronized spontaneous Ca^{2+} oscillation.

Introduction

The mitogen-activated protein kinase (MAPK) cascade is a ubiquitous serine/threonine kinase cascade that has been classically studied as a critical biochemical pathway involved in cell proliferation and differentiation[1]. MAPKs constitute a superfamily of three related kinases that are activated by various extracellular stimuli including the extracellular signal regulated kinases (ERKs), the Jun N-terminal kinases (JNKs), and p38 kinases (p38)[2,3]. ERK, JNK, and p38 can all be activated by a variety of stimuli, but these kinases are differentially affected by certain signals. For example, ERKs are most highly activated in response to mitogenic stimulation, whereas JNKs and p38 show greater activation in response to cellular stress[4,5]. The pathway leading to ERK activation by growth factors and other mitogens has been studied extensively. The first step involves activation of membrane-associated tyrosine kinases, followed by the sequential activation of Ras and Raf. Raf then phosphorylates the mitogen-activated protein kinase kinase (MEK), which in turn activates ERK[6,7]. Although this cascade is mainly studied in mitotic cell regulation, its components are actually most abundantly expressed in postmitotic neurons of the developed nervous system[8]. The hippocampus region, which is commonly used as a model to study synaptic plasticity, has highly expressed ERK[9,10]. What are the physiologic roles of this cascade in mature neurons? PD98059, a specific inhibitor of MEK, the enzyme that activates ERK[11], has been shown to block induction of LTP in area CA1 of the hippocampus[12] and attenuates multiple forms of synaptic plasticity in rat dentate gyrus *in vitro*[13]. However, the regulation mechanism or physiological role of this cascade in the activity-dependent synaptic connections between neurons is not clear.

Calcium plays an important role in regulating a great variety of neuronal processes, especially in the transmitter release and synaptic connection. Oscillations in cytoplasmic calcium have been observed in a wide variety of neuronal cell types including cortical and hippocampal neurons[14–16]. In primary cultured hippocampal neurons, after one week in culture, networks of interconnected neurons are formed. At approximately 9 d *in vitro*, some networks show spontaneous synchronized Ca^{2+} oscillations[15,17]. These oscillations are believed to encode information in neural circuits[18,19] and might play an important role during physiological or pathological events[20,21]. Many studies have implied that the MAPK cascade might participate in $[Ca^{2+}]_i$ regulation[22–24]. Here we used PD98059, a commercially available inhibitor of MEK, and SB202474, a negative control, to explore whether this cascade participates in the regulation of spontaneous synchronized Ca^{2+} oscillations.

Materials and methods

Drugs Dulbecco's modified Eagle's medium (DMEM) media, neurobasal medium, fetal bovine serum, B27 supplements, 0.25% trypsin-EDTA, and poly-*D*-lysine for cell culture were from Invitrogen (Carlsbad, CA, USA). Equine serum and *L*-glutamine were from Hyclone (Logan, UT, USA). PD98059 and SB202474 were purchased from Calbiochem (La Jolla, CA, USA) and were dissolved in dimethyl sulfoxide. Fluo-4-AM was from Molecular Probes (Eugene, OR, USA). Other reagents were purchased from Sigma (St Louis, MO, USA).

Hippocampal cell culture and experiment Hippocampal neurons from embryonic rats (E18) were obtained according to the method previously described[25]. In brief, hippocampal tissues from 18-d-old fetal rats were dissected and treated with 0.25% trypsin in Ca^{2+}-Mg^{2+}-free HBSS at 37 °C for 15 min; they were then dissociated by trituration with a glass Pasteur pipette and plated in 35 mm culture dishes with glass bottoms (MatTek, Ashland, MA) for culture and subsequent microscopy. The glass surface in each dish (~15 mm diameter) was pretreated with poly-*D*-lysine for 2 h (500 μg/mL in borate buffer), washed three times, and air-dried before cell plating. Approximately 75 000 cells were plated in the glass area of each dish in DMEM containing 5% fetal bovine serum and 5% horse serum. On the second day after plating, the culture medium was replaced by serum-free Neurobasal medium containing 2% B27 supplement and 500 μmol/L glutamine for reduced glial growth. Cells were maintained in a CO_2 incubator at 37 °C, and one-half volume of the culture medium was replaced with fresh Neurobasal medium every 3 d. The experiments were carried out on cultures after 7 d.

Ca^{2+} imaging Hippocampal cells were loaded with 4 μmol/L Fluo-4-AM in Krebs-Ringer's saline (recording solution) (150 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 10 mmol/L glucose, and 10 mmol/L HEPES, pH 7.4)[19] at 37 °C for 30 min, followed by three washes and a 15-min incubation period for further de-esterification of Fluo-4-AM before imaging. Cells grown on the glass bottom in 35 mm dishes were directly imaged on a Nikon (Tokyo, Japan) TE300 inverted microscope using a 40×numerical aperture, 1.30 oil immersion Plan Fluor objective. A Lambda DG-4 highspeed wavelength switcher (Sutter Instruments, Novato, CA) was used for Fluo-4 excitation at 480 nm, and a cooled CCD camera (CoolSnap FX; Roper Scientific, Princeton, NJ) was used for image acquisition. MetaFluor imaging software (Universal Imaging, Downingtown, PA) was used for hardware control, image acquisition, and image analysis. The time-lapse recording of Ca^{2+} signals in hippocampal neurons was carried out for a 2-min control period before and a 6-min period after the application of different chemicals. The sampling rate was one frame every 2 s. The exposure time was 50 ms when CCD binning of 4×4 was used.

Quantitative analysis of synchronized Ca^{2+} spikes Quantitative measurements of changes in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were done by obtaining the average Fluo-4 fluorescence intensity of a 3×3 pixel analysis box placed at the center of the cell body; the intensity values were then subtracted from the average background intensity measured in cell-free regions. Changes of $[Ca^{2+}]_i$ in each cell were then represented by the changes of relative Fluo-4 fluorescence ($\Delta F/F_0$) where F_0 was the baseline intensity obtained from the 2 min control period. Ca^{2+} spikes were defined as rapid elevation of $\Delta F/F_0$ equal to or >20%. Under our imaging settings, fields of 3–10 neurons were typically recorded and subsequently analyzed. To determine the frequency and amplitude of Ca^{2+} spikes, we counted the number of Ca^{2+} spikes and the average amplitude of these spikes over a 2-min period of the recording as a defined time point. As a result, the 2-min control period yielded only one frequency and one amplitude value, whereas the experiment period (6 min) resulted in three frequency and amplitude values at different time points after bath application of a specific molecule. To the changes in the spike frequency and amplitude, these three frequency and amplitude values after the drug application were normalized to the control frequency or amplitude values respectively and expressed as percentages, with a value of 100% indicating no change. We quantified and examined the changes in the spike frequency and amplitude through the entire 6 min period after bath application. In order to assess the baseline changes after drug application, we calculated the average $\Delta F/F_0$ values of the rock bottom of each spike.

Bath application of different drugs To prevent adverse effects of high concentrations of drugs, a 2×working concentration of the drug was made in Krebs-Ringer's solution and was applied to the cells to achieve the desired final concentration through 1:1 dilution (*v*/*v*). Specifically, we first recorded Ca^{2+} activities for a 2-min control period in 1 mL of Krebs-Ringer's solution, removed 0.5 mL from the bath, added 0.5 mL of the 2×solution, and subsequently recorded for 6 min to examine the effects on spontaneous Ca^{2+} spikes. For the control, we simply carried out the same procedure to apply Krebs-Ringer's solution to determine that there was no artifact of this application method.

Statistical analysis Data from at least three dishes from different batches of cultures were pooled together and analyzed for statistically significant differences using the paired Student's *t*-test. Compiled data are expressed and graphed as mean±SEM, with *n* denoting the number of neurons studied for each treatment. Differences were considered significant if a P value was <0.05.

Results

Synchronized spontaneous Ca^{2+} spikes and the mechanisms in cultured hippocampal networks We prepared low-density hippocampal neurons culture as described by Banker *et al*[25]. After at least one week in culture, many hippocampal neurons formed local networks which usually contained 3–10 neurons (Figure 1A, left panel). Spontaneous synaptic activities of these neurons were examined by Ca^{2+} imaging using the calcium-sensitive dye Fluo-4[26] (Figure 1A, right panel). We observed periodic, spontaneous spike elevations of $[Ca^{2+}]_i$ and these spikes appeared to be primarily synchronized among the local group of cells (Figure 1B) without removing or reducing Mg^{2+} in medium.

The mechanisms underlying the synchronized spontaneous Ca^{2+} spikes in hippocampal networks have been studied extensively, but the results from different published reports are confusing. This may be caused by the variety of preparations used for experiments. For example, Leinekugel *et al* reported that the synchronized spontaneous Ca^{2+} spikes were mediated by the synergistic excitatory actions of gamma-aminobutyric acid ($GABA_A$) and *N*-methyl-*D*-aspartate (NMDA) receptors in the neonatal hippocampus[27], whereas Tanaka *et al* reported that the oscillation of Ca^{2+} was mainly mediated by non-NMDA-type glutamatergic transmission[15]. To confirm the Ca^{2+} spikes observed in our culture were driven by particular receptors, we applied different antagonists of these receptors to our cultures. We found that a non-selective antagonist of NMDA and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors kynurenic acid 1 mmol/L completely blocked the spikes immediately after application (Figure 2A). The NMDA receptor antagonist APV at 50 μmol/L only partially inhibited the spike amplitude (Figure 2B), whereas AMPA/kainate receptors antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX) at 20 μmol/L completely and immediately blocked the spikes (Figure 2B, 2C). The addition of the $GABA_A$ receptor antagonist bicuculline had a mixed effect on the Ca^{2+} oscillations, which caused an increase in amplitude but a decrease in frequency (Figure 2D). Subsequently adding kynurenic acid or DNQX completely blocked the spikes (Figure 2D–F). These findings suggest that the oscillations we observed are similar to those

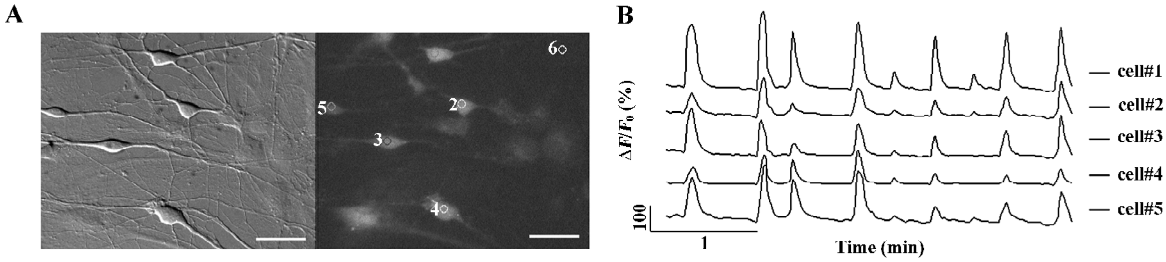

Figure 1. Synchronized spontaneous Ca^{2+} spikes in cultured hippocampal neurons. (A) The DIC image on the left shows five interconnected hippocampal neurons. The fluorescent image on the right shows the same region of hippocampal neurons with the DIC image loaded with Fluo-4. Scale bar, 40 μm. (B) Traces depict synchronized spontaneous Ca^{2+} transients in these five neurons.

observed by Tanaka *et al*, which were mainly mediated by non-NMDA-type glutamatergic transmission.

PD98059 acutely inhibited synchronized spontaneous Ca^{2+} spikes at a higher concentration To test whether the MAPK cascade is required to maintain Ca^{2+} oscillations, we used the MEK inhibitor PD98059 to block MAPK activation in hippocampal neurons. As reported by Dudley *et al*, PD98059 exerts it inhibition effect on MEK at concentrations from 1 to 100 μmol/L, with the IC_{50} value at approximately 10 μmol/L[28]. We chose 10, 30, and 60 μmol/L concentrations to test the effect on Ca^{2+} oscillations. We found that PD98059 at 10 μmol/L had no significant effect on Ca^{2+} spike frequency and only slightly decreased the Ca^{2+} spike amplitude (Figure 3A,3D,3E). PD98059 30 μmol/L significantly inhibited the Ca^{2+} spikes immediately after application (Figure 3B). Six minutes after application, the spike frequency was decreased to 25.38%±7.40% (mean±SEM, *n*=16) of that of the control period (Figure 3D) and the spike amplitude was 25.16%±6.99% (*n*=16) of that of the control period (Figure 3E). Application of 60 μmol/L PD98059 caused a more rapid and severe inhibition of the Ca^{2+} spikes (Figure 3C). Six minutes after application the spike frequency was decreased to 14.53%±

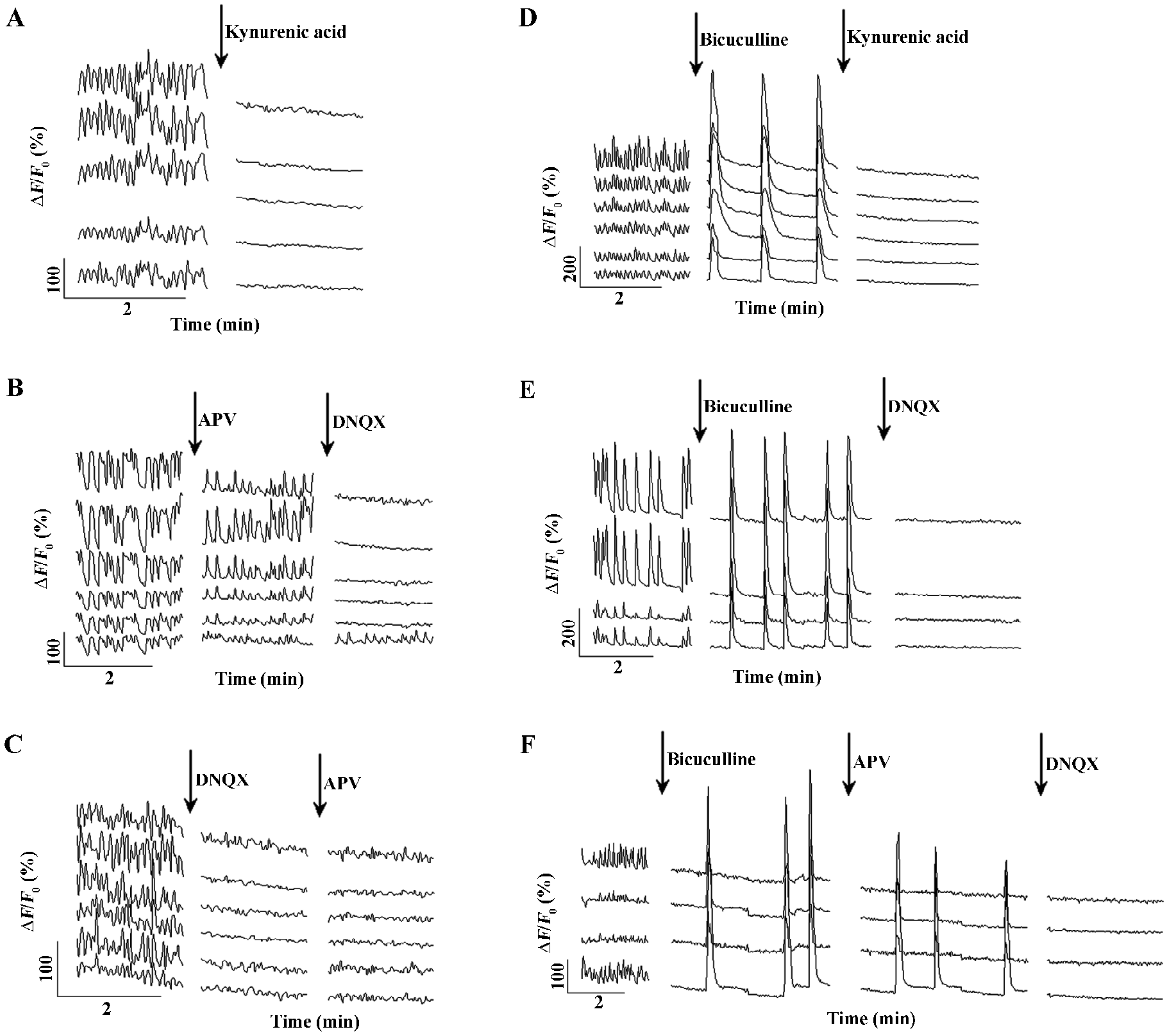

Figure 2. Role of glutamatergic and GABAergic neurons in calcium oscillation. Traces show synchronized Ca^{2+} spikes in 4–6 neurons randomly selected from a group of synchronically firing cells. The time gap indicated by arrows is approximately 30 s, during which time different agonists were added. (A) 1 mmol/L kynurenic acid; (B) 50 μmol/L APV for 2 min, followed by 20 μmol/L DNQX; (C) 20 μmol/L DNQX for 2 min followed by 50 μmol/L APV; (D) 50 μmol/L bicuculline for 2 min followed by 1mmol/L kynurenic acid; (E) 50 μmol/L bicuculline for 2 min followed by 20 μmol/L DNQX; and (F) 50 μmol/L bicuculline for 2 min followed by 50 μmol/L APV, then 20 μmol/L DNQX. Each response was repeated at least three times.

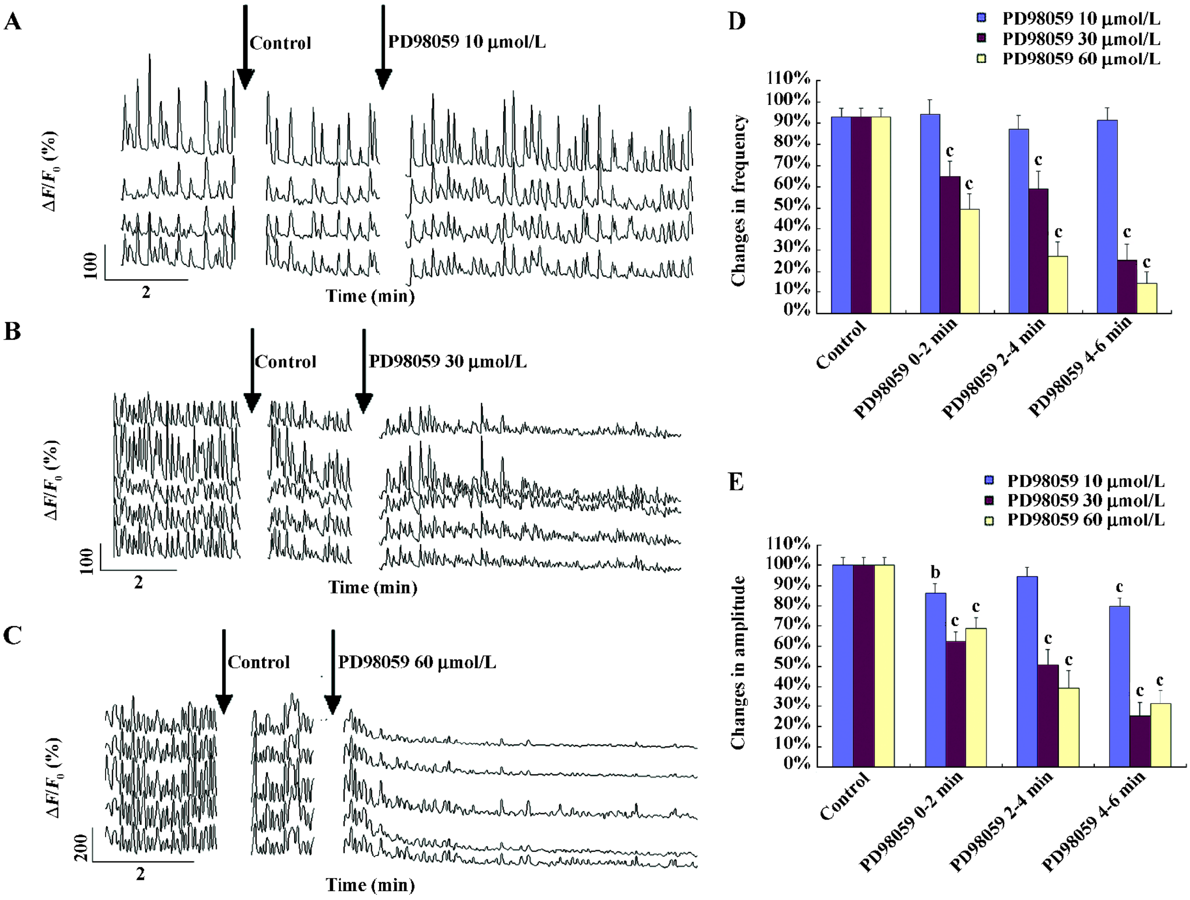

Figure 3. Acute inhibition of the synchronized Ca^{2+} oscillations by PD98059. (A–C) Traces show synchronized Ca^{2+} spikes in several neurons randomly selected from a group of synchronically firing cells. The three parts represent Ca^{2+} spikes in these cells under control conditions, after application of control medium, and after bath application of 10 μmol/L (A), 30 μmol/L (B) and 60 μmol/L (C) PD98059. Changes in the frequency (D) and mean amplitude (E) of the synchronized Ca^{2+} spikes every 2 min after bath application of medium (control) or different concentrations of PD98059. Data are presented as the mean±SEM. n=16 neurons studied for each trial. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control.

5.34% (n=16) of that of the control period (Figure 3D) and the spike amplitude was 31.81%±6.27% (n=16) of that of the control period (Figure 3E). No significant difference was found with the control period, indicating that no artifact was produced by the bath application method. Overall, these results demonstrate that PD98059 rapidly inhibits the Ca^{2+} spikes in a dose-dependent manner.

The effect of SB202474 on synchronized spontaneous Ca^{2+} spikes SB202474 is an inactive structural analog of PD98059. We also tested its effect on the Ca^{2+} spikes to confirm whether or not the inhibition effect of PD98059 on Ca^{2+} spikes was through inhibition of the MAPK cascade. We found that 10 μmol/L SB202474 had no inhibition effect on the Ca^{2+} oscillations frequency, but it had a small enhancement effect on the frequency immediately after application. As a consequence of the frequency increase, the amplitude of the spikes decreased (Figure 4A,4C,4D). Application of 60 μmol/L SB202474 had no significant effect on frequency or amplitude of the Ca^{2+} spikes (Figure 4B,4C,4D).

Discussion

We prepared the hippocampal culture based mainly on the method described by Banker *et al*[25]. We used serum-free Neurobasal medium with B27 supplement to reduce glia cell growth and increase neuron survival. As described previously, in Neurobasal/B27 medium, glia growth is reduced to less than 0.5%, resulting in a nearly pure population of neurons[29]. We used the microtubule-associated protein (MAP2) and 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) double-staining to detect the neuron proportion in our culture. We found that there were

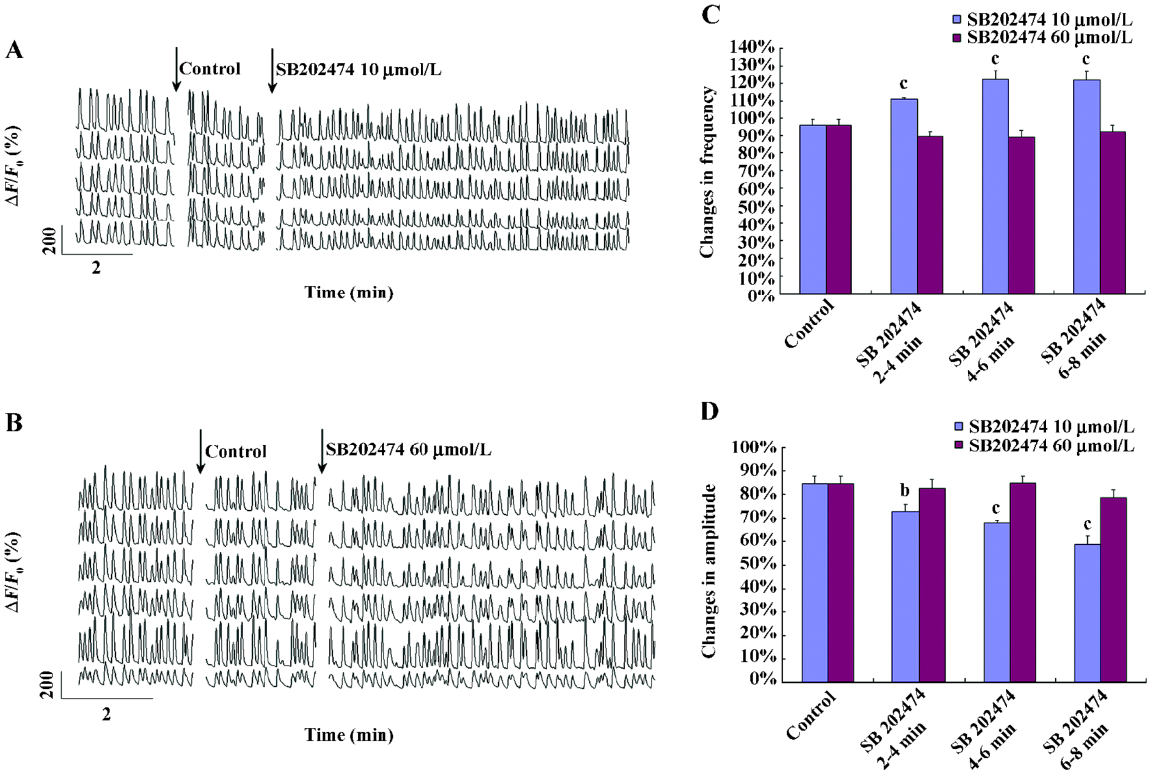

Figure 4. SB202474 had no effect on synchronized Ca^{2+} oscillations. (A, B) Traces show synchronized Ca^{2+} spikes in several neurons randomly selected from a group of synchronically firing cells. The three parts represent Ca^{2+} spikes in these cells under control conditions, after application of control medium, and after bath application of 10 µmol/L (A) and 60 µmol/L (B) SB202474. Changes in the frequency (C) and mean amplitude (D) of the synchronized Ca^{2+} spikes every 2 min after bath application of medium (control) or different concentrations of SB202474. Data are presented as the mean±SEM. $n=16$ neurons studied for each trial. ${}^{b}P<0.05$, ${}^{c}P<0.01$ *vs* control.

more than 85% neurons in our culture, and the other cells were glia cells including astrocytes and oligocytes. In this culture, glia cells were spread beneath the neuron network. The synchronized spontaneous Ca^{2+} oscillations we observed were confined to the neuron networks. Although glia cells sometimes were observed irregularly $[Ca^{2+}]_i$ elevation, they did not directly participate in the Ca^{2+} oscillation of neuron networks. We could deduce that glia cells might act as a supporter in maintaining and modifying the synchronized spontaneous Ca^{2+} oscillation in neuron networks. We detected the mechanism of the Ca^{2+} oscillation in our culture, and found that it was mainly mediated by non-NMDA-type glutamatergic transmission. NMDA-type glutamatergic transmission only partially inhibited the spike amplitude. GABAergic synaptic transmission might act as a regulator of the Ca^{2+} oscillation.

PD98059 has long been used as a specific MEK inhibitor to study the involvement of the ERK pathway on cellular events as diverse as growth and differentiation, cell death and survival, and synaptic plasticity[30]. Our results show that PD98059 acutely inhibits the synchronized Ca^{2+} oscillations in a dose-dependent manner. When the concentration (10 µmol/L) is too low to inhibit MAPK activation[31], PD98059 has little effect on the Ca^{2+} spikes. However, at higher concentrations (30 µmol/L and 60 µmol/L) PD98059 significantly and acutely inhibits synchronized Ca^{2+} oscillations. We also showed that PD98059 inhibited the synchronized Ca^{2+} oscillations mainly through inhibition of MEK. This conclusion is based on the observation that SB202474, a structural analog of PD98059, which is usually used as a negative control for MAPK inhibitor studies, has no effect on the Ca^{2+} spike at a higher concentration (60 µmol/L). Although SB202474 has a strange effect on the Ca^{2+} oscillations at a lower concentration (10 µmol/L), by slightly increasing the frequency of the Ca^{2+} spikes and decreasing the amplitude of the spikes, it has no effect at a higher concentration (60 µmol/L). The effect of SB202474 on Ca^{2+} spikes at a lower concentration might result from its nonspecific activation of the Ca^{2+} channel, an idea we will try to explain in future studies. The salient point of this study is that we can conclude that

SB202474 does not inhibit Ca^{2+} spikes, but activates Ca^{2+} spikes at a lower concentration. As SB202474 is an inactive analog of PD98059, these observations indicate that PD98059 inhibits Ca^{2+} oscillations mainly through the inhibition of MEK, but not its side-effect on Ca^{2+} channel[30,32].

Many studies have implied that the MAPK cascade might participate in [Ca^{2+}]$_i$ regulation[22,23]. The synchronized spontaneous Ca^{2+} spikes in networked neurons represent the periodic firing of action potentials, which are believed to play a major role in the development and plasticity of neuronal circuitry[19], and the encoded information of the spontaneous Ca^{2+} oscillations was reported to lie in their frequency or amplitude[33]. Therefore, the inhibitory effect of PD98059 on the frequency and amplitude of spontaneous Ca^{2+} oscillations reported here implied that the MAPK cascade was required to maintain the spontaneous Ca^{2+} oscillations in developing hippocampal neurons. We know that MAPKs are a family of serine/threonine protein kinases which have classically been studied as regulators of cell proliferation and differentiation. The most important and well-known member of the MAPK family is ERK, which is initiated by growth factor receptor signaling. ERKs are extensively expressed in dendrites and somas of pyramidal neurons of the adult nervous system and can be activated by several neurotransmitters in neuronal culture system[34]. These points suggest that MAPKs might be excellent candidates for regulation of synaptic plasticity in post-mitotic neurons. MAPKs have been reported to regulate glutamate release and participate in the introduction of LTP[12]. Previous findings have shown that the MAPK cascade regulates synaptic transmission, and our work substantiates this by providing the time-course of PD98059 actions on synaptic transmission (the synchronized Ca^{2+} transients). It is also well known that Ca^{2+} plays an important role in the epileptiform discharge[35,36]. The synchronized spontaneous Ca^{2+} oscillations in the neuron network[37] are usually considered a kind of spontaneous epileptiform activity. Zhao *et al* reported that ERK1/2 was required for the induction of group I metabotropic glutamate receptor-mediated epileptiform discharges. Murray *et al* reported that PD98059 protected hippocampal neurons from seizure-like events[38]. Our results provide further evidence for the effect of PD98059 on the hippocampal network. The inhibitory effect of PD98059 on synchronized spontaneous Ca^{2+} oscillations through MAPK might be used to develop drugs for epileptiform therapy.

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