Intracellular Calcium Oscillation in Cultured Rat Hippocampal Neurons:
A Model for Glutamatergic Neurotransmission

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Received October 16, 1995    Accepted November 13, 1995

ABSTRACT—Neurons can form a synaptic network in culture and show spontaneous oscillation of intracellular Ca²⁺ concentration ([Ca²⁺]). In the present study, spontaneous oscillation of [Ca²⁺], was characterized in cultured hippocampal neurons. The oscillation was blocked completely by tetrodotoxin, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and nicardipine, while DL-2-amino-5-phosphonovaleric acid (APV) showed only a partial depression of the increase in [Ca²⁺]. These results suggest that the oscillation in [Ca²⁺], is mainly mediated by non-N-methyl-D-aspartate (NMDA) type glutamatergic transmission. The oscillation of [Ca²⁺], may be a good model for analyzing glutamatergic transmission and synapse formation.

Keywords: Glutamatergic neurotransmission, Synapse formation, non-NMDA receptor

It is widely known that oscillation of intracellular Ca²⁺ concentration ([Ca²⁺]) is observed in several neuronal preparations in vitro, such as cultured hippocampal (1, 2) and cortical neurons (3), and the slice preparation of hypothalamic suprachiasmatic nucleus (4). Such intracellular Ca²⁺ dynamics are synchronized to the burst of action potentials and seem to be due to Ca²⁺ influxes during the spike bursts (5). Especially, the dissociated embryonic neurons can develop morphologically and functionally in culture, form a synaptic network, and show the oscillation of [Ca²⁺]. For the analysis of the oscillation in cultured neurons, the Mg²⁺-depleted condition (1, 2) or Mg²⁺-depleted and glycine-containing condition (5) has been used presumably to avoid voltage-dependent block of N-methyl-D-aspartate (NMDA)-receptor channels by Mg²⁺ (6). Lowering extracellular Mg²⁺ concentration induces spontaneous NMDA receptor-dependent postsynaptic potential and epileptiform firing in the cortical slice preparation (7, 8). Neuronal activity may be facilitated by the Mg²⁺-free condition. However, in the brain, extracellular Mg²⁺ is present generally in mM order and is thought to have important roles in the control of transmitter release (9). In the present study, we report the new findings that the spontaneous oscillatory changes of [Ca²⁺], occur even in the presence of a physiological concentration of Mg²⁺. The pharmacological characterization revealed that non-NMDA type glutamatergic transmission primary contributes to the oscillation of [Ca²⁺],, which contrasts with the results under the Mg²⁺-free condition (1, 5).

The hippocampal neurons from embryonic day 18 Wistar rat brain were dissociated with digestion of trypsin as described previously (10). They were plated in poly-L-lysine-coated glass coverslips (0.3 cm²) at a density of 200,000 cells/cm² and cultured in modified Eagle’s minimum essential medium containing 10% fetal bovine serum.

The neurons were perfused with HEPES-buffered basal salt solution having the following composition: 130.0 mM NaCl, 5.0 mM KCl, 1.8 mM CaCl₂, 10 mM glucose, 1.3 mM MgCl₂ and 20 mM HEPES-NaOH (pH 7.4). Changes of [Ca²⁺], were detected by the microfluorometrical technique with fura-2 according to the method described previously (10). Calibration of [Ca²⁺], was based on chelation with EGTA in PIPES-KOH buffered solution (1, 11).

Tetrodotoxin (TTX) and DL-2-amino-5-phosphonovaleric acid (APV) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) was purchased from Tocris Neuramin (Bristol, England). Nicardipine was a kind gift from Yamanouchi Pharmaceut. Co. (Tokyo).

Eleven to 14 days after the cell dissociation, hippocampal neurons showed synchronous and periodical oscillation of [Ca²⁺], in Mg²⁺-containing solution. Such oscillation was not observed when the duration of culture was

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less than 10 days. The typical pattern of oscillation is shown in Fig. 1A, and the calculated \([Ca^{2+}]_i\) at the peak and the bottom were 108 ± 8.5 and 58 ± 4.6 nM (N=52 neurons), respectively. The averaged frequency was 3.2 ± 0.3 (mean ± S.E.M.) min\(^{-1}\). These values for each neuron were obtained over a period of 5 min. Although this type of oscillation usually continued for more than 1 hr, the frequency was often changed during the observation period. The periodicity, or the interval between the peaks, was not regular and varied greatly among the neuronal groups. Therefore, we did not analyze the frequency of the oscillation quantitatively to evaluate the effect of drugs.

The application of TTX (1 μM) reversibly abolished the spontaneous activity. However, the basal level of \([Ca^{2+}]_i\) (56.8 ± 1.9 nM, N=33) was not significantly affected (Fig. 1A). Reversible depression of the oscillation was also observed during the perfusion of \(Ca^{2+}\)-free solution supplemented with 0.1 mM EGTA, and the basal level of \([Ca^{2+}]_i\) was decreased to 46.3 ± 2.6 nM (N=19, Fig. 1B). This characteristic of the oscillation described above was distinct from that of the \([Ca^{2+}]_i\) oscillation in astrocytes, which is insensitive to TTX and independent of extracellular \(Ca^{2+}\) (12). These results suggest that the propagation of action potential and synaptic transmission are necessary for the periodical spontaneous \([Ca^{2+}]_i\) oscillation between neurons.

Then we tested whether spontaneous increase of \([Ca^{2+}]_i\) is operated by synaptically released glutamate. APV, a specific blocker of NMDA type receptor, slightly attenuated the peak increase of \([Ca^{2+}]_i\) concentration-dependent (10–100 μM), and statistical significance was detected at 100 μM (Fig. 2Aa). However, even at 100 μM, APV could not completely abolish the periodic activity (Fig. 3). CNQX, a selective blocker of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) /kainate type glutamate receptor, also suppressed the spontaneous increase in \([Ca^{2+}]_i\) concentration-dependently (3–30 μM). In contrast to the results with APV, CNQX completely abolished the oscillation at 30 μM (Fig. 2Ab). These data are summarized in Fig. 3. CNQX at 30 μM also decreased the minimum value of \([Ca^{2+}]_i\) significantly. Figure 2Ac shows that the \([Ca^{2+}]_i\) oscillation remaining in the presence of 100 μM APV was
abolished by an additional exposure to 3 μM CNQX. These results suggest that the spontaneous increase in [Ca²⁺]ᵢ is mediated by glutamatergic synaptic transmission.

As mentioned above, CNQX blocked the oscillation completely while APV showed only a partial depression of the increase in [Ca²⁺]ᵢ. This is in contrast to the previous results in the Mg²⁺-free condition, in which either APV (1) or CNQX (5) alone inhibits the oscillation of [Ca²⁺]ᵢ completely. Therefore, we analyzed the expression of the oscillation in the absence of Mg²⁺. The Mg²⁺-free condition tended to shift the oscillation ampli-
tude upward (peak and bottom [Ca$$^{2+}$$]$_i$, 158±27 and 106±20 nM, respectively; mean±S.E.M., N=8). In this condition, 50 μM APV and 10 μM CNQX showed 34.8±3.6% (significant P<0.05) and 52.5±3.4% (significant P<0.01) inhibition of the amplitude, respectively. They showed the similar inhibition and a tendency to decrease the minimum value of [Ca$$^{2+}$$]$_i$ even in the Mg$^{2+}$-free condition. These results suggest that the oscillation has low sensitivity to APV and that the Mg$^{2+}$-free condition is not necessary for the induction of the Ca$^{2+}$ oscillation. The characteristics of the oscillation were different from those reported previously (1).

The NMDA-receptor channel is highly modulated by the voltage-dependent blockade of a physiological concentration of Mg$^{2+}$ (6), and the fast depolarizing responses at most excitatory synapses are usually operated by AMPA receptor channels. Several studies showed that if a neuron is depolarized by AMPA receptor activation or the influence of inhibitory postsynaptic potentials is reduced so as to reduce the Mg$^{2+}$ block, then NMDA receptors can be synaptically activated. Our present results indicate that the synaptic activation of AMPA receptor alone is enough to initiate the increase of [Ca$$^{2+}$$]$_i$.

The activation of glutamate receptor channels elicits membrane depolarization that will promote Ca$$^{2+}$$ influxes through voltage-gated Ca$^{2+}$ channels (VGCCs). During application for 6 min of 10 mM nicardipine, an L-type VGCC blocker, the spontaneous increase in [Ca$$^{2+}$$]$_i$ disappeared, and the basal level of [Ca$$^{2+}$$]$_i$ decreased to 51.3±2.6 nM (N=28, Fig. 2B). The result indicates that the Ca$$^{2+}$$ influx through L-type VGCC rather than NMDA channel contributes primarily to the increment of [Ca$$^{2+}$$]$_i$. This also coincides with the previous findings that the major Ca$$^{2+}$$ influx is mediated not by NMDA channels but by VGCC in hippocampal CA1 pyramidal cells (13) and that voltage-clamp abolishes the [Ca$$^{2+}$$]$_i$ oscillation although bursts of inward current can be observed (5). L-Type Ca$^{2+}$ channels cluster at the base of dendrites in hippocampal pyramidal cells (14). On the other hand, both NMDA and non-NMDA type glutamate receptors are concentrated at synaptic sites along dendrites (15). Therefore, direct entry of Ca$$^{2+}$$ through VGCC appears to contribute mainly to the increase of [Ca$$^{2+}$$]$_i$ in the cell body.

The oscillation of [Ca$$^{2+}$$]$_i$ in the presence of Mg$^{2+}$ is available as a new model for the analysis of non-NMDA type glutamatergic transmission, because the amplitude of oscillation can reflect the glutamatergic synaptic excitability followed by Ca$$^{2+}$$ influx. Moreover, it may help to examine the mechanism of synapse formation.

In conclusion, the spontaneous oscillation in [Ca$$^{2+}$$]$_i$ was mediated by the glutamatergic transmission between neurons. The activation of non-NMDA type glutamate receptor was important for the initiation of spontaneous increase in [Ca$$^{2+}$$]$_i$. Influx not that through NMDA-receptor channels but that through dihydropyridine-sensitive L-type VGCC primarily contributed to the spontaneous rise in [Ca$$^{2+}$$]$_i$. The oscillation of [Ca$$^{2+}$$]$_i$ may be a good model for analyzing glutamatergic transmission and synapse formation.

Fig. 3. Concentration-dependent effects of APV or CNQX on the peak increase and the bottom values of [Ca$$^{2+}$$]$_i$. The level of the top and bottom of the column indicate the peak and bottom values of [Ca$$^{2+}$$]$_i$, respectively. CNQX was more potent and effective than APV in decreasing the peak. CNQX but not APV decreased the baseline value. Asterisks indicate significant differences (*P<0.05, **P<0.01) from the control value by Tukey's multiple test. Vertical bars represent S.E.M. Data were obtained from 26 cells.
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