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Correlation between mEPSC Amplitude and Rise Time upon the Blockade of AMPA Receptor Desensitization at Hippocampal Synapses

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Conventional views of synaptic transmission generally overlook the possibility of "postfusional-control" the regulation of the speed or completeness of transmitter release upon vesicular fusion. However, such regulation often occurs in non-neuronal cells where the dynamics of fusion-pore opening is critical for the speed of transmitter release. In case of synapses, the slower the transmitter release, the smaller the size and rate-of-rise of postsynaptic responses would be expected if postsynaptic neurotransmitter receptors were not saturated. This prediction was tested at hippocampal synapses where postsynaptic AMPA-type glutamate receptors (AMPAR) were not generally saturated. Here, we found that the small miniature excitatory postsynaptic currents (mEPSCs) showed significantly slower rise times than the large mEPSCs when the sucrose-induced mEPSCs recorded in cyclothiazide (CTZ), a blocker for AMPAR desensitization, were sorted by size. The slow rise time of the small mEPSCs might result from slow release through a non-expanding fusion pore, consistent with postfusional control of neurotransmitter release at central synapses.

Key Words: Fusion pore, Neurotransmitter release, Postfusional control, mEPSC

INTRODUCTION

Dr. Bernard Katz proposed that neurotransmitters were stored in small packets whose sizes were relatively homogenous, and that such packets were released in a probabilistic way upon the arrival of action potentials (Katz, 1969). This quantal theory of neurotransmitter release has not been proven yet, but it has been widely accepted mainly because it well explained a great deal of experimental data and because no one has found any clear evidence against it. Currently, the small packets are believed to represent synaptic vesicles, and all of neurotransmitters stored in synaptic vesicles are thought to be released at once upon the fusion of synaptic vesicles. In this classical vesicle recycling pathway, synaptic vesicles collapse completely into the surface membrane and become internalized with variable duration at a distant location from the active zone and mediated by clathrin-meidated endocytosis and processing through endosomes (Heuser, 1973; Koenig, 1996).

An alternative model, the 'kiss-and-run' hypothesis, proposes that synaptic vesicles may transiently fuse with the plasma membrane and release their contents through a partially open fusion pore without merging of vesicle and plasma membrane (Ceccarelli & Hurlbut, 1980; Almers & Tse, 1990; Neher, 1993; Fesce et al, 1994). Such fusion pores have been shown to exhibit two modes of operation as shown in large dense core vesicles of endocrine cells (Bruns & Jahn, 1995); 1) some of fusion pores do not expand (a non-expanding fusion pore), 2) others expand rapidly (a rapidly expanding fusion pore). While transmitters are released almost instantaneously through the rapidly expanding fusion pore, transmitter release can be slowed down due to the non-expanding fusion pore (Bruns & Jahn, 1995). The fusion pore operation could be modulated by kinases, phosphatases, and calcium levels, thereby regulating the speed of transmitter release (Scepek et al, 1998; Ales et al, 1999).

However, the modulation of transmitter release through the dynamics of fusion pore opening has not been tested in case of synaptic vesicles mainly due to technical difficulties (but cf. Khanin et al, 1994). It is extremely difficult to locate any sensors or probes right in front of presynaptic release sites because corresponding postsynaptic spines directly appose the release sites. One way to deal with this problem would be to use postsynaptic neurotransmitter receptors as a sensor for transmitter release. The kinetics of currents mediated by postsynaptic neurotransmitter receptors then could represent that of transmitter release such that slow release arising from the non-expanding fusion pore might induce small amplitudes of postsynaptic responses with slow rise times.

In the present study, we examined postsynaptic AMPA-type glutamate receptor-mediated responses in response to the fusion of single vesicles at hippocampal synapses, and compared their size and kinetics. We found that, in the presence of CTZ, the small responses exhibited slower rise

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ABBREVIATIONS: CTZ, cyclothiazide; mEPSC, miniature excitatory postsynaptic currents; CON, control.

times than the large responses, consistent with the postfusional regulation of neurotransmitter release.

METHODS

Brain slices

Rats (2~8 day-old) were sacrificed by decapitation and the brains were cooled in ice-cold, modified artificial cerebrospinal fluid (aCSF) containing (in mM): sucrose, 194; NaCl, 20; KCl, 3.5; $MgCl_2$, 1.3; NaH_2PO_4, 1.25; $NaHCO_3$, 26; glucose, 11 and gassed with 95% O_2/5% CO_2 at room temperature. Transverse hippocampal slices (300 mm) were cut in ice-cold modified aCSF using an automated vibroslicer. Slices were then transferred into the recording chamber, submerged and superfused continuously at 2~4 ml/min with aCSF containing (in mM): NaCl, 120; KCl, 3.5; $MgCl_2$, 1.3; $CaCl_2$, 2~2.5; NaH_2PO_4, 1.25; $NaHCO_3$, 26; glucose, 11; picrotoxin, 0.1, and gassed with 95% O_2/5% CO_2 at room temperature. A cut was made between regions CA3 and CA1 to prevent epileptiform activity.

Electrophysiology

Whole-cell voltage-clamp recordings were made under differential interference contrast (DIC)-enhanced visual guidance from neurons 3~4 cell layers below the surface of slices at room temperature. Miniature EPSCs were recorded with a patch electrode (1~4 MΩ tip resistance, no fire polishing or Sylgard coating) in the whole cell recording mode (Axopatch 1D). Pipette solution contained (in mM): cesium-gluconate, 100; EGTA, 0.6 or 10; HEPES, 10; CsCl, 10; NaCl, 5; TEAOH, 20; Mg-ATP, 4; Na-GTP, 0.3 (pH 7.3 with CsOH). The series resistance, which was not compensated, was typically within 10 MΩ. Miniature EPSCs were filtered at 1~2 kHz, digitized at 5 kHz and stored in a microcomputer. AMPA receptor-mediated mEPSC amplitudes recorded at −60 to −90 mV were determined by averaging the current over a 1 ms window centered on the peak response and subtracting a baseline estimate from the same record. Kinetics of the rising and decaying phases were described by measuring 10~90% rise time and 90~50% decay time. A sharply-defined change in superfusing solutions was achieved by means of a three-way stop cock that switched between inflow of normal and drug-containing aCSF. This allowed precise control of the duration of antagonist application during repeated trials. All averaged values are given as mean±SEM and were compared statistically by paired *t*-test (criterion for significance, $p<0.05$), unless otherwise noted.

Outside-out patch recordings and ultrafast solution changes

We recorded AMPAR responses from patches taken from somata of CA1 pyramidal cells in slices from 5~8 day-old rats. External solutions were gravity-fed to each lumen of theta glass tubing pulled to a tip diameter of around 200 mm. Fast solution changes (10~90% rise time $<200\,\mu s$) were elicited by rapid movement of the interface between solutions, driven by a piezoelectric translator (LSS-3100, Burleigh, Fisher, New York) (Clements et al, 1992). Control external solution contained 160 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 10 mM HEPES, 2 mM $MgCl_2$, 50 mM D-AP_5, 2 mM picrotoxin and 1 mM tetrodotoxin at pH 7.3. Internal solution contained 150 mM CsCl, 10 mM HEPES and 10 mM EGTA at pH 7.2.

RESULTS

The major goal of the present study was to detect slow release possibly from a non-expanding fusion pore by using postsynaptic neurotransmitter receptors as a sensor for neurotransmitter release. Because neurotransmitters are thought to be released in a millisecond scale (Clements et al, 1992), we chose to use AMPA-type glutamate receptors, of which activation kinetics has been shown to be extremely fast. AMPARs, however, tend to desensitize very fast, which prevents AMPARs from gauging glutamate release properly. Due to possible desensitization of AMPARs by slow release from a non-expanding fusion pore, the size of the desensitized responses might be too small to detect in our experimental conditions. To overcome this problem, we used CTZ, a potent inhibitor for AMPAR desensitization in all the experiments.

Experiments in outside-out patches confirmed that the duration of glutamate transient was well represented by rising phases of AMPAR currents in the presence of CTZ and that CTZ enhanced the activation of AMPAR currents at low glutamate concentrations (Dzubay & Jahr, 1999). Pulses of 100 μM glutamate evoked AMPAR currents whose

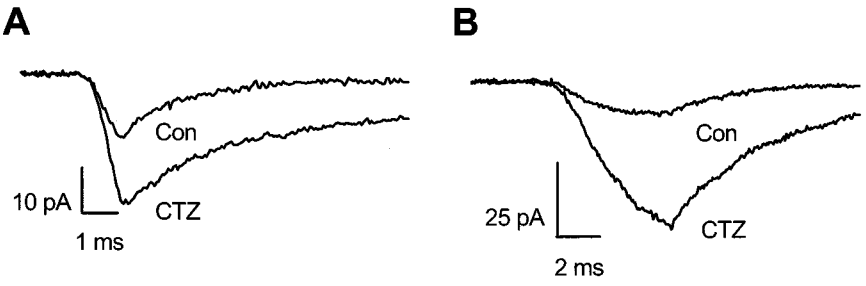

Fig. 1. CTZ blocks AMPAR desensitization and enhances AMPA receptor function. CTZ potentiates glutamate responses of outside-out membrane patches from CA1 neurons. AMPAR current responses of outside-out pateches to 1 ms (A) or 5.5 ms (B) pulses of 100 μM glutamate.

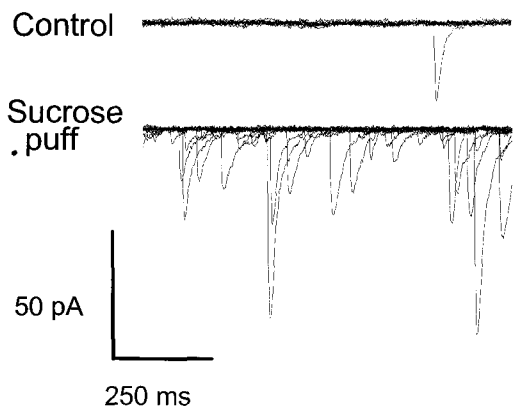

Fig. 2. Local application of hypertonic solution increases incidence of mEPSCs. Superimposed, representative current traces showing that incidence of mEPSCs was induced by pressure application of 0.3 M sucrose. Slices were pre-equilibrated with 100 μM cyclothiazide at least 15 min before a start of whole-cell patch-clamp recordings. Membrane potentials were held at −80 or −90 mV. 1 μM TTX and 50 μM Cd^{2+} were included in all solutions. The puffer pipette (tip resistance >5 MΩ) contained the same solution as the external fluid (including any drugs), but with addition of sucrose, and was located ~100 μM from cell soma. Pressure application was maintained for a period of 20~40 sec. The effect of sucrose application was limited to a local region, as judged by disappearance of responses upon moving the pipette tip by only 10~20 μm.

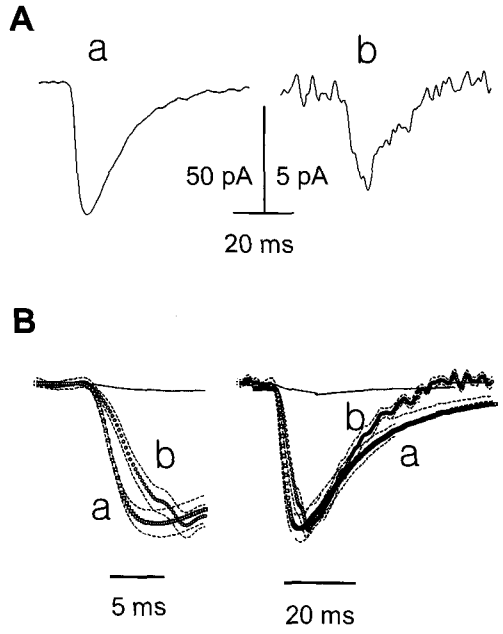

Fig. 3. CTZ reveals small mEPSCs exhibiting slow rise time. A, Representative current traces on a faster time scale, providing comparison of the kinetics of small EPSCs (smallest 30% of mEPSC amplitudes sorted by size) and large EPSCs (largest 20% of mEPSCs). The threshold value for event detection was 3.5-fold greater than the standard deviation of the background noise. B, Comparison of kinetics of small and large mEPSCs, scaled to the same peak amplitude. Averaged current traces assembled by aligning individual current traces at the point of maximal rising slope. Shown are mean current (solid line)±1 SEM (dashed traces). a and b represent large and small mEPSCs, respectively.

rise times tracked the duration of the glutamate transient (Fig. 1). As the glutamate pulse was prolonged (from 1 to 5.5 ms), the 10~90% rise time for control AMPA current increased from 0.80±0.05 ms to 3.5±0.5 ms (n=5), and from 0.83±0.05 ms to 3.93±0.15 ms in CTZ (n=4). In pooled data, 100 μM CTZ enhanced the responses to 1 ms or 5.5 ms glutamate pulses by 2.28±0.29 fold and 3.78±0.38-fold, respectively. Thus, the rise times of AMPAR currents measured in the presence of CTZ would be expected to be a good indicator for the duration of glutamate transient.

Having confirmed the usefulness of AMPARs as a sensor for glutamate receptor, we used local application of hypertonic solution to record mEPSCs, AMPAR-mediated synaptic currents due to the fusion of single vesicles (Malgaroli & Tsien, 1992). Hippocampal brain slices were presaturated with 100 μM CTZ for at least 15 min before initiation of whole-cell recordings. When aCSF solution containing 0.3 M sucrose was applied onto a dendrite through a puffer pipette located ~100 μm from cell soma, the frequency of mEPSCs was greatly increased (Fig. 2). The mEPSCs were completely inhibited by CNQX (n=3, data not shown). The amplitudes of sucrose-induced mEPSCs in the absence and presence of CTZ averaged 13.9±2.4 pA and 18.3±2.7 pA, respectively ($p>0.3$, unpaired t-test).

When the sucrose-induced mEPSCs recorded in the presence of CTZ were sorted by size, there was a systematic variation in their kinetic properties. The small mEPSCs showed considerably slower rise times and faster decay times than the large mEPSCs (Fig. 3 and 4). As summarized in Fig. 5, rise time of small mEPSCs (7.0±0.9 ms) was significantly slower than for large mEPSCs (2.4±0.2

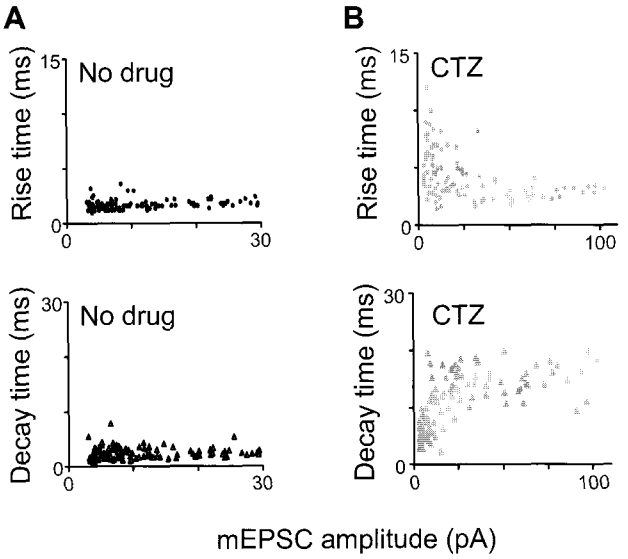

Fig. 4. Kinetic properties of individual EPSCs plotted against event amplitude. A, properties of mEPSCs collected while puffing 0.3 M sucrose onto a dendrite in the absence of CTZ (N=120). Rise and decay times of mEPSCs failed to exhibit significant correlations with event amplitudes. For the measurement of rise and decay time, current traces were repetitively filtered using a Gaussian filter available in pClamp6. B, kinetic properties of EPSCs recorded in the presence of CTZ (N=110). Small EPSCs rise more slowly, but decay more quickly than large mEPSCs.

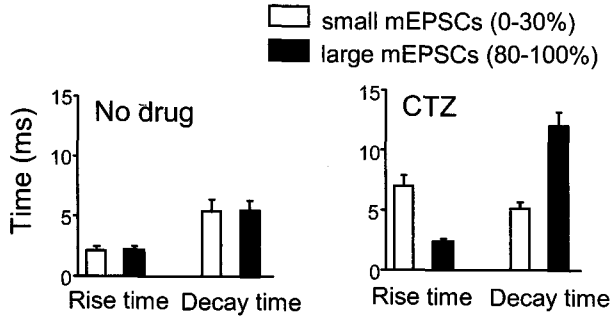

Fig. 5. Summary of kinetic data. Left, Summary of kinetic data in the absence of CTZ (n=4, left). Note that small and large mEPSCs showed no significant kinetic differences in their rise and decay times between (rise times of 2.2 ± 0.3 and 2.1 ± 0.4 ms for large and small mEPSCs, $p>0.4$; decay times of 5.5 ± 0.8 and 5.4 ± 1.0 ms for large and small mEPSCs, $p>0.9$). Right, Summary of kinetic data in the presence of 100 μM CTZ (n=7).

ms) ($p<0.002$), but the decay time was faster for small mEPSCs (5.1 ± 0.6 ms) than for large ones (12.1 ± 1.2 ms) ($p<0.0001$). Similar contrasts were observed for spontaneously occurring mEPSCs in the presence of CTZ, although the event frequency was much lower. For large mEPSCs, decay times in the presence of CTZ were significantly longer than those recorded in its absence ($p<0.05$), whereas decay times of small mEPSCs recorded in the absence or presence of CTZ were not significantly different ($p>0.7$), consistent with the notion that their decay was controlled by a factor other than desensitization.

The slow rise and brisk decay of the small mEPSCs gave them an unconventional waveform, consistent with a slow escape of glutamate from single vesicles and its abrupt termination. In contrast, sucrose-induced mEPSCs in the absence of CTZ failed to show any significant correlation between mEPSC amplitude and rise or decay time (Figs. 4 and 5). In Fig. 4, relationship between mEPSC amplitude and rise or decay time were examined in individual mEPSCs, revealing no significant correlations. Furthermore, averaged rise or decay time of small mEPSCs (see Fig. 5 legend for the definition of small and large mEPSCs) was not significantly different from that of large mEPSCs (Fig. 5). These findings are consistent with our expectation that the majority of the small responses with slow kinetics are not detectable without CTZ.

Taken together, the observations in Figs. 4 and 5 suggest that CTZ can reveal transmission with distinctive kinetics at hippocampal synapses, reflecting novel quantal glutamate release properties consistent with a non-expanding fusion pore.

DISCUSSION

Our findings support a possible role of fusion pores in hippocampal synaptic transmission. The small mEPSCs with unusually slow kinetics was revealed by administration of CTZ. Because mEPSCs is believed to be synaptic responses due to the fusion of single vesicles, the slow kinetics of the small mEPSCs was most likely to be induced by existence of non-expanding fusion pores which hindered intravesicular glutamate from being released onto postsynaptic AMPARs.

Our data do not support the hypothesis that the slow rise time of the small mEPSCs reflects spillover of glutamate from neighboring boutons in the face of failures at the immediately presynaptic terminal (Kullmann & Asztely, 1998; Rusakov & Kullmann, 1998). In this case, decay times as well as rise times of the small mEPSCs are supposed to be prolonged since it takes longer for glutamate not only to diffuse onto receptors but also to be cleared out. However, decay times of the small mEPSCs were faster than those of the large mEPSCs, arguing against the spillover hypothesis.

The slowness of the small mEPSCs could not be readily accounted for by increased detection of distal dendritic inputs shaped by cable filtering (Hausser et al, 2000). The voltage-clamp conditions were optimized and stable (see Methods), and the unitary synaptic currents were extremely small. Furthermore, the decay times of the small mEPSCs were faster than those of the large mEPSCs. This finding is opposite to what one can expect from the dendritic cable filtering. If the slow rise times of the small mEPSCs were due to the dendritic filtering, then the decay times would be also slowed down. Thus, our results do not easily reconcile with the dendritic filtering hypothesis.

Currently, it remains to be elucidated whether the two modes of fusion pore operation (non-expanding vs rapidly expanding) can be inter-convertible through the actions of kinases, phosphatase or calcium levels as suggested in endocrine cells. If so, the proposed mechanism offers significant functional advantages for both synaptic transmission and synaptic plasticity. A change in the mode of gating of presynaptic fusion pores represents a precisely targeted, switch-like action for which there is ample precedent in non-neuronal cells (Rahamimoff & Fernandez, 1997; Wang et al, 2001). The regulation of peak cleft glutamate concentrations through the modulation of fusion pore dynamics would allow efficient, stepwise increases in AMPAR transmission, difficult to achieve by other cell biological mechanisms (Liao et al, 1999).

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