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A Single Packet of Transmitter Does Not Saturate Postsynaptic Glutamate Receptors

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Summary

Neurotransmitter is stored in synaptic vesicles and released by exocytosis into the synaptic cleft. One of the fundamental questions in central synaptic transmission is whether a quantal packet of transmitter saturates postsynaptic receptors. To address this question, we loaded the excitatory transmitter L-glutamate via whole-cell recording pipettes into the giant nerve terminal, the calyx of Held, in rat brainstem slices. This caused marked potentiations of both quantal and action potential-evoked EPSCs mediated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or *N*-methyl-D-aspartate (NMDA) receptors. These results directly demonstrate that neither AMPA nor NMDA receptors are saturated by a single packet of transmitter, and indicate that vesicular transmitter content is an important determinant of synaptic efficacy.

Introduction

It has long been proposed that a quantal packet of transmitter saturates postsynaptic receptors at the central synapse. This saturation hypothesis is based on the findings that the quantal size deduced from the equally spaced peaks in the amplitude histogram of nerve-evoked responses has small and nonincremental variation (Jack et al., 1981; Larkman et al., 1991; Jonas et al., 1993). This hypothesis is also supported by the estimation that the vesicular transmitter content largely exceeds the number of postsynaptic receptors activated by each transmitter packet (for a review, Frerking and Wilson, 1996). Recently, however, the saturation hypothesis was challenged at putative single-site synapses in the cerebellum (Silver et al., 1996b) and in the hippocampus (Gulyas et al., 1993; Liu et al., 1999; McAllister and Stevens, 2000) because of large variations in the quantal size compared with those previously deduced from evoked responses (for reviews, Frerking and Wilson, 1996; Auger and Marty, 2000).

Compared with AMPA receptors, NMDA receptors have much higher affinity to glutamate (Patneau and Mayer, 1990). At the hippocampal synapse, the AMPA/NMDA ratio of EPSCs remained constant when release probability was altered (Perkel and Nicoll, 1993; Tong and Jahr, 1994). These observations have led to a proposal that a packet of transmitter may saturate both types of receptors (Tong and Jahr, 1994). In contrast, at the putative single-site synapse in cortical (Umemiya et al., 1999) and hippocampal (McAllister and Stevens, 2000) cell culture, it has been suggested that neither AMPA nor NMDA receptors is saturated by a transmitter packet because the AMPA/NMDA ratio of miniature (m) EPSCs is constant despite their large size variation. Nonsaturation of postsynaptic NMDA receptors has also been proposed at the hippocampal CA1 synapse, because the NMDA receptor-mediated Ca^{2+} transients at the putative single-site synapse were facilitated by the paired pulse stimulation (Mainen et al., 1999).

Whether or not quantal transmitter saturates postsynaptic receptors is a fundamental question in central synaptic transmission because this determines whether an increase in vesicular transmitter content or multivesicular release at a release site (Auger et al., 1998) can enhance the synaptic efficacy. If transmitter quanta saturate postsynaptic receptors, an increase in vesicular transmitter content has no effect on the synaptic efficacy. Therefore, the most direct test for the saturation hypothesis is to increase the vesicular transmitter content and examine whether this increases the quantal amplitude. Such a test can be made at the calyx of Held synapse in auditory brainstem, where various molecules can be loaded into the giant presynaptic terminal via whole-cell patch pipettes, while recording simultaneously from the presynaptic terminal and the postsynaptic cell (Hori et al., 1999; Takahashi et al., 2000). Although this nerve terminal is large, electron microscopic studies show that the synaptic contacts that individual active zones make with the postsynaptic membrane are similar to those found in other regions of the mammalian nervous system (Lenn and Reese, 1966), with each active zone separated by 0.4 μm (Meinrenken et al., 2002). Thus this synapse can be a good model for testing the saturation hypothesis. Using this preparation, we manipulated the presynaptic cytoplasmic concentration of the excitatory transmitter L-glutamate, and examined its effect on the quantal and evoked EPSCs.

Results

Washout of Presynaptic L-Glutamate Causes EPSC Rundown

Paired whole-cell recordings were made from a giant nerve terminal, the calyx of Held, and a postsynaptic principal cell in the medial nucleus of the trapezoid body (MNTB) in rat brainstem slices (Borst et al., 1995; Hori et al., 1999; Takahashi et al., 2000). Non-NMDA-EPSCs were evoked by presynaptic action potentials in the presence of D(-)-2-amino-5-phosphonopentanoic acid (D-AP5, 25 μM) (Figure 1A). Evoked and spontaneous EPSCs could be abolished by the AMPA receptor antag-

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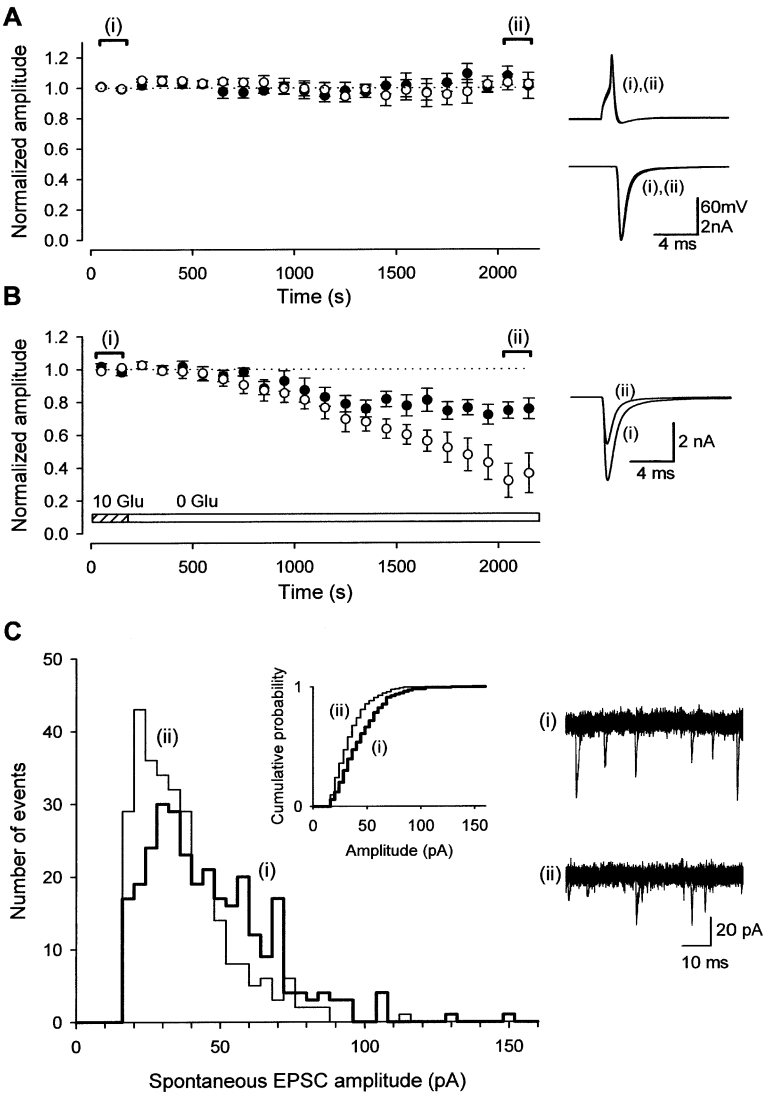

Figure 1. EPSC Rundown Caused by Washout of Presynaptic Cytoplasmic Glutamate

(A) Stability in the mean amplitude of action potential-evoked (open circles) and spontaneous (filled circles) EPSCs recorded from an MNTB neuron with a presynaptic whole-cell pipette containing 1–10 mM L-glutamate (n = 4). Data at each period (abscissa) represent mean amplitude of EPSCs from four experiments normalized to the initial two points (ordinate) both in (A) and (B). Error bars indicate SEMs in this and following Figures. Data derived from experiments in normal (n = 3) and Mg^{2+}-free (n = 1) aCSFs at −70 mV postsynaptic holding potential. The inset illustrates sample records of presynaptic action potentials and EPSCs. Records from two periods (i and ii) are superimposed.

(B) Presynaptic L-glutamate concentration was changed from 10 mM (hatched bar) to 0 mM (open bar) via pipette perfusion. Mean amplitudes of evoked (open circles) and spontaneous (filled circles) EPSCs. Each data is normalized to the amplitude before switch. Sample records are averaged evoked EPSCs before (i) and after (ii) switch (superimposed). Before L-glutamate infusion, the mean amplitude of evoked EPSCs was 4.80 ± 1.0 nA and that of spontaneous EPSCs was 43.1 ± 3.3 pA (n = 4). Mean frequency of spontaneous EPSCs was 3.47 ± 1.0 Hz.

(C) Representative amplitude histograms of spontaneous EPSCs recorded from a postsynaptic MNTB cell before (i) and after (ii) washout of glutamate (297 events each). In inset, spontaneous EPSCs are shown in ten consecutive records (superimposed) before (i) and after (ii) switch. Cumulative amplitude histograms (upper column) in the same cell before (thick lines) and after (thin lines) L-glutamate washout showing significantly different amplitude profiles ($p < 0.01$ in Kolomogolov-Smirnov test). The c.v. of spontaneous EPSC amplitude in four cells was 0.45 ± 0.06 before and 0.39 ± 0.04 after switch.

onist GYKI52466 (100 μM), indicating that these EPSCs are mediated by AMPA receptors (Futai et al., 2001). Tetrodotoxin (TTX, 1 μM) affected neither the frequency (93% ± 7%, mean ± SEM, n = 6 cells) nor the amplitude (103% ± 2%) of spontaneous EPSCs, indicating that these are equivalent to mEPSCs representing quantal events arising from transmitter liberated by single synaptic vesicles. In paired recordings with no glutamate in the presynaptic pipette, EPSCs gradually undergo "rundown" (Chuhma and Ohmori, 1998; Sakaba and Neher, 2001). When presynaptic pipettes contained L-glutamate (1–10 mM), however, the mean amplitude of evoked (open circles) and spontaneous (filled circles) EPSCs remained stable for more than 30 min (Figure 1A). The rundown of EPSCs could be reproduced by infusing glutamate-free solution into the presynaptic terminal via pipette perfusion (Figure 1B; Hori et al., 1999; Takahashi et al., 2000). After infusing glutamate-free solution, the amplitude of evoked EPSCs gradually decreased, and 30 min afterward reached 37% ± 11% (n = 4) of control (Figure 1B). Concomitantly spontaneous EPSCs decreased both in mean amplitude (to 75% ± 6%) and mean frequency (to 58% ± 14%). The amplitude histogram of spontaneous EPSCs shifted toward small amplitude (Figure 1C) with small events merging into the noise level. Washout of glutamate from synaptic vesicles can increase the number of undetectable quanta, i.e., poorly filled vesicles, thereby reducing apparent frequency of spontaneous EPSCs (Figure 1C). Concomitant reductions in amplitude and frequency of mEPSCs have also been reported for the effects of an AMPA receptor antagonist or a vesicle transporter blocker (Zhou et al., 2000). This mechanism explains a greater reduction in the mean amplitude of evoked EPSCs (including undetectable quanta) compared with that of spontaneous EPSCs (excluding undetectable events) (Figure 1B).

Presynaptic L-Glutamate Loading Potentiates Spontaneous and Evoked EPSCs

Given that the reduction of presynaptic cytoplasmic glutamate concentration reduced mean quantal amplitude, might increasing the glutamate concentration increase it? The quantal amplitude will not increase if postsynaptic AMPA receptors are saturated by transmitter quanta.

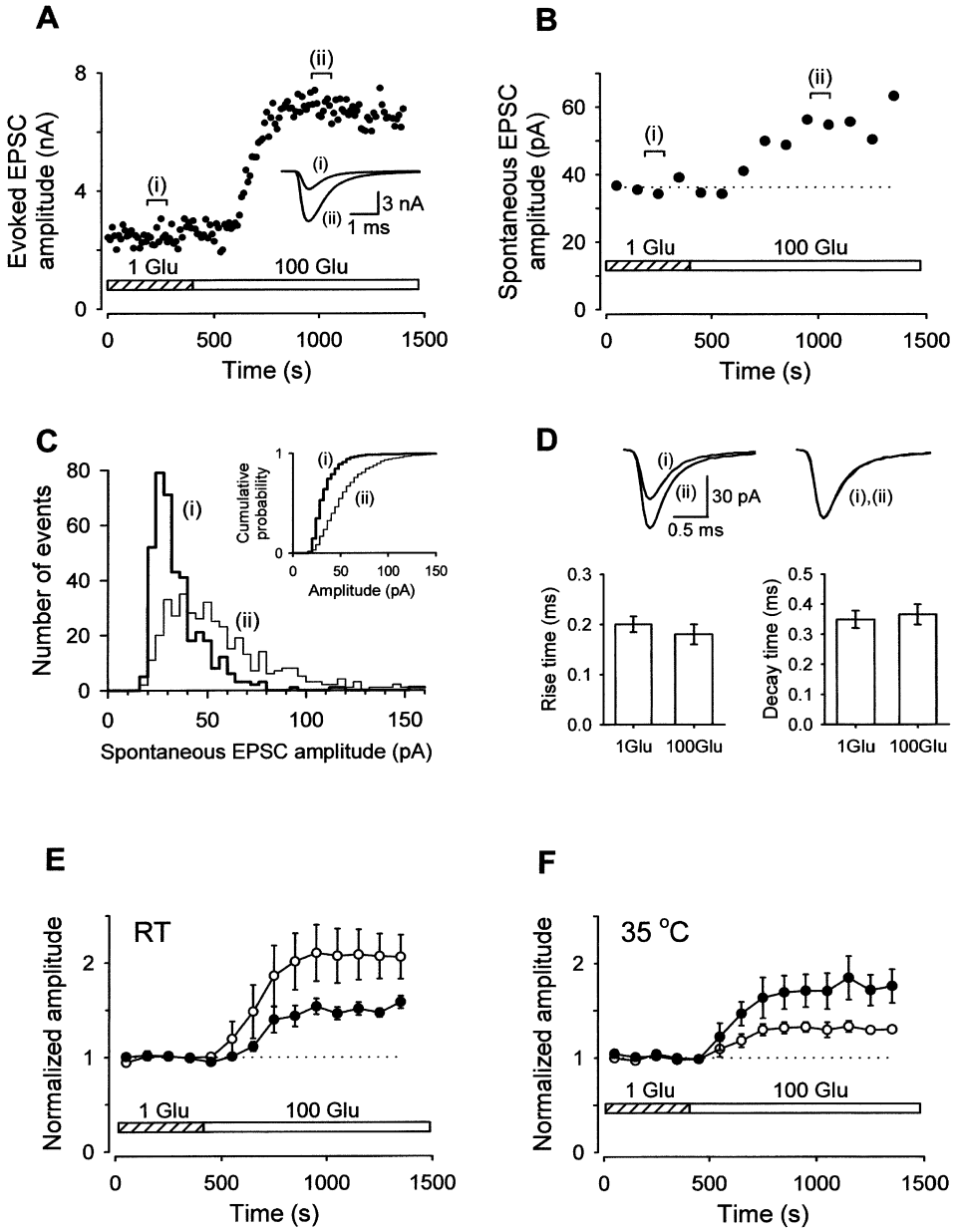

Figure 2. Potentiation of Evoked and Spontaneous EPSCs Caused by Presynaptic L-Glutamate Infusion

(A) Evoked EPSCs potentiated after switching L-glutamate concentration from 1 mM to 100 mM via pipette perfusion. Sample records are averaged EPSCs before (i) and after (ii) switch (superimposed).

(B) Concomitant potentiation of spontaneous EPSCs. Each data point represents mean amplitude of spontaneous EPSCs during 100 s periods.

(C) Amplitude histograms of spontaneous EPSCs recorded from the same neuron before (i) and after (ii) switch (390 events each). In cumulative amplitude histograms, the profiles before and after switch were significantly different (Kolomogolov-Smirnov test, $p < 0.01$).

(D) Kinetics of spontaneous EPSCs before (1 Glu) and after (100 Glu) L-glutamate infusion. Averaged spontaneous EPSCs (left) and those normalized in amplitude (right) before (i) and after (ii) switch are superimposed. No significant change in the rise time (10%–90%; 0.20 ± 0.02 ms before and 0.18 ± 0.02 ms after switch, $p > 0.1$, $n = 5$) or in the decay time (e-fold; 0.35 ± 0.03 ms before and 0.37 ± 0.03 ms after switch, $p > 0.7$, $n = 5$).

(E) Summary data from five synapses at room temperature (RT). Mean amplitudes of evoked (open circles) and spontaneous (filled circles) EPSCs before switch were 2.55 ± 0.34 nA and 33.9 ± 2.2 pA, respectively.

(F) Potentiation of spontaneous and evoked EPSCs at physiological temperature (35°C). Mean amplitudes of evoked (open circles) and spontaneous (filled circles) EPSCs derived from four synapses recorded in normal aCSF solution. Mean amplitude of spontaneous EPSCs before switch was 34.9 ± 3.2 pA ($n = 4$).

To answer this question, we infused 100 mM L-glutamate into the presynaptic terminal via pipette perfusion while recording evoked and spontaneous EPSCs. After a stable epoch of EPSCs recorded with 1 mM L-glutamate in a presynaptic pipette, 100 mM L-glutamate was infused into the pipette (Figure 2A). This caused a dra-

matic increase in the amplitudes of evoked and spontaneous EPSCs (Figures 2A and 2B) with no significant change in the frequency of spontaneous EPSCs (133% ± 40%, n = 5). After L-glutamate infusion, the amplitude profile of spontaneous EPSCs shifted toward larger events (Figure 2C). Concomitantly, the skewness decreased (by 39% ± 6%, n = 5, $p < 0.05$) as reported at a cholinergic synapse overexpressed with vesicular acetylcholine transporter (Song et al., 1997), suggesting that skewness arose, at least in part, from incompletely filled vesicles. However, the coefficient of variation (c.v.) (0.42 ± 0.02, n = 5) remained similar (105% ± 3%, n = 5). At five synapses, the mean magnitude of potentiation was 108% ± 28% for evoked EPSCs and 50% ± 7% for spontaneous EPSCs (Figure 2E). Despite the marked increase in the amplitude of spontaneous EPSCs, neither their rise time (10%–90%) nor decay time (e-fold) significantly changed (Figure 2D), suggesting that increased amount of transmitter in each vesicle increased occupancy of receptors apposing the release site rather than activating remote receptors. These results strongly suggest that quantal packets of transmitter do not normally saturate postsynaptic AMPA receptors.

Nonsaturation of Postsynaptic AMPA Receptors at Physiological Temperature

Although a single packet of transmitter does not saturate postsynaptic AMPA receptors at room temperature, it may saturate the receptors at physiological temperature at which reaction rates of the transmitter-receptor interaction are faster (Silver et al., 1996a). To examine this, we made experiments at 35°C, at which the mean amplitude of evoked EPSCs (5.53 ± 0.44 nA with 1 mM presynaptic L-glutamate, n = 4) was larger than that at room temperature (2.55 ± 0.34 nA, n = 5, $p < 0.005$, unpaired t test), presumably because of higher release probability. Presynaptic infusion of 100 mM L-glutamate potentiated the mean amplitude of spontaneous EPSCs by 75% ± 19% (n = 4, Figure 2F), indicating that quantal transmitter does not saturate postsynaptic AMPA receptors at physiological temperature. The evoked EPSCs were also potentiated (by 32% ± 6%, n = 4), but much less in magnitude than that at room temperature.

The Mean Quantal Amplitude Is Dependent upon the Presynaptic Cytosolic L-Glutamate Concentration

The calyceal nerve terminals were loaded with various concentrations of L-glutamate via presynaptic whole-cell pipettes, and spontaneous mEPSCs were recorded in the presence of TTX (Figure 3). When the presynaptic L-glutamate concentration was higher, the mean amplitude of mEPSCs was larger. The mean amplitude of mEPSCs recorded with a single postsynaptic pipette (42.9 ± 4.3 pA, n = 9, open circle) fell between those recorded with 1 mM and 10 mM presynaptic L-glutamate, suggesting the range of endogenous L-glutamate concentration in the calyx of Held nerve terminal. Similar values have been estimated for crayfish motor axons (2–7 mM; Shupliakov et al., 1995) and goldfish retinal cells (1–10 mM; Marc et al., 1990). At higher presynaptic L-glutamate concentrations, mEPSCs were significantly larger (50 mM, $p < 0.05$; 100 mM, $p < 0.005$, unpaired t test), supporting further the nonsaturation of postsynaptic AMPA receptors by a single quantum.

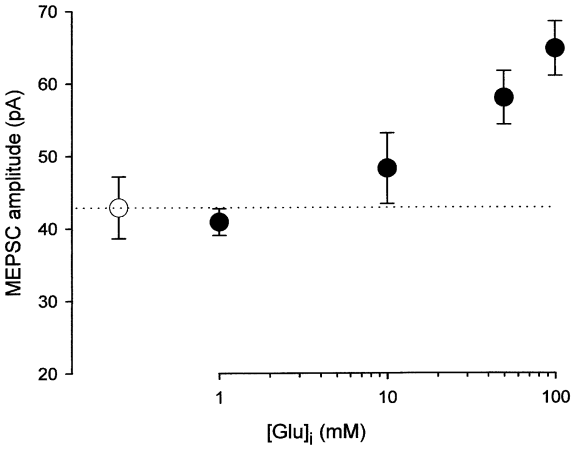

Figure 3. Dependence of Mean Quantal Amplitude on Presynaptic Cytoplasmic L-Glutamate Concentration

In simultaneous pre- and postsynaptic whole-cell recordings, presynaptic pipettes contained 1–100 mM L-glutamate. MEPSCs were recorded in the presence of TTX (1 μM). Data were sampled more than 10 min after membrane rupture. Filled circles represent the mean amplitudes of mEPSCs from 4 to 6 synapses (from 200 to 400 events each). Dashed line and an open circle indicate the mean amplitude of spontaneous mEPSCs recorded from MNTB neurons with a single postsynaptic pipette (n = 9).

Simultaneous Release of Quanta from Multiple Sites Can Saturate Postsynaptic AMPA Receptors

While a single packet of transmitter does not saturate postsynaptic AMPA receptors, simultaneous release of many packets of transmitter from multiple sites might saturate them. Large EPSCs were evoked at high release probability by presynaptic Ca^{2+} currents induced by depolarizing square pulses after blocking sodium and potassium conductance (Wu and Borst, 1999; Sun and Wu, 2001). Increasing the duration of presynaptic depolarizing pulses from 1 ms to 2 ms increased the amplitude of EPSCs, but longer pulse duration (5 ms) had no further effect (Figure 4A), suggesting that postsynaptic AMPA receptors might be saturated by transmitter released by 2 ms pulse (Wu and Borst, 1999; Sun and Wu, 2001). The decay times of these EPSCs were slow (time constant, 4.4 ± 0.8 ms, n = 4), suggesting sustained high concentration of transmitter in the synaptic cleft (Silver et al., 1996b). When we infused 100 mM L-glutamate into the nerve terminal, EPSCs evoked by 2 ms pulses remained similar in amplitude, whereas spontaneous mEPSCs underwent potentiation by 46% ± 8% (n = 4, Figure 4B). These results suggest that massively released transmitter quanta saturate postsynaptic AMPA receptors, whereas a single packet of transmitter cannot. Transmitter simultaneously released from multiple sites may overlap with each other resulting in a sustained high concentration in the synaptic cleft, thereby saturating postsynaptic receptors, as described at the cholinesterase-treated neuromuscular junction (Hartzell et al., 1975) and at the cerebellar mossy fiber synapse (Silver et al., 1996b). This may explain the smaller magni-

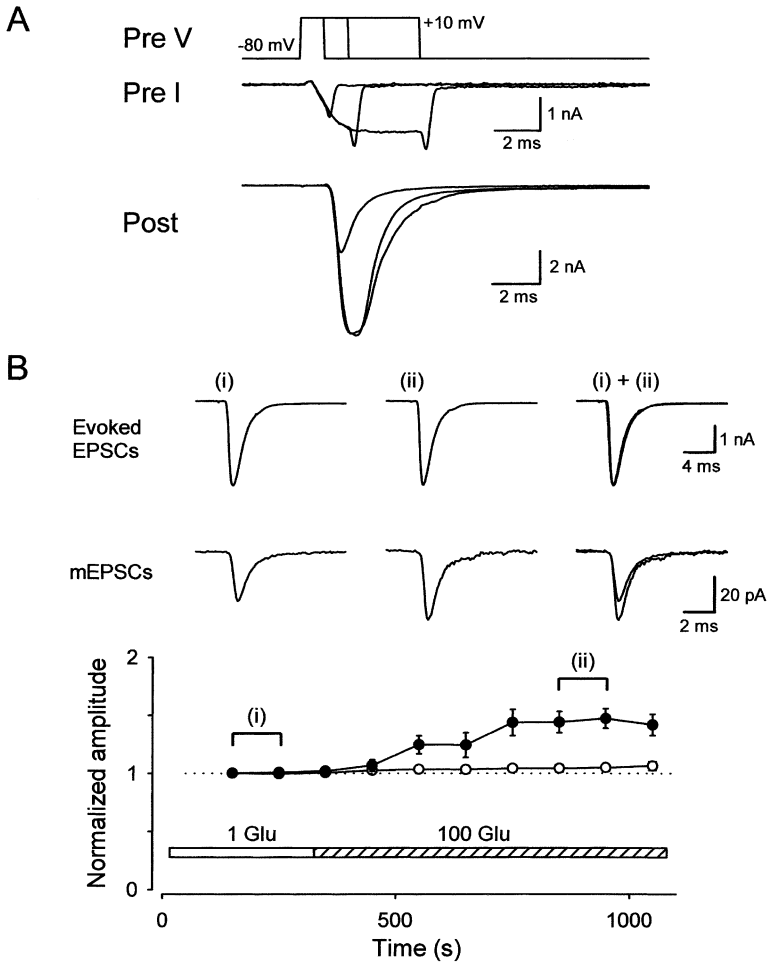

Figure 4. Effects of Presynaptic L-Glutamate Loadings on EPSCs Evoked at High Release Probability

(A) EPSCs (Post) evoked by presynaptic Ca^{2+} currents (Pre I) elicited by depolarizing pulses of 1, 2, and 5 ms duration from the holding potential of −80 mV to +10 mV (Pre V) in the presence of TTX (superimposed). EPSCs were evoked at 0.05 Hz. The holding potential of postsynaptic cell was −70 mV.

(B) Presynaptic loading of 100 mM L-glutamate had no effect on EPSCs evoked by 2 ms pulses at 0.05 Hz (open circles), but potentiated mEPSCs (filled circles). Sample traces are evoked EPSCs and spontaneous mEPSCs before (i) and after (ii) 100 mM L-glutamate infusion (superimposed). In this particular cell, postsynaptic potential was held at −20 mV for better voltage-clamp performance for 2 s when EPSCs were evoked. But for this cell, postsynaptic holding potential was −70 mV. Spontaneous EPSCs were recorded at the holding potential of −70 mV for 5 s periods between the evoked EPSCs. In time plot, each data point of evoked EPSCs derived from five consecutive records.

tude of potentiation of evoked EPSCs compared with that of spontaneous EPSCs after L-glutamate infusion at physiological temperature (Figure 2F, also see below).

Presynaptic Loading of L-Glutamate Increases Quantal Amplitude and Quantal Content

After the L-glutamate infusion, at the room temperature, the magnitude of potentiation of evoked EPSCs was significantly larger than that of spontaneous EPSCs (Figure 2E). To examine whether the quantal content (number of quanta released by a presynaptic action potential) might increase after L-glutamate loading, we made quantal analysis in low Ca^{2+} (0.5 mM)/high Mg^{2+} (5 mM) aCSF solution. At the low release probability, evoked EPSC amplitude can be adequately described by the Poisson's law, and the mean quantal content can be estimated from stochastic failures of EPSCs (Sahara and Takahashi, 2001). In the low Ca^{2+}/high Mg^{2+} solution, infusion of 100 mM L-glutamate potentiated evoked EPSCs (mean amplitude, 23.0 ± 2.0 pA, n = 4) by 89% ± 24% and spontaneous EPSCs by 65% ± 15% (n = 4, Figure 5, measured from the last 3 points), whereas the mean quantal content (*Np*) increased from 0.69 ± 0.03 to 0.85 ± 0.05 (23% ± 5% increase, n = 4, $p < 0.05$, paired t test, Figure 5), suggesting that the release probability (*p*) and/or the number of releasable vesicles (*N*) increased by L-glutamate loading. Transmitter contents in individual vesicles are thought to vary widely (Bekkers et al., 1990). Thus increased cytosolic L-glutamate might recruit previously empty vesicles, thereby contributing to an increase in the effective number of releasable vesicles (*N*). However, it is also possible that L-glutamate increased release probability (*p*) via an unknown mechanism. Although the increase in quantal content well explains the difference in the magnitude of potentiation between evoked and spontaneous EPSCs in the low Ca^{2+} solution, greater difference observed in normal solution (Figure 2E) may additionally be caused by an overlap of quantal transmitter released from multiple sites (Silver et al., 1996b).

A Single Packet of Transmitter Does Not Saturate Postsynaptic NMDA Receptors

Compared with AMPA receptors, NMDA receptors have by two orders of magnitude higher affinity to L-glutamate (Patneau and Mayer, 1990), therefore they can be more readily saturated by transmitter glutamate (Clements et al., 1992). We examined whether quantal packets of transmitter saturate postsynaptic NMDA receptors at this synapse. Because of the small amplitudes and slow kinetics, spontaneous NMDA-EPSCs cannot be measured reliably after blocking AMPA-EPSCs. We therefore

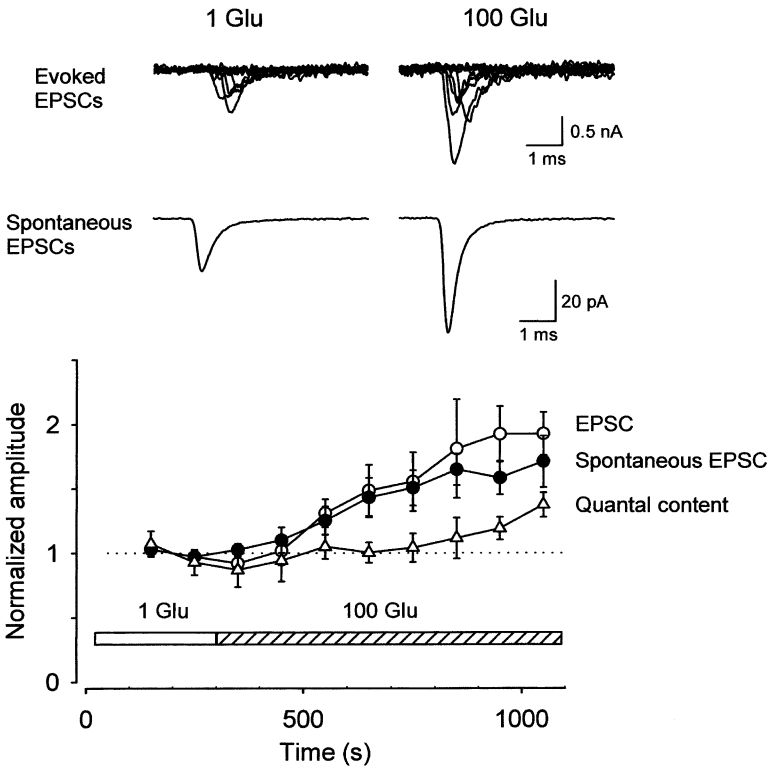

Figure 5. Effects of Presynaptic L-Glutamate Loadings on EPSCs Evoked at Low Release Probability

Evoked and spontaneous EPSCs recorded in 0.5 mM Ca^{2+}/5 mM Mg^{2+} aCSF solution before (1 Glu) and after infusion of 100 mM L-glutamate (100 Glu). Sample records are EPSCs evoked by presynaptic action potentials (consecutive events at 1 Hz, superimposed) and averaged spontaneous EPSCs obtained from a postsynaptic MNTB cell. Mean amplitudes of evoked (open circles) and spontaneous (filled circles) EPSCs at four synapses are shown in time plots. Mean quantal content (*Np*, open triangles) was calculated from $Np = -\ln$ (rate of failure) (del Castillo and Katz, 1954).

extracted NMDA-EPSCs by a subtraction method using D-AP5. After recording stable EPSCs in nominally Mg^{2+}-free solution, NMDA receptors were blocked by D-AP5 (50 μM) and AMPA-EPSCs were isolated (Figure 6A, ii). We then washed out D-AP5 and let the NMDA component recover to the original level. After a stable epoch of EPSCs, we infused 100 mM L-glutamate into the nerve terminal. This caused marked and parallel potentiations of evoked AMPA-EPSCs (by 109% ± 25%) and NMDA-EPSCs (127% ± 25%, n = 5, Figure 6A, iv). In experiments where the NMDA components were reliably resolved in spontaneous EPSCs, infusion of 100 mM L-glutamate increased the NMDA component by 90% ± 26% in parallel with the AMPA component (increased by 66% ± 19%, n = 4) of spontaneous EPSCs with no significant change in their kinetics (Figure 6B). These results suggest that a quantal packet of transmitter does not saturate postsynaptic NMDA receptors.

Discussion

Presynaptic loading of L-glutamate at the calyx of Held synapse increased quantal amplitudes of AMPA- and NMDA-EPSCs by 66% and 90%, indicating that a single packet of transmitter can occupy AMPA and NMDA receptors at most 60% and 53%, respectively. While the affinity of NMDA receptors to glutamate at equilibrium state is much higher than that of AMPA receptors (Patneau and Mayer, 1990), the binding rates of these receptors are comparable (Clements et al., 1998). The amount of transmitter bound to postsynaptic AMPA receptors is less than 10% of transmitter liberated by a vesicle (Frerking and Wilson, 1996; Sahara and Takahashi, 2001), implying that glutamate released into the synaptic cleft is rapidly cleared away by diffusion (Holmes, 1995; Diamond and Jahr, 1997) or binding to glutamate transporters (Diamond and Jahr, 1997). Parallel potentiations of AMPA- and NMDA-EPSCs observed in this study after presynaptic L-glutamate loadings suggest that the fraction of receptors activated by transmitter is determined by binding steps in nonequilibrium states rather than the affinity of receptors in equilibrium states (Perkel and Nicoll, 1993; Holmes, 1995). Consistently, cyclothiazide, which increases the apparent affinity of AMPA receptors in equilibrium states (Patneau et al., 1993), has no appreciable effect on the mean amplitude of AMPA-mEPSCs at this synapse (Ishikawa and Takahashi, 2001).

Presynaptic L-glutamate loadings increased the amplitude of quantal AMPA-EPSCs and NMDA-EPSCs, but had no significant effect on their kinetics, suggesting that increased vesicular glutamate activated postsynaptic receptors not far from a release site. Electron microscopic studies show that this calyceal synapse comprises hundreds of discrete synaptic contacts, each consisting of an active zone and a postsynaptic density. At this synapse, the size of each synaptic contact (mean radius, 125 nm; Meinrenken et al., 2002) is comparable with that of the hippocampal glutamatergic synapse (Rusakov et al., 1998; Harris and Stevens, 1989), and individual active zones are well separated (by 0.4 μm; Meinrenken et al., 2002) as at the hippocampal synapse (by 0.46 μm; Rusakov et al., 1998). Therefore, if vesicular glutamate spills over to neighboring active zones, it would be detected as slowed synaptic current kinetics.

Our results indicate that the vesicular transmitter content depends on the presynaptic cytoplasmic concentration, the normal range of which is 1–10 mM at this

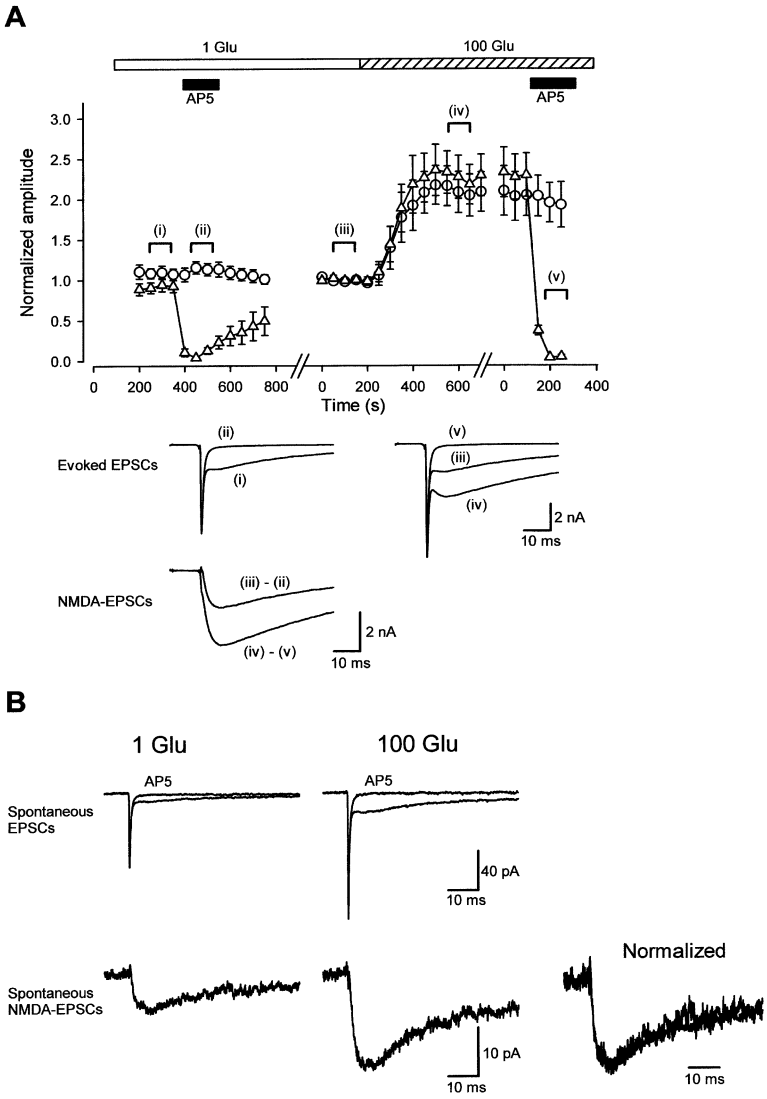

Figure 6. Parallel Potentiation of NMDA-EPSCs with AMPA-EPSCs after Presynaptic L-Glutamate Loadings

(A) Normalized amplitudes of NMDA-EPSCs (open triangles) and AMPA-EPSCs (open circles) evoked by presynaptic action potentials in 0 mM Mg^{2+} solution at −70 mV postsynaptic holding potential (from five experiments). Each data point derived from five consecutive records. D-AP5 (50 μM) selectively and reversibly blocked NMDA-EPSCs (i–iii). Presynaptic infusion of 100 mM L-glutamate potentiated NMDA- and AMPA-EPSCs in parallel (iv). Potentiated NMDA-EPSCs were blocked again by D-AP5 (v). Sample records of evoked EPSCs at each period are shown (superimposed). Sample traces at the lower panel are NMDA-EPSCs extracted as an AP5-sensitive component before (iii–ii) and after (vi–v) the infusion of 100 mM L-glutamate (superimposed). Normalized data in time plot are interrupted at the infusion of 100 mM L-glutamate, and also at the second D-AP5 application. Mean amplitude of evoked NMDA-EPSCs before L-glutamate infusion was 0.99 ± 0.3 nA (n = 5).

(B) Sample records of spontaneous EPSCs before (1 Glu) and 600–800 s after the 100 mM L-glutamate infusion (100 Glu). Averaged currents before and after AP5 applications are superimposed in the upper panel. Currents in the lower panel are spontaneous NMDA-EPSCs extracted as D-AP5-sensitive components from averaged currents before and after the 100 mM L-glutamate infusion. Rightmost traces in this panel are spontaneous NMDA-EPSCs before and after the L-glutamate infusion normalized in amplitude and superimposed. Mean amplitude of spontaneous NMDA-EPSCs before L-glutamate infusion was 4.03 ± 1.2 pA (n = 4).

synapse. Because glutamate is concentrated in synaptic vesicles at 60–150 mM (Burger et al., 1989) by a vesicular glutamate transporter having the affinity of 1–2 mM (Tabb and Ueda, 1991), 10- to 100-fold increase in cytoplasmic glutamate concentration will enhance vesicular filling by activating the transporter. Conversely, when the cytoplasmic glutamate concentration is lowered, glutamate may be leaked out of vesicles by the reverse transport (Burger et al., 1991). However, glutamate may also leak in or out of vesicles passively as suggested for synaptic vesicles isolated from Torpedo electric organs (Carpenter et al., 1980) and for incorporated false transmitters into synaptic vesicles from cytoplasm at cultured synapses (Dan et al., 1994). It remains to be determined which mechanism underlies the incorporation of loaded glutamate into synaptic vesicles. It might be further argued that nonvesicular leakage of glutamate from nerve terminals (Jabaudon et al., 1999) may induce potentiation of EPSCs by a postsynaptic mechanism. However, at this synapse, this is highly unlikely because bath-applied L-glutamate (0.01–1 mM) did not increase the amplitude of EPSCs, but instead decreased it (at above 0.1 mM) presumably via desensitization of postsynaptic glutamate receptors (our unpublished observations). Presynaptic cytoplasmic glutamate concentration is thought to be maintained by glutamine transported via astrocytes, which take up glutamate from the synaptic cleft using glutamate transporters and convert it to glutamine (Laake et al., 1995). If the efficacy of this transport system changes, it may be reflected in synaptic efficacy via changes in the quantal amplitude.

By injecting L-glutamate into nerve terminals, the present study has directly demonstrated that neither AMPA nor NMDA receptors are saturated by a packet of transmitter glutamate. Although this type of experiment is possible only at the giant nerve terminal, a similar approach can also be made at small synapses by using the overexpression of vesicle transporters, as at the cholinergic synapse (Song et al., 1997). This would determine whether our conclusion drawn at the calyceal synapse is applicable to small spiny synapses. While the nonsaturation of postsynaptic glutamate receptor has been suggested at various central synapses (Gulyas et al., 1993; Silver et al., 1996b; Liu et al., 1999; Umemiya

et al., 1999; Mainen et al., 1999; McAllister and Stevens, 2000), our results at the calyx of Held synapse provide direct evidence for this issue. Thus vesicular glutamate content may be an important determinant of synaptic efficacy at many central glutamatergic synapses.

The calyx of Held synapse is formed by a single presynaptic fiber making multiple synaptic contacts with a postsynaptic target cell (Lenn and Reese, 1966). This structure allows single presynaptic impulse to evoke large supra-threshold EPSPs to ensure phase-locked transmission for sound localization. At this synapse, synaptic efficacy affects the maximal frequency of transmission at which postsynaptic action potentials can be accurately evoked (Futai et al., 2001; Iwasaki and Takahashi, 2001). Our present results indicate that postsynaptic glutamate receptors are not saturated by individual quanta but can be saturated at a high release probability. This mechanism would provide a wide dynamic range for the efficacy of synaptic transmission.

Experimental Procedures

Recordings

Transverse slices (150 μm) of superior olivary complex were prepared from 14- to 15-day-old Wistar rats after decapitation under halothane anesthesia. Slices were superfused with artificial cerebrospinal fluid (aCSF) composed of 125 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 0.5 mM ascorbic acid, 3 mM myo-inositol, 2 mM Na-pyruvate, and 4 mM lactate (pH 7.3 with 95% O_2/5% CO_2, 310 mOsm). The aCSF routinely contained bicuculline methiodide (10 μM) and strychnine hydrochloride (0.5 μM) to block GABAergic and glycinergic synaptic currents, respectively. For recording NMDA-EPSCs, $MgCl_2$ in the aCSF was omitted and glycine (10 μM) was supplemented. Recordings were made at room temperature (22°C–26°C) unless otherwise noted. Principal cells in the MNTB and the presynaptic terminal, the calyx of Held, were visualized under upright microscope (Axioskop, Zeiss) using a 60× water immersion objective (Olympus). Whole-cell current-clamp recordings were made from calyceal nerve terminals simultaneously with voltage-clamp recordings from postsynaptic principal cells (Borst et al., 1995; Futai et al., 2001) using Axopatch 200B amplifiers. Patch pipettes for postsynaptic recordings were filled with 110 mM CsF, 30 mM CsCl, 10 mM HEPES, 5 mM EGTA, 1 mM $MgCl_2$, and 5 mM N-(2, 6-diethylphenylcarbamoylmethyl)-triethyl-ammonium chloride (QX-314, pH 7.3 adjusted with CsOH, 290–300 mOsm). Presynaptic pipette solutions contained 105 mM potassium gluconate, 30 mM KCl, 10 mM HEPES, 0.5 mM EGTA, 1 mM $MgCl_2$, 12 mM phosphocreatinine (Na salt), 3 mM ATP (Mg salt), 0.5 mM GTP (Na salt, pH adjusted to 7.3 with KOH, 290–300 mOsm). When L-glutamate was included in the pipette solution, osmolarity was balanced by mixing a solution containing 105 mM potassium gluconate with another solution containing 9 mM potassium gluconate and 100 mM potassium L-glutamate at various proportions. EPSCs were evoked by presynaptic action potentials elicited in nerve terminals by a depolarizing pulse of 1 ms duration at 0.1 Hz unless otherwise noted. Spontaneous mEPSCs were recorded in the presence of TTX (1 μM). In these experiments, mEPSCs were occasionally induced by steady depolarization (to −40 mV) of the presynaptic terminals (Sahara and Takahashi, 2001). The mean amplitude and amplitude profile of mEPSCs induced by presynaptic depolarization were essentially the same as those of spontaneous mEPSCs (Sahara and Takahashi, 2001). For recording presynaptic Ca^{2+} currents, the aCSF contained 1 μM TTX, 10 mM tetraethylammonium (TEA) chloride, and 0.5 mM 4-aminopyridine, and the presynaptic pipette solution contained 30 mM CsCl, 1 mM Cs glutamate, 104 mM Cs gluconate, 10 mM HEPES, 0.5 mM EGTA, 1 mM $MgCl_2$, 12 mM phosphocreatine (Na salt), 3 mM ATP (Mg salt), and 0.5 mM GTP (Na salt). For the internal perfusate with 100 mM L-glutamate, 99 mM Cs gluconate was replaced by Cs glutamate. Presynaptic Ca^{2+} currents were evoked by depolarizing pulses from −80 mV to +10 mV applied every 20 s. The postsynaptic and presynaptic pipettes had resistances of 2–3 MΩ and 6–8 MΩ, respectively. The series resistance of the postsynaptic recordings was typically 4–8 MΩ and was monitored continuously. The postsynaptic holding potential was −70 mV unless otherwise noted. The liquid junction potential between pipette and external solution was not corrected for.

Pipette Perfusion

Pipette solutions containing different glutamate concentrations were infused into calyces through a plastic tube installed in a presynaptic patch pipette as reported previously (Hori et al., 1999). Briefly, the tube was fabricated from an Eppendorf yellow tip heated and pulled for an outer tip diameter of 50–70 μm. After back-filling with the desired solution, the tube was inserted into a patch pipette with the tip of the former being 500–600 μm behind that of the latter. After obtaining control responses, the solution in the tube was ejected using positive pressure applied manually through a syringe.

Data Analysis

Synaptic currents were low-pass filtered at 5 kHz and stored on a DAT tape (sampling rate 48 kHz). Data were digitized at 20–50 kHz by using a CED1401 interface (Cambridge Electronic Design) or Digidata 1320 (Axon instruments) and analyzed offline using Axograph (Axon). The template method was used for capturing spontaneous and miniature EPSCs, and events were aligned at their peak for average. In the glutamate loading experiments, the mean amplitude of synaptic currents was measured 500–800 s after glutamate infusion and compared with that before infusion. For amplitude-time plots, each data point for evoked EPSCs is an average of ten consecutive records, and that for spontaneous EPSCs is an average of events during 100 s, unless otherwise noted. Baseline was determined from data during the period of 200 s before L-glutamate infusion. All values are given as mean ± SEM, and significance of difference was evaluated by Student's paired t test unless otherwise noted.

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