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Synaptic Refractory Period Provides a Measure of Probability of Release in the Hippocampus

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Summary

Despite extensive research, much controversy remains regarding the locus of expression of long-term potentiation (LTP) in area CA1 of the hippocampus, specifically, whether LTP is accompanied by an increase in the probability of release (p_r) of synaptic vesicles. We have developed a novel method for assaying p_r, which utilizes the synaptic refractory period—a brief 5–6 ms period following release during which the synapse is incapable of transmission (Stevens and Wang, 1995). We show that this assay is sensitive to a battery of manipulations that affect p_r but find no change following either NMDA receptor-dependent LTP or long-term depression (LTD).

Introduction

Long-term potentiation (LTP) is a use-dependent increase in synaptic efficacy that may play an important mechanistic role in learning and memory. In area CA1 of the hippocampus, the essential induction mechanisms underlying LTP have largely been determined and involve calcium entry through NMDA receptors on the postsynaptic cell (Bliss and Collingridge, 1993; Nicoll and Malenka, 1995). The site of expression for LTP, however, has remained controversial, and it is still unclear whether LTP is due to an increase in the postsynaptic responsiveness to a quantum of released glutamate (q), to an increase in the presynaptic probability of release (p_r), or to an increase in the number of active synapses (n; Kullmann and Siegelbaum, 1995; Nicoll and Malenka, 1995).

A number of approaches have been utilized specifically to determine whether or not LTP is accompanied by an increase in p_r. Paired-pulse facilitation (PPF), a phenomenon that is sensitive to changes in the presynaptic probability of release (Manabe et al., 1993; Dobrunz and Stevens, 1997), does not appear to change with LTP (Manabe et al., 1993; Asztely et al., 1996; but see Schultz et al., 1994). It could be argued, however, that LTP might alter the probability of release in a novel way that does not interact with PPF. A second method for determining a change in p_r has been to look at the relative change in the AMPA receptor- versus the NMDA receptor-mediated components of synaptic responses following LTP. While an increase in probability of release should affect both components equally, many reports show little or no change in the NMDA component following LTP (Muller and Lynch, 1988; Kauer et al., 1988; Asztely et al., 1992; Perkel and Nicoll, 1993; Kullmann, 1994; Selig et al., 1995; but see Clark and Collingridge, 1995; O'Conner et al., 1995), arguing against an increase in p_r alone accounting for LTP. However, it has been proposed that the spillover of glutamate from one synapse to another could, in part, be responsible for this observation (Kullmann et al., 1996).

A third method for examining changes in p_r during LTP utilizes the NMDA receptor open channel blocker, MK-801. MK-801 causes a use-dependent decrease in the NMDA receptor-mediated excitatory postsynaptic current (EPSC), the rate of this decrease being proportional to p_r (Hessler et al., 1993; Rosenmund et al., 1993). In theory, this assay is a fairly direct measure of p_r, but whether there is a change in the rate of decline of the NMDA receptor-mediated EPSC in the presence of MK-801 following LTP is controversial (Manabe and Nicoll, 1994; Kullmann et al., 1996). Furthermore, changes in NMDA receptor properties could cause a change in the MK-801 decay rate and, therefore, could be mistaken for a change in p_r. Finally, several groups have attempted to look directly at quantal parameters by recording from only one or a few release sites (Foster and McNaughton, 1991; Kullmann and Nicoll, 1992; Liao et al., 1992; Voronin, 1994; Stevens and Wang, 1994; Bolshakov and Siegelbaum, 1995; Isaac et al., 1996; Stricker et al., 1996). Conclusions from these experiments have varied, ranging from LTP being due solely to a change in p_r to LTP being due to changes in q and n.

Because of this continuing debate and the limitations inherent in each of the approaches described above, we have continued to work on developing new methods that allow estimates of p_r. In this paper, we describe a novel method of estimating p_r, which is based on the finding that individual synapses exhibit a short absolute refractory period following transmitter release, during which the synapse is incapable of transmission (Stevens and Wang, 1995). This method, based on comparing the sizes of EPSCs in response to paired-pulse stimulation at different intervals, is independent of PPF, does not rely on a measurement of the NMDA receptor-mediated EPSC, and also is independent of the number of synapses being sampled. To test the usefulness of this novel assay of p_r, we first performed a series of manipulations that change p_r in a predictable fashion. We then used this method to study changes in p_r during NMDA receptor-dependent LTP and its counterpart, LTD.

Results

We began by investigating the properties of synaptic transmission at short paired-pulse intervals while recording EPSCs from putative single release sites. Consistent with published results (Stevens and Wang, 1995), at longer intervals (>20 ms) the potency, defined as the

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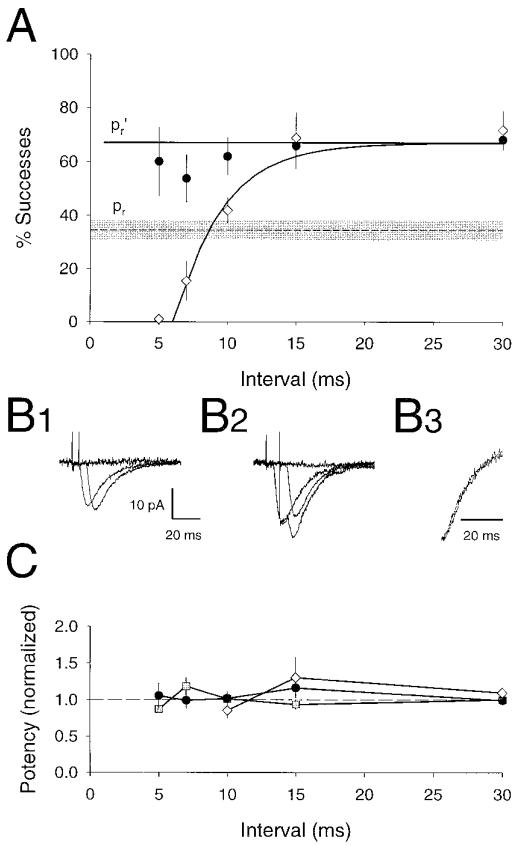

Figure 1. Minimal Stimulation of Single Sites Reveals a Synaptic Refractory Period

(A) Shown is the probability of release (p_r) of the second of a pair of EPSCs plotted against the paired-pulse interval, depending on whether the initial stimulus resulted in a success (open diamonds) or a failure (closed circles; n = 4; average of 41 trials per interval from each cell). The dashed line is the mean p_r (34% ± 4%) of the initial EPSCs (shaded area provides SEM). The horizontal line (p_r') is the facilitated release probability determined by averaging data at 15 and 30 ms intervals. The curved line is the average of the best fit from each individual experiment for data following an initial success.

(B) Traces from a single experiment.

(B_1) Average of all trials at a 5 ms interpulse interval that responded with an EPSC to the first or to the second stimulus or a failure to both.

(B_2) Average of all trials at a 10 ms interpulse interval that responded with an EPSC to the first, to the second, or to both stimuli or with a failure to both.

(B_3) Aligned decay phase of EPSCs in B_1 illustrates that no responses to both stimuli were hidden in the EPSCs.

(C) Potency (mean amplitude of successes only) normalized to the initial EPSC potency as a function of the paired-pulse interval for the second EPSCs following either a success (open diamonds) or a failure (closed circles). The shaded squares are the potency for the initial EPSCs. The data points for responses following a success for 5 and 7 ms were not included, due to the extremely small sample size.

amplitude of the responses when they occur, were equal for the first and second stimuli, indicating that we were recording from a single site. Additionally, there was no difference in p_r following a success or a failure on the first stimulus (Figure 1A)—in either case, the subsequent response was facilitated (p_r'), presumably due to residual calcium in the terminal (Zucker, 1989). At short (5 ms) paired-pulse intervals, we observed that when release of neurotransmitter occurred in response to the first stimulus, no response was elicited by the second stimulus; that is, the synapse exhibited an absolute refractory period lasting several milliseconds (Figures 1A and 1B). When a failure occurred in response to the first stimulus, the subsequent EPSC responded with a facilitated probability of release comparable to that seen at longer intervals.

This synaptic refractory period is not due to an inability to identify responses at short intervals. Even at intervals of 5 ms, EPSCs in response to the first pulse could be distinguished from EPSCs in response to the second stimulus by the clear onset of the EPSC (Figure $1B_1$). At longer intervals in which EPSCs occasionally occurred in response to both stimuli, the response to the second could be clearly identified as a deflection on the falling phase of the first EPSC (Figure $1B_2$). Finally, if these double responses were occurring at shorter intervals but were being misidentified as single responses to the first pulse, we would expect to see a difference in the decay of the EPSC, which was not observed (Figure $1B_3$).

The recovery curve following a response to the first pulse was best fit by an exponential curve with an offset (t_0) of 6.2 ± 0.5 ms and a time constant of 3.4 ± 0.7 ms. These values are consistent with previously reported data (Stevens and Wang, 1995). Figure 1C shows measurements of the potency normalized to the potency of the first response. There was no significant difference (repeated measures ANOVA) in the potency of the second response following a failure or following a success down to 10 ms. This indicates that, at least at intervals of 10 ms or longer, AMPA receptor desensitization is not causing a decrease in the EPSC amplitude.

These data are most consistent with a scenario where, following a synaptic release, there is a 5–6 ms refractory period during which the synapse cannot transmit. Assuming that this synaptic refractory period is a common feature of all excitatory synapses on CA1 cells, we predicted that if we were recording from a population of synapses, following an EPSC some proportion of the synapses would be in this refractory period and that this fraction should reflect the probability of release. For example, if $p_r = 1.0$, all of the synapses would release and therefore would be refractory at 5 ms. The amplitude of an evoked EPSC at that time would be zero (Figure $2A_1$, dashed line). As p_r decreases, fewer synapses would be refractory, leaving more synapses available to release at 5 ms. Figure 2 shows a simulation of the expected amplitude of an EPSC generated by 40 release sites (i.e., 40 synapses, assuming one release site per synapse) in response to the second of paired stimuli given at various short intervals. Figure $2A_1$ shows the simulated curves when p_r ranges from 0.3–0.5. When we normalize these curves to the EPSC amplitude at a 30 ms interstimulus interval (Figure $2A_2$), it becomes clear that the amplitude at 5 ms is equivalent to $1 - p_r$ (a derivation of this is provided in the Experimental Procedures). Simulations changing the number of synapses (i.e., release sites) over a similar range, shown in

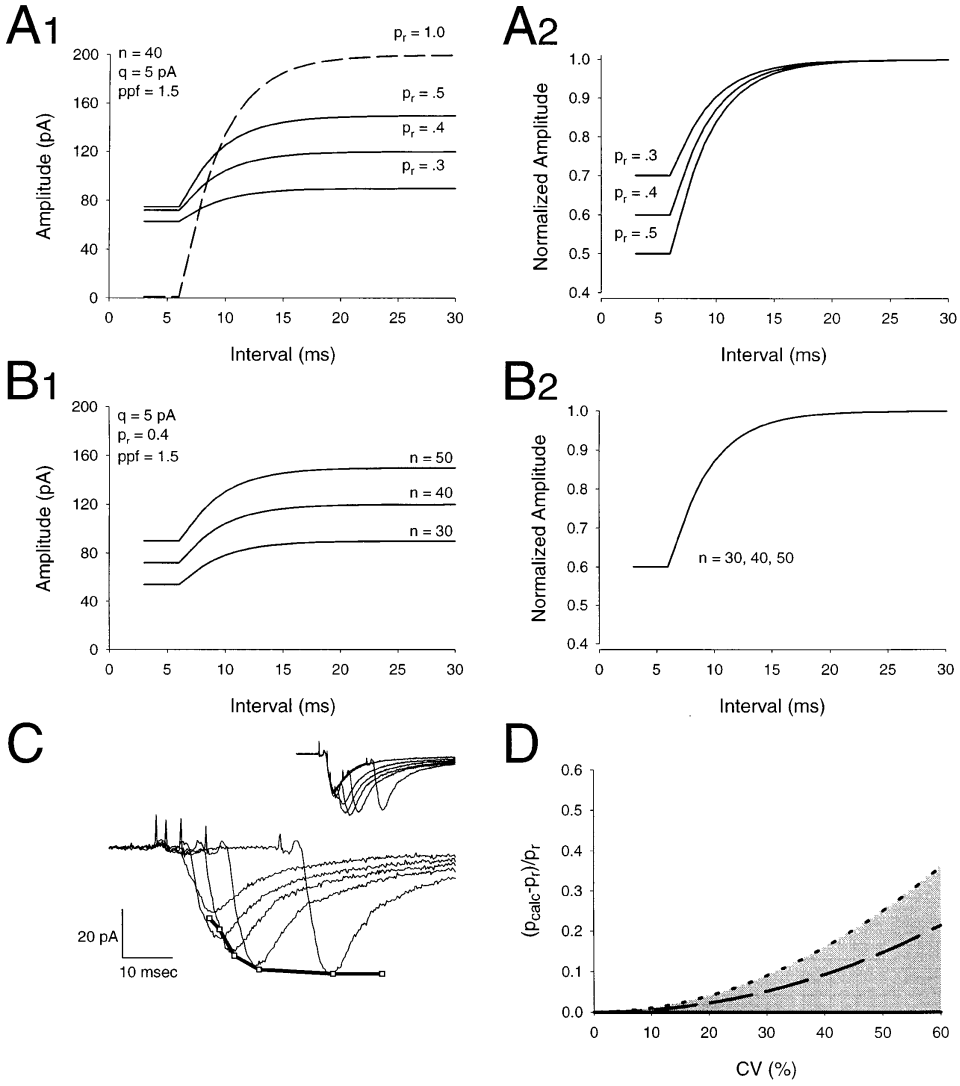

Figure 2. Computer Simulations Illustrate the Sensitivity of Paired-Pulse Intervals to Changes in p_r for Multiple Site Recordings

(A) Calculated amplitude (A_1) of a paired EPSC as a function of paired-pulse interval for various initial values of p_r with constant values of n (40), q (5 pA), and paired-pulse facilitation (1.5). The dashed line is the extreme example in which all of the synapses release on the first pulse. In (A_2), amplitude normalized to the amplitude at 30 ms reveals the relationship between p_r and the normalized amplitude of paired pulses at short intervals.

(B) Changes in n affect the amplitude of the second EPSC (B_1) but do not affect the normalized curve (B_2).

(C) Actual data is fit well by the calculated curve. Second EPSCs elicited at various intervals are well fit by a curve in which only q and p_r were allowed to vary. The inset shows the raw data prior to subtraction of the initial EPSCs. Data are the averages of 20 trials at each interval.

(D) Estimate of a possible error in the calculation of p_r (p_{calc}) due to the variance in p_r from site to site. The variance between synapses will result in a possible overestimation of p_r depending on the relationship between paired-pulse facilitation and p_r. This error is bound by the two extreme cases, in which PPF is constant across all synapses (dotted line) and PPF is inversely proportional to p_r (solid line at zero). The dashed line is the error, given a recent estimate for the PPF function (Dobrunz and Stevens, 1997).

Figure 2B_1, did not differ following normalization (Figure 2B_2). Similarly, changing the magnitude of PPF or the quantal size (i.e., q) had no effect on the normalized curve (data not shown).

As a first test of the validity of this approach when simultaneously assaying multiple synapses, we recorded standard "large" EPSCs in response to paired-pulse stimulation at variable intervals. As shown in Figure 2C, the EPSCs (after subtraction of the first response) could be fit quite well ($r^2 = 0.975$, $p < 0.01$) with a curve that used the time constants measured in Figure 1 and that was generated by allowing only p_r and q to vary.

The release probability is unlikely to be uniform at all synapses (Hessler et al., 1993; Isaacson and Hille, 1997; Rosenmund et. al., 1993; Murthy et al., 1997), a fact that may have important effects on the interpretation of quantal measurements (Faber and Korn, 1991). The effect that intersite variance in p_r has on our analysis is shown in Figure 2D. If the amount of PPF is inversely

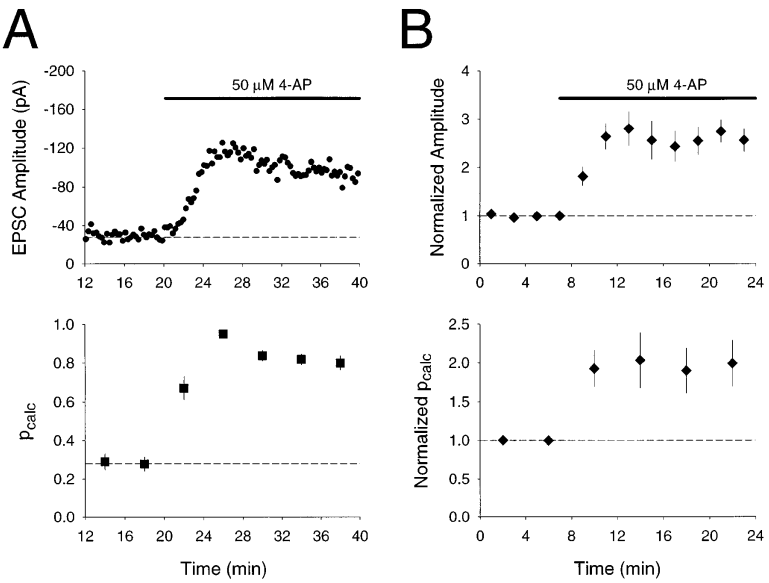

Figure 3. The Potassium Channel Blocker 4-AP Causes an Increase in the Calculated p_r

(A) Individual experiment showing the effects of application of 50 μM 4-AP on the amplitude of the initial EPSCs (upper graph) and on the calculated p_r (lower graph). The dashed lines give the average of all baseline data. The squares are averages of 15 individual series of trials.

(B) Average of five individual experiments showing changes in both amplitude and p_{calc} following 4-AP application. The mean initial p_{calc} value for these experiments was 0.48 ± 0.06.

proportional to p_r across all synapses, the variance in p_r will have no effect on our measure (Figure 2D, horizontal line on x-axis). On the other hand, if the facilitation function is less than inverse, we will tend to overestimate the true p_r. The maximal possible error (Figure 2D, dotted line) would arise in the unlikely condition that the magnitude of PPF is constant at all synapses despite their initial p_r. A recent estimate for the facilitation function (Dobrunz and Stevens, 1997) results in the dashed line. Even then, when the coefficient of variation of p_r is 50%, our error in calculating p_r is only 15%.

The refractory period for synaptic transmission at a single synapse has been attributed to a refractory period for vesicle exocytosis (Stevens and Wang, 1995). However, our data do not allow us to rule out contributions from other mechanisms, such as postsynaptic receptor desensitization. Importantly, for the purposes of this study, it is not critical to delineate the mechanism(s) responsible for the refractory period. That is, this method of measuring p_r based on the paired-pulse ratio of EPSCs is only dependent on the existence of a refractory period and is independent of the underlying mechanisms responsible for it.

To test directly the applicability of using the refractory period to assay p_r, we determined whether this measurement is sensitive to experimental manipulations of p_r. We assayed p_r by interleaving EPSCs evoked with paired pulses separated by 5 ms and 30 ms. Our estimate of p_r, p_{calc}, was then determined by:

$$p_{calc} = 1 - \frac{EPSC(5)}{EPSC(30)}$$

where EPSC(5) and EPSC(30) are the amplitudes of the paired pulse at 5 and 30 ms, respectively, after subtracting the EPSC evoked by the first pulse.

Initially, we examined the effects of 4-aminopyridine (4-AP), a drug that enhances transmitter release by blocking presynaptic potassium currents (Llinás et al., 1976; Heuser et al., 1979). At a concentration of 50 μM, 4-AP caused a marked enhancement in the EPSC amplitude (Figure 3A) and, similarly, a large increase in p_{calc}. This increase in p_{calc} was seen in five out of five cells (Figure 3B) and averaged 197.6% ± 28.6% of baseline. A similar increase in EPSC amplitude and in p_{calc} was observed following application of the A_1 adenosine receptor antagonist, 8-cyclopentyl-1,3-dimethylxanthine (CPT; 10 μM, applied in the presence of a basal level of 0.5 μM adenosine; Figure 4A; n = 4). Cadmium, a nonspecific Ca^{2+} channel blocker, caused a large decrease in the size of the EPSC and also caused a decrease in our calculation of p_r (Figure 4B; n = 4). In contrast, increasing the stimulus strength, which increases n but should not affect p_r, did not cause a change in p_{calc} (Figure 4C; n = 6).

We were concerned that perhaps our calculation was simply assaying for changes in PPF, as has been done previously (Manabe et al., 1993). To test this, we examined the effects of applying the membrane-permeant calcium buffer EGTA-AM (200 μM; Figure 4D). This caused a decrease in the amplitude of the EPSC and, unlike other pharmacological manipulations that decrease p_r, also decreased (rather than increased) PPF (1.78 ± 0.07 before and 1.32 ± 0.09 after application; n = 4). This decrease in both PPF and release probability is consistent with the ability of EGTA-AM to buffer calcium in the presynaptic terminal (Borst and Sakmann, 1996; Castillo et al., 1996). Importantly, p_{calc} also decreased during EGTA-AM application, demonstrating that this measure is not simply reflective of changes in PPF.

Having established that our assay is sensitive to changes in p_r, we examined what effects NMDA receptor-dependent LTP and LTD have on p_{calc}. Figure 5A shows a typical example of LTP induced by a pairing protocol. The EPSC amplitude increased by 75%, yet there was no change in p_{calc} (0.34 ± 0.04, baseline; 0.36 ± 0.02, LTP). A summary of six cells is shown in Figure 5B. Following LTP, the amplitude increased to 216% ± 30% of baseline but p_{calc} remained constant (102% ± 7% of baseline), as did PPF (99% ± 6%). In two of these

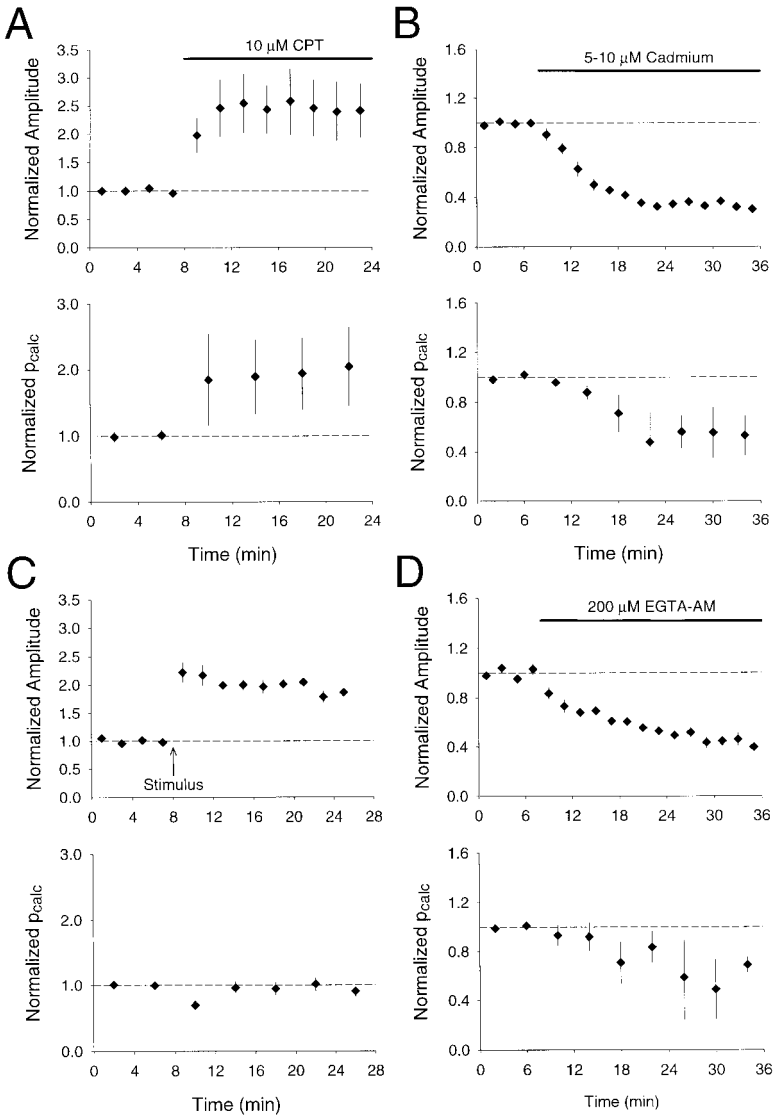

Figure 4. p_{calc} Changes Following Manipulations that Change p_r but Is Insensitive to Changes in n

(A) Application of 10 μM CPT in the presence of a basal level of 0.5 μM adenosine increases both the EPSC amplitude and p_{calc} ($n = 4$). Due to the adenosine, the initial p_{calc} value was lower (0.34 ± 0.11).

(B) Application of 5–10 mM cadmium decreases both the EPSC amplitude and p_{calc} ($n = 4$). The mean initial p_{calc} value was 0.50 ± 0.08.

(C) Changing n by increasing the stimulus intensity has no effect on p_{calc} ($n = 6$). The mean initial p_{calc} value was 0.52 ± 0.07.

(D) Application of 200 μM EGTA-AM, which decreases PPF, causes a decrease in p_{calc}, indicating that p_{calc} is not simply reflecting changes in PPF ($n = 4$). The mean initial p_{calc} value was 0.47 ± 0.12.

cells, a tetanus (100 Hz for 1 s, given twice) was used to induce LTP with similar results to those obtained when LTP was induced using a pairing protocol.

LTD also had no effect on p_{calc} ($n = 6$; Figure 5C). Prolonged low frequency stimulation paired with depolarization to −40 mV, which elicits an NMDA receptor-dependent LTD, caused a depression in the EPSC amplitude to 72% ± 4% of baseline; p_{calc}, on the other hand, remained at 100% ± 5% of baseline.

A summary of all of our experiments is shown in Figure 6. All of the experimental manipulations of p_r caused a significant change in p_{calc}. A regression analysis of the individual experiments gave a highly significant correlation ($r^2 = 0.82$, $p \ll 0.01$, degrees of freedom = 15). LTP and LTD, on the other hand, had no effect on this assay of p_r. This suggests that these forms of LTP and LTD are not due to a change in the probability of release, but are more likely due to changes in q and/or in n.

It is theoretically possible that if there is a very high variability in p_r across synapses, LTP could be due to a selective increase in p_r only at synapses with low p_r. This would cause a reduction in the variability across synapses and a concomitant reduction in the error due to that variability. However, to account for our data, it would be necessary to have a coefficient of variation (CV) of p_r of 100% or greater in the baseline and a CV of near 0% following LTP. This would also require that the magnitude of PPF be nearly constant across all synapses, a suggestion that is incompatible with recently published experiments examining PPF at putative single release sites (Dobrunz and Stevens, 1997). Nevertheless, because of the ongoing debate concerning the role of increases in p_r during LTP, we performed additional experiments that addressed this issue.

We reasoned that if LTP is due primarily to an increase in p_r, then synapses with high p_r should exhibit LTP that is smaller than synapses with lower p_r. This prediction has been tested previously by a number of investigators, and the experiments have yielded confusing results. Several groups found that raising extracellular Ca^{2+} had no effect on the magnitude of LTP (Muller and Lynch, 1989; Asztely et al., 1994; Isaac et al., 1996). On the other

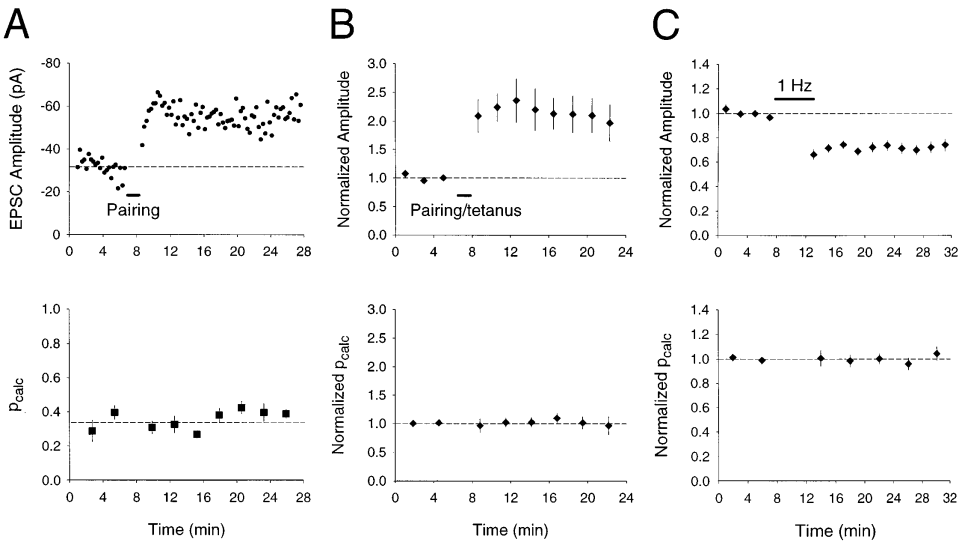

Figure 5. Long-Term Potentiation and Long-Term Depression Have No Effect on p_{calc}

(A) Individual experiment showing changes in the amplitude of the initial EPSCs and the calculation of p_r following pairing-induced LTP (100 stimuli at 1 Hz with the cell depolarized to 0 mv). The dashed lines give the average of all baseline data. Squares are averages of 10 individual series of trials.

(B) Average of six individual experiments shows changes in amplitude but not in p_{calc} following LTP induction. In two of these experiments, LTP was induced by a 100 Hz tetanus with the cell held at 0 mv.

(C) Average of six individual experiments shows changes in amplitude but not p_{calc} following induction of NMDA receptor-dependent LTD. LTD was induced by pairing prolonged low frequency stimulation (5 min at 1 Hz) with the cell held at -40 mv. The mean initial p_{calc} value for the plasticity experiments was 0.50 ± 0.05.

hand, Schulz (1997) reported a significant decrease in the magnitude of LTP when elicited in high extracellular Ca^{2+}. It also has been argued that LTP cannot be elicited in neonatal slices under normal conditions, because p_r is close to 1, but that it can be elicited if p_r is lowered experimentally (Bolshakov and Siegelbaum, 1995). To readdress this issue, we raised p_r by first applying 4-AP to the slice. We then performed a pairing protocol to elicit LTP using perforated-patch recording techniques.

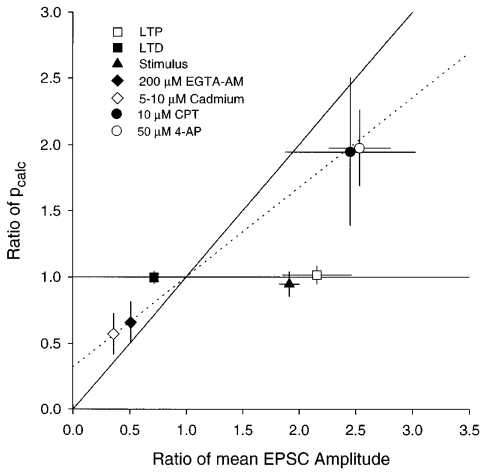

Figure 6. Summary of all Manipulations Plotted as the Ratio of p_{calc} (Following Manipulation to Baseline) to the Ratio of EPSC Amplitude

The dashed line is a linear regression through all individual control experiments in which p_r was manipulated.

It was necessary to use perforated-patch recording because the ability to induce LTP often washes out during standard whole-cell recording (Malinow and Tsien, 1990), and a true test of the influence of raising p_r on LTP required that we compare the saturated level of LTP reached under high p_r versus control conditions. Figure 7A shows a comparison of the LTP elicited in cells recorded in our standard experimental conditions and the LTP elicited while perfusing the slice with 4-AP (50 μM), which increases the mean p_r by 2-fold. It can be seen that the LTP was essentially identical in these two conditions (377% ± 47% versus 390% ± 29% of baseline in control versus 4-AP conditions).

We also performed the converse experiment. That is, if LTP is primarily due to an increase in p_r, experimental manipulations that increase p_r should have less of an effect at synapses that have undergone LTP than at control synapses. Indeed, it has been demonstrated that synapses with low p_r are more sensitive to manipulations that increase p_r, such as 4-AP, than high p_r synapses (Hessler et al., 1993). To compare directly the effects of 4-AP on potentiated versus control synapses in the same preparation, we recorded field excitatory postsynaptic potentials (EPSPs) in response to stimulation of two independent pathways. In one pathway, we induced LTP repeatedly (Figure 7B, arrows) until the LTP was saturated (i.e., a subsequent tetanus caused no further increase in the EPSP). This protocol induced robust LTP (257% ± 23%; n = 6). After turning the stimulus strength down, so that the potentiated and control field EPSPs were of similar size, we added 4-AP (50 μM) to the bath. Similar to its effects on whole-cell EPSCs, this

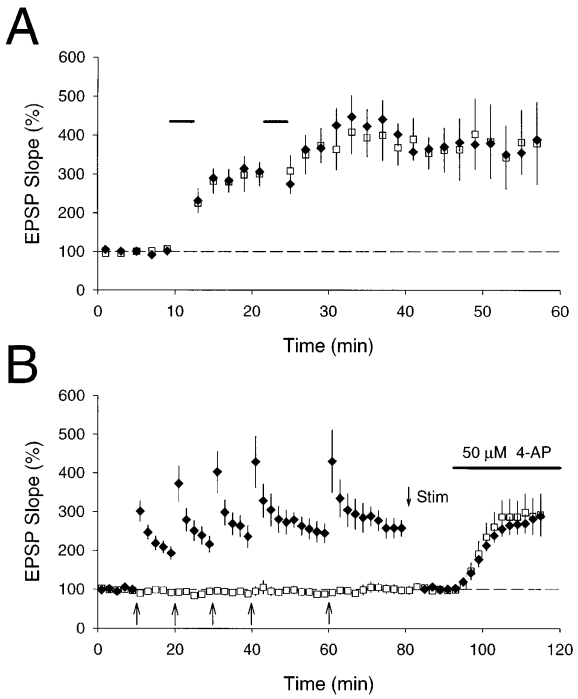

Figure 7. Long-Term Potentiation Does Not Interact with a Manipulation Increasing p_r

(A) Using perforated-patch recordings, a similar magnitude of LTP occurs under control conditions (open squares, n = 4) and in the presence of 50 μM 4-AP (closed diamonds; n = 4). LTP was elicited with two episodes of pairing (100 stimuli at 1 Hz; cell depolarized to 10 mV).

(B) A summary of six field experiments showing that application of 50 μM 4-AP has a similar effect on a test pathway in which LTP has been saturated and an independent control pathway. LTP was saturated by repeated 100 Hz tetani (each arrow is two 100 Hz tetani for 1 s separated by 15 s).

concentration of 4-AP caused a more than 2-fold increase in the field EPSPs. More importantly, the effect of 4-AP on the two paths was indistinguishable (285% ± 26% versus 293% ± 47% for LTP and control paths, respectively; not significant, paired t test). This demonstrated lack of an interaction between LTP and p_r in this and the preceding experiment provides further evidence against a significant role for a change in p_r underlying LTP.

Discussion

We have confirmed (Stevens and Wang, 1995) that following transmitter release at putative single release sites, there is a brief refractory period during which the synapse cannot transmit. Utilizing this observation, we developed a novel measure that assays the probability of release from a population of synapses by comparing the change in synaptic strength elicited by paired stimuli given at short and longer interstimulus intervals. This method of calculating p_r was sensitive to an array of manipulations that are known to modify p_r but did not change following the generation of NMDA receptor-dependent LTP or LTD.

The existence of a synaptic refractory period following release has previously been proposed (Betz, 1970; Korn et al., 1984; Triller and Korn, 1985) and is a necessary correlate of the one-vesicle hypothesis, which states that following an action potential no more than one vesicle can be released from an individual release site (Korn et al., 1982, 1984). Indeed, evidence has been presented (Stevens and Wang, 1995) consistent with a release-dependent process lasting up to 10 ms, during which subsequent exocytosis at that release site cannot occur. While our data are consistent with a presynaptic locus for this refractory period, we cannot rule out that the refractoriness may be due to a postsynaptic mechanism resulting in EPSCs so small that they are classified as failures. For instance, the postsynaptic receptors may still be bound by the glutamate released on the first pulse. These receptors would have to be in a desensitized state, since the synaptic conductance should be complete within a few milliseconds (Jonas and Sprusston, 1994). However, the reported time constants for entry into and recovery from desensitization in hippocampal pyramidal neurons (Colquhoun et al., 1992) are too slow to account for either the decay of a single EPSC or our measured refractory period. Whatever the mechanism(s) responsible for the refractory period, an attractive feature of our method of calculating p_r is that the underlying mechanism is immaterial; only the observation that a synapse does not transmit twice within 5 ms pertains.

Although we have demonstrated that our assay is sensitive to changes in p_r, the change in p_{calc} with pharmacological manipulations did not correlate perfectly with the change in EPSC amplitude—that is, the slope of the regression line in Figure 6 is <1. There are a number of possible reasons for this modest discrepancy. First, as has been pointed out, variance in p_r across synapses may cause an overestimate in our measure of p_r if PPF is less than inversely correlated with p_r. Furthermore, it is likely that manipulations that increase p_r preferentially act on lower p_r synapses and thus decrease the CV of p_r. This would result in an underestimate of the true change in p_r. How much variance there is between synapses is unclear. Recent studies utilizing FM1-43 in cultured hippocampal cells provide varying estimates ranging from 33% (see Figure 4 in Isaacson and Hille, 1997) to >50% (Murthy et al., 1997). Second, our method assumes that the probability of release following a failure at 5 ms is the same as the probability of release at 30 ms. This may not be the case at all synapses, for a number of reasons. For instance, it is possible that facilitation is not a step function but develops over time, or there may be additional refractory mechanisms involved that are not dependent on the release of a vesicle (e.g., calcium channel inactivation or an extended period of action potential refractoriness in some axons). Third, it is possible that a change in p_r may not account for the entire change in EPSC amplitude, although it seems unlikely that this would be true for all of our pharmacological manipulations. Finally, our model assumes independence between release sites, which if not true may also cause an underestimation of the true changes in p_r.

Despite these potential sources of error, all of our control pharmacological manipulations showed highly

significant changes in p_{calc}, whereas LTP and LTD showed no change at all. Furthermore, there was no difference between the initial p_{calc} of the control experiments (0.48 ± 0.05; excluding the CPT experiments, which were performed in a basal level of adenosine and therefore had a lower p_{calc}) and the LTP/LTD experiments (0.50 ± 0.05), indicating that the degree of error was similar in both groups. Indeed, if the error we observed with known changes in p_r is taken into account, we end up with a corrected p_{calc} of 0.34 ± 0.03, a value that is quite similar to the estimates of p_r in the literature from paired recordings or with minimal stimulation (Malinow, 1991; Allen and Stevens; 1994; Stevens and Wang; 1995; Raastad and Lipowski, 1996; Isaac et al., 1996; Dobrunz and Stevens, 1997).

While the most straightforward explanation of these results is that LTP is not accompanied by an increase in p_r, an alternative explanation can be put forward to account for our results. For instance, a reduction in the error associated with p_{calc} following LTP might counteract an actual change in p_r. However, this scenario seems improbable. To counteract a change in p_r sufficient to account for LTP, the initial error would have to start at >100% and, following LTP, be reduced to virtually 0%.

Although we were confident that our assay accurately measures changes in p_r when they occur, we performed additional experiments that directly tested two straightforward predictions of the hypothesis that LTP is due primarily to an increase in p_r. First, we asked whether the magnitude of LTP is less at synapses that have a high p_r. In agreement with previous work from our laboratory (Isaac et al., 1996), we found that the magnitude of LTP was unaffected by significantly increasing p_r. Second, we asked whether application of 4-AP, a manipulation that increases p_r and has been shown to have less of an effect at high p_r synapses (Hessler et al., 1993), has less of an effect at synapses expressing LTP when compared with control synapses in the same preparation. Consistent with a previous report that examined the effects of increasing extracellular calcium on potentiated versus control synapses (Muller and Lynch, 1989), there was no difference in the effects of 4-AP on the two sets of synapses. These results provide additional evidence that LTP at synapses on CA1 pyramidal cells is not due to significant increases in the probability of transmitter release.

While the present results argue against an important role for changes in p_r contributing to the expression of LTP (and LTD), this set of experiments does not rule out the involvement of other presynaptic mechanisms. For example, LTP could involve the activation of presynaptically silent synapses (Malenka and Nicoll, 1997), a mechanism that would cause an increase in n. Similarly, an increase in quantal size due to modification of postsynaptic receptor number and/or function is not readily distinguishable from an increase in the amount of transmitter in a vesicle at a synapse whose receptors were not saturated by exocytosis of the contents of a single vesicle. Currently, we favor a model in which postsynaptic glutamate receptor function and number is modulated during LTP, perhaps accompanied by structural changes that ultimately could affect both sides of the synapse.

Experimental Procedures

Hippocampal slices (400 μm) were prepared from 2- to 4-week-old Sprague-Dawley rats, allowed to recover for a minimum of 1 hr, transferred to poly-D-lysine coated coverslips, and visualized under a Zeiss Axioskop using a 40× objective. The slices were perfused at room temperature with a standard external solution containing 119 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgSO_4$, 10.0 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 11 mM glucose, and 0.1 mM picrotoxin. During the experiments illustrated in Figures 2–7, 1 μM CNQX was included to reduce EPSC variance. The CA3 region was removed to prevent epileptiform activity. Field, whole-cell, and perforated-patch recording techniques were performed as previously described (Selig et al., 1995; Isaac et al., 1996). Whole-cell recording pipettes (2–4 MΩ) were filled with a solution containing 107.5 mM Cs gluconate, 20 mM HEPES, 0.2 mM EGTA, 8 mM NaCl, 10 mM TEA Cl, 4 mM Mg ATP, and 0.3 mM GTP (pH 7.2 with CsOH, osmolarity adjusted to 270–280). Perforated patch recordings were made using amphotericin-B (Rae et al., 1991). The perforated-patch solution contained 117.5 mM Cs gluconate, 20 mM HEPES, 0.2 mM EGTA, and 0.48 mg/ml amphotericin-B (pH 7.2). Cells were held at −65 to −75 mV during the recordings. Series resistance was monitored online throughout the experiment. Stimulation of Schaffer collateral/commissural afferents (0.25 Hz, whole cell; 0.033 Hz, field; perforated patch) was performed using either a stainless steel or platinum–iridium bipolar electrode.

Minimal stimulation recordings were performed by reducing stimulus strength until most stimuli resulted in synaptic failures. Single sites were determined on the basis of having a uniform onset latency and waveform and by comparing the potency of single responses to the potency of paired responses at 30 ms (Stevens and Wang, 1995). Successes and failures to both initial and paired pulses were assayed visually. A scaled average EPSC to single stimuli was subtracted from the EPSCs elicited by paired stimuli at short (5–10 ms) intervals to determine whether any detectable EPSC occurred in response to the second stimulus.

Probability of release was estimated by interleaving paired pulses at 5 ms and 30 ms with a single pulse. The ratio of the amplitude at 5 ms to the amplitude at 30 ms (after subtracting the initial response) was subtracted from 1 to give p_{calc}. This calculation was performed on each series of stimuli and averaged into 2.5–4 minute bins.

The minimal stimulation data in Figure 1A was fit with the following functions: S(t), the time-dependent probability of release following a success, and F(t), the time-dependent probability of release following a failure, where t is the interpulse interval. For the time range of 5–30 ms, $F(t) = p_r'$, the maximal facilitated probability of release, and:

$$S(t) = (1 - e^{\frac{t - t_0}{\tau_s}}) \cdot p_r'$$

where t_0 is the absolute refractory period, and τ_s is the time constant of recovery.

The curve to fit multiple synapses is a simple summation. A synapse that initially fails will add q·F(t). A synapse that releases will add q·S(t). Since the number of synapses that release on a given stimulus is equal to the product of n and p and the number that fail is the product of n and (1 − p), the EPSC amplitude at an interval t is:

$$EPSC(t) = n \cdot (1 - p_r) \cdot q \cdot F(t) + n \cdot p_r \cdot q \cdot S(t)$$

At very short intervals, all of the synapses that have released are refractory and S(t) = 0, so:

$$EPSC(5) = n \cdot (1 - p_r) \cdot q \cdot p_r'$$

At longer intervals:

$$EPSC(30) = n \cdot p_r \cdot q \cdot S(30) + n \cdot (1 - p_r) \cdot q \cdot p_r'$$

However, at this interval, the synapses that have released are no longer refractory and $S(30) = p_r'$. Therefore:

$$EPSC(30) = n \cdot q \cdot p_r'$$

Taking the ratio of EPSC(5) to EPSC(30) gives:

$$\frac{\text{EPSC(5)}}{\text{EPSC(30)}} = \frac{(1-p_r)\cdot n\cdot q\cdot p_r'}{n\cdot q\cdot p_r'} = (1-p_r)$$

Finally, solving for p_r leaves:

$$p_r = 1 - \frac{\text{EPSC(5)}}{\text{EPSC(30)}}$$

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