Neuron, Vol. 22, 139–146, January, 1999, Copyright ©1999 by Cell Press

Augmentation Is a Potentiation of the Exocytotic Process

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Summary

Short-term synaptic enhancement is caused by an increase in the probability with which synaptic terminals release transmitter in response to presynaptic action potentials. Since exocytosed vesicles are drawn from a readily releasable pool of packaged transmitter, enhancement must result either from an increase in the size of the pool or an elevation in the fraction of releasable vesicles that undergoes exocytosis with each action potential. We show here that at least one major component of enhancement, augmentation, is not caused by an increase in the size of the readily releasable pool but is instead associated with an increase in the efficiency with which action potentials induce the exocytosis of readily releasable vesicles.

Introduction

Several decades ago, biochemists and electrophysiologists identified a readily releasable supply of neurotransmitter in synaptic terminals (Liley and North, 1953; Birks and MacIntosh, 1961; Elmqvist and Quastel, 1965). These pioneering workers noticed that the amplitudes of successive synaptic responses to trains of action potentials were not constant. Rather, synaptic strength typically would increase briefly during the first part of a high-frequency nerve stimulation and then would eventually decrease to a low level (Birks and MacIntosh, 1961; Brooks and Thies, 1962; Hubbard, 1963; Elmqvist and Quastel, 1965). The synapses were found to recover their original strength after a sufficient amount of rest, and thus these temporary changes in synaptic efficacy are now collectively referred to as "short-term plasticity" (reviewed by Zucker, 1989). Most short-term plastic changes result from a modulation of the probability with which individual synaptic terminals release quantal packets of transmitter in response to an action potential and are therefore presynaptic in nature (Del Castillo and Katz, 1954; Birks and MacIntosh, 1961; Magleby and Zengel, 1976).

The original investigators noticed that after the induction of a significant amount of depression, the synaptic strength becomes proportional to the length of time between the individual stimuli of sufficiently rapid stimulations; that is, the overall rate of release is not changed by increasing the frequency of the stimulation (Perry, 1953; Birks and MacIntosh, 1961; Elmqvist and Quastel, 1965; Abbott et al., 1997). During the early part of a presynaptic tetanus, however, they found the rate of transmitter release to be highly dependent on stimulation frequency (Birks and MacIntosh, 1961; Brooks and Thies, 1962; Elmqvist and Quastel, 1965). Synapses stimulated at the higher frequencies initially release transmitter at a higher rate but then also depress more quickly. Measurements of this relationship led the early analysts to postulate the existence of a presynaptic readily releasable pool of transmitter that would supply the quantal packets of transmitter for use in physiological signaling (Liley and North, 1953; Birks and MacIntosh, 1961; Elmqvist and Quastel, 1965). The readily releasable pool at hippocampal synapses has been defined more recently, and a number of its properties have been described (Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996; Dobrunz and Stevens, 1997; Murthy and Stevens, 1998; Stevens and Sullivan, 1998; Stevens and Wesseling, 1998).

Depletion of the readily releasable pool results in depression, but what causes the transient strengthening typically seen at the beginning of a high-frequency stimulation? The phenomenon is known to be driven by the residual calcium that accumulates in the presynaptic terminal during action potential activity (Kamiya and Zucker, 1994; Delaney and Tank, 1994). But the relationship between this short-term enhancement and the readily releasable pool has never been established. A prominent component of enhancement is "augmentation" (Magleby and Zengel, 1976; Zengel et al., 1980; reviewed by Fisher et al., 1997). Several investigators have speculated that augmentation might reflect a transient increase in the size of the readily releasable pool (Llinás et al., 1985; Lin and Llinás, 1993; Delaney and Tank, 1994). But, it could just as well result from a potentiation of the release machinery itself that then causes an increase in the average fraction of available quanta released by an action potential.

By inducing augmentation and then measuring the size of the readily releasable pool, we show here that augmentation is not associated with an increase in the size of the readily releasable pool. It is instead caused by an increase in the average fraction of the pool that can be released by a single action potential.

Results

The strategy adopted here depends upon two special features of our experimental preparation that allowed us concurrently (1) to monitor the size of the readily releasable pool from a population of synapses with a procedure that does not itself induce augmentation, and (2) to independently induce augmentation (via bursts of action potentials) in the same synapses. Although exocytosis of synaptic vesicles normally occurs in response to calcium influx into the presynaptic terminal, the contents of the pool can also be released in a calcium-independent manner when the terminals are challenged with a suitable hypertonic solution (Rosenmund and Stevens, 1996; Stevens and Wesseling, 1998). Such

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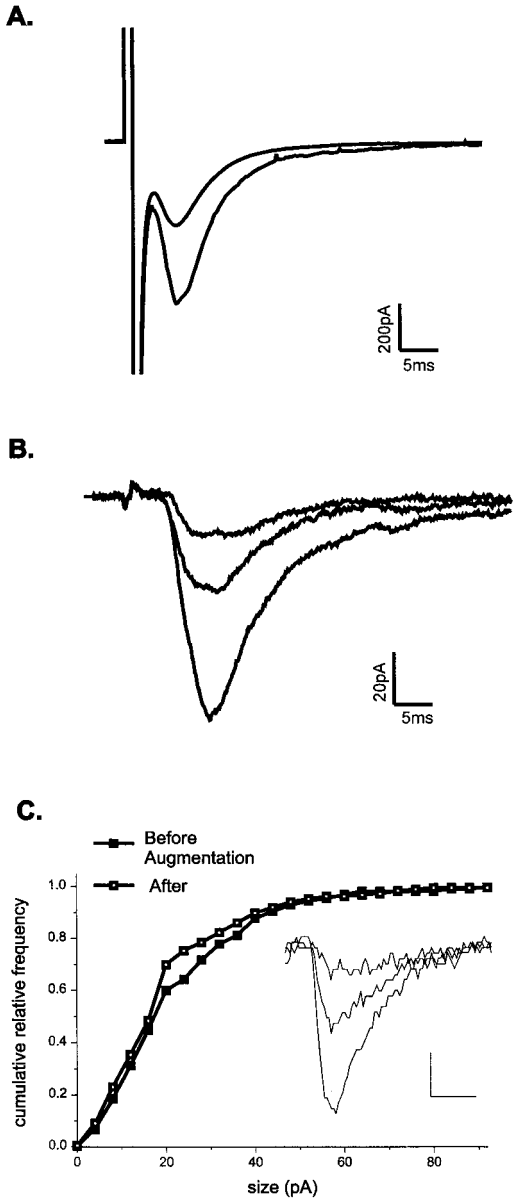

Figure 1. Identification of Augmentation in the Synapses of Isolated, Cultured Neurons

(A) Autapses. Synaptic enhancement was induced in solitary isolated cells by firing high-frequency trains of action potentials (9 Hz for 10 s) under low calcium (0.25 mM) conditions. Starting 1 s after the rapid stimulation, 30 additional action potentials were induced at low frequency (1 every 2 s). Displayed is the average response to the second low-frequency stimulus (3 s after high-frequency stimulation, large deflection) and the average response to the last 15 low-frequency stimuli (30–60 s after induction of augmentation; average from two cells, six trials).

(B) Cell pairs. Similar experiment as described for (A), except done on concurrently patch-clamped cell pairs. Action potentials were induced in one cell and postsynaptic responses recorded in another. Displayed is the average response to the low-frequency stimuli, 1 s, 7 s, and 27 s after high-frequency stimulation (average of seven trials).

(C) Spontaneous events are unchanged in size. The sizes of the miniature excitatory postsynaptic currents (mEPSCs) were measured in the autapses shown in (A) and were found to have the same size during the 5 s before high-frequency stimulation as they did during the first 2 s afterward. Plotted are the cumulative amplitude histograms of all the mEPSCs versus size before and after the same high-frequency stimulation that left the synaptic current enhanced for several seconds The inset depicts three overlaid examples of mEPSCs (scale bar, 20 pA by 4 ms).

an osmotic shock does not affect the postsynaptic quantal response size (Bekkers et al., 1990; Stevens and Tsujimoto, 1995; Stevens and Wesseling, 1998), and so the contents of the pool can be measured up to a proportionality constant by simply recording the synaptic currents evoked by hypertonic challenge. We were able to compare the size of the readily releasable pool of synapses when they were and were not augmented by applying this treatment to solitary, isolated hippocampal neurons grown in tissue culture on small "islands" (Segal and Furshpan, 1990; Bekkers and Stevens, 1991). Because these cells receive synaptic input only from themselves (autapses), action potentials evoked in the neuron cell body activate all of the synapses that contribute to the response to hyperosmotic challenge. By combining electrical and hypertonic stimulation of these neurons, we were thus able to monitor the size of the readily releasable pool in a synaptic population that was known to be augmented.

A key point is that we conducted the experiments for this study at low calcium concentrations in order to isolate augmentation from short-term depression (Magleby and Zengel, 1976). Under normal conditions, a hippocampal synapse stimulated at high frequency first transiently strengthens (short-term enhancement) and then eventually weakens (short-term depression). These two phenomena are caused by distinct underlying processes since enhancement can be isolated from depression under conditions that reduce the initial probability of release (Katz, 1969). Perhaps the best-characterized way to decrease the probability of release is to lower the calcium concentration in the saline outside of the synaptic terminal (Dodge and Rahamimoff, 1967). By reducing the amount of calcium that ordinarily flows into the terminal and triggers exocytosis (Augustine and Charlton, 1986), this treatment effectively lowers the fraction of vesicles within the readily releasable pool that undergo fusion following each action potential while leaving the number of vesicles in the pool essentially unchanged (Rosenmund and Stevens, 1996). During high-frequency stimulation with a sufficiently low calcium concentration, the synaptic strength increases, but little or no depression occurs as it does when the synapse is bathed in a physiological saline (Katz, 1969; Magleby, 1973a, 1973b; Magleby and Zengel, 1976; Zengel et al., 1980; reviewed by Zucker, 1989).

Induction of Augmentation in Cultured Neurons

Figure 1 shows that robust augmentation can be induced in synapses formed by cultured hippocampal neurons. Using a low extracellular calcium concentration (250 μM), we patch clamped solitary neurons on isolated islands and evoked action potentials at 9 Hz for 10 s. Since these cells synapse upon themselves, the unclamped, regenerative currents associated with the action potentials tend to obscure the postsynaptic responses. The autaptic responses to the first few stimuli were thus partially buried within this action potential

artifact, but after 90 stimuli, the synaptic response had increased enough in size to be a prominent feature of the records. Starting 1 s after the high-frequency stimulation, action potentials were evoked 30 more times, 1 every 2 s. During the low-frequency stimulation, the synaptic part of the evoked response remained large for several seconds and then eventually declined to baseline over the next 30 s (Figure 1). The large, slowly decaying component of short-term enhancement is characteristic of augmentation (Magleby and Zengel, 1976; Zengel et al., 1980). The high-frequency stimulation did not change the size of the spontaneous miniature excitatory postsynaptic currents (mEPSCs): mEPSCs had an average peak amplitude of 26 pA (with a coefficient of variation of 0.66) before and 24 pA (CV = 0.68) during the first 2 s after the high-frequency stimulation (Figure 1C). The constancy of mEPSC size demonstrates that this enhancement is, as expected, a presynaptic event. We thus conclude that our electrical stimulation produces typical augmentation in cultured autaptic connections.

We next sought to resolve the time course over which the augmented synaptic responses return to baseline amplitudes. In this case, the solitary neurons—at the same time both presynaptic and postsynaptic—have a disadvantage compared to more conventional synaptic connections between two different cells. With low extracellular calcium concentrations, autaptic currents are typically so small that their size cannot be accurately measured because the presynaptic regenerative currents that are associated with the action potential dominate the recordings (see Figure 1A). Therefore, we chose to measure the time course of relaxation of augmentation in synapses between pairs of neurons grown on similarly isolated cell culture islands—a situation in which the amplitude of small postsynaptic currents can be accurately measured.

By simultaneously patch clamping synaptically coupled pairs of neurons, we were able to quantitate the accumulation and decay of short-term enhancement (Figure 2). For each pair of cells, we recorded the postsynaptic currents of one cell as we induced a high-frequency train of action potentials in the other (9 Hz for 15 s). On average, the 9 Hz stimulation of the presynaptic cell caused the synaptic strength to increase 7-fold (four cell pairs; Figure 2B). As in the autapses, this increase was temporary (see below).

To study the time course of decay of augmentation, an action potential was fired in the presynaptic cell once every 2 s for 60 s following the end of the high-frequency stimulation. The average quantal content of each response was small enough that there was a considerable amount of trial-to-trial variability in the postsynaptic response. The experiments were thus repeated at least seven times for each synaptic connection studied.

The sequential postsynaptic responses recorded at low frequency declined steadily until they had returned to their initial size within 30 s (Figure 2A), an observation indicating that this low rate of stimulation was not fast enough to induce a substantial amount of augmentation on its own. In the first second after the high-frequency stimulation, the response declined about 40%. This rapid, initial component of decline of synaptic strength

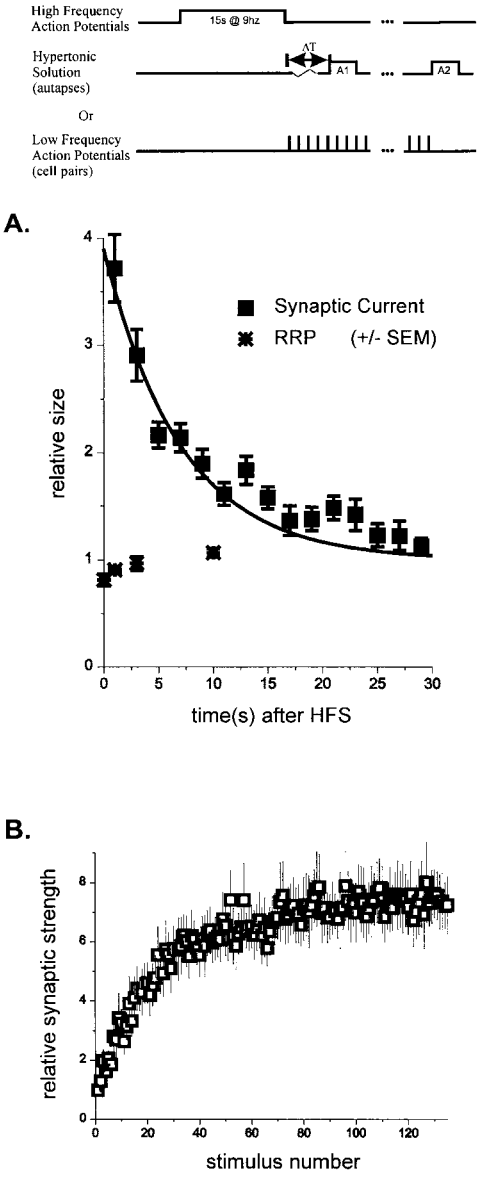

Figure 2. Augmentation Does Not Correlate with an Increase in the Size of the Readily Releasable Pool

Augmentation was studied in two preparations as diagramed at the top. Following high-frequency stimulation (9 Hz, 15 s), presynaptic action potentials were evoked at 0.5 Hz for 60 s in cell pairs. The resulting postsynaptic responses were integrated and normalized by the average responses to the last 15 low-frequency stimuli (between 30 and 60 s after the cessation of high-frequency stimulation). The normalized responses to the first 15 low-frequency action potentials are plotted versus time after the high-frequency stimulation in (A) (squares; ±SEM; four cells, 36 trials). The data are overlaid with a single decaying exponential with a time constant of 7 s (solid line). The identically normalized responses to the high-frequency action potentials are plotted versus the stimulus number in (B). In separate experiments, solitary isolated neurons were stimulated with the same high-frequency protocol. The relative pool size was measured at various times afterward and is also plotted in (A) versus time (snowflakes; average ± SEM; three cells, eight trials).

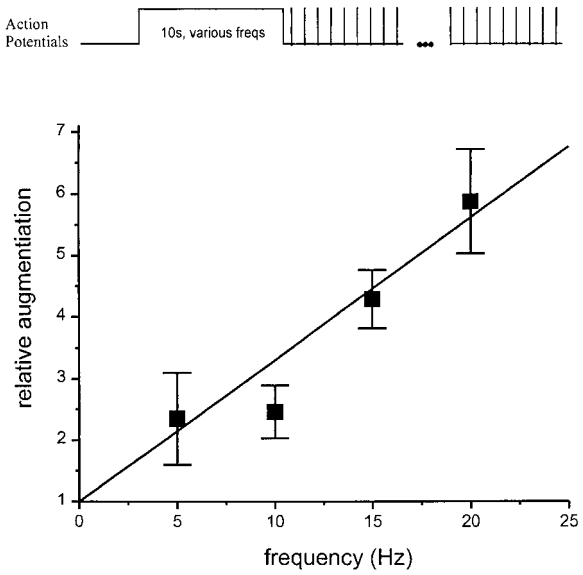

Figure 3. Relationship between Augmentation and Frequency of Stimulation

Following 10 s of high-frequency stimulation (5 Hz to 20 Hz), presynaptic action potentials were evoked at 0.5 Hz for 60 s in cell pairs. The first two responses to the low-frequency stimulation (within 3 s of high-frequency stimulation) were averaged and normalized by the average responses to the last 15 low-frequency stimuli (between 30 and 60 s after the cessation of high-frequency stimulation) and plotted against the frequency of stimulation (average from six cells ± SEM). The data are overlaid with a straight line (relative augmentation = 0.23 s × frequency + 1).

reflects the decay of the fastest components of enhancement, termed "facilitation." The decaying response measured over the next 30 s is well fitted by a single exponential with a time constant of 7 s. This is the defining time course of the intermediate component of enhancement, called "augmentation" (Magleby and Zengel, 1976; Zengel et al., 1980). Since the synaptic responses returned to baseline within 30 s, practically no posttetanic potentiation was induced by our stimulation protocol (Magleby, 1973b).

Augmentation in these synapses accumulates linearly with stimulation frequency (Figure 3), just as it does at the neuromuscular junction (Magleby and Zengel, 1976; Delaney and Tank, 1994; Tank et al., 1995), and the effect is a rather large one. In experiments much like the ones detailed above, short-term enhancement was induced in six cell pairs with high-frequency trains of action potentials (10 s), and then the decay of augmentation was monitored with low-frequency stimulation (one action potential every 2 s for 60 s, starting 1 s after the high-frequency stimulation). This time, the frequency of the rapid stimulation was varied from trial to trial (between 5 and 20 Hz), and the experiments were conducted with an even lower extracellular calcium concentration (150 μM) to avoid the small amount of depression that accumulates at 20 Hz in 250 μM Ca^{2+}. Relative augmentation was calculated for six cell pairs as the average of the first two responses to the low-frequency stimulation (evoked during the first 3 s immediately after rapid stimulation) compared to the average of the final 15 responses (30–60 s after the induction of enhancement). The linear relationship (see Figure 3) between the amount of augmentation that we measure and the frequency of the inducing stimulation is characteristic of augmentation in the other types of synapses where it has been studied (Delaney and Tank, 1994; Tank et al., 1995).

The Size of the Readily Releasable Pool Does Not Increase with Augmentation

We next sought to test the hypothesis that augmentation reflects a short-term increase in the size of the readily releasable pool by measuring the pool size in autapses during the decay of augmentation. Solitary neurons on isolated islands were patch clamped and, as in the experiments detailed above, action potentials were first evoked at 9 Hz for 15 s (250 μM Ca^{2+}). This time, the isolated neurons were probed with two 4 s long hypertonic solution challenges that served to monitor the size of the readily releasable pool. The first challenge was presented at various times soon after the cessation of high-frequency stimulation and was used to measure the relative size of the readily releasable pools at augmented synapses. The second challenge was applied 60 s after the end of the first one and provided an estimate for the resting size of the pool (Stevens and Tsujimoto, 1995; Stevens and Wesseling, 1998).

Figure 2A shows that augmentation is not associated with an increase in the size of the readily releasable pool. Immediately after the induction of augmentation, the pool was actually a bit smaller than it was 60 s later. If anything, the high-frequency train of action potentials served to reduce the population of readily releasable vesicles, even while it increased the size of the synaptic current by a large amount.

The Rate of Exocytosis from the Readily Releasable Pool Is Increased with Augmentation

The average quantity of neurotransmitter released in response to single action potentials is increased at augmented synapses. Since this increase cannot be accounted for by a larger readily releasable pool, augmentation must involve an increase in the efficiency with which action potentials trigger the exocytosis of already available vesicles. We have tested this notion by examining the time it takes for hypertonic solution to act. We explain below how an increase in vesicle fusion efficiency could be expected to shift the onset of exocytosis produced by rapid application of hypertonic solution to earlier times.

When synapses are subjected to a step change in tonicity of the bathing solution, the rate at which neurotransmitter is released does not jump instantaneously to its maximum value but rather increases smoothly after a delay of about half a second, as illustrated in Figure 4A. What causes a synapse to dump its readily releasable pool in response to an osmotic shock with this time course? Because the spontaneous release rate at a synapse is low—about one release per synapse per minute (Geppert et al., 1994)—there must be a high-energy barrier to vesicular fusion in place during periods of rest. Although the mechanism by which hypertonic solution produces release is unknown, the treatment

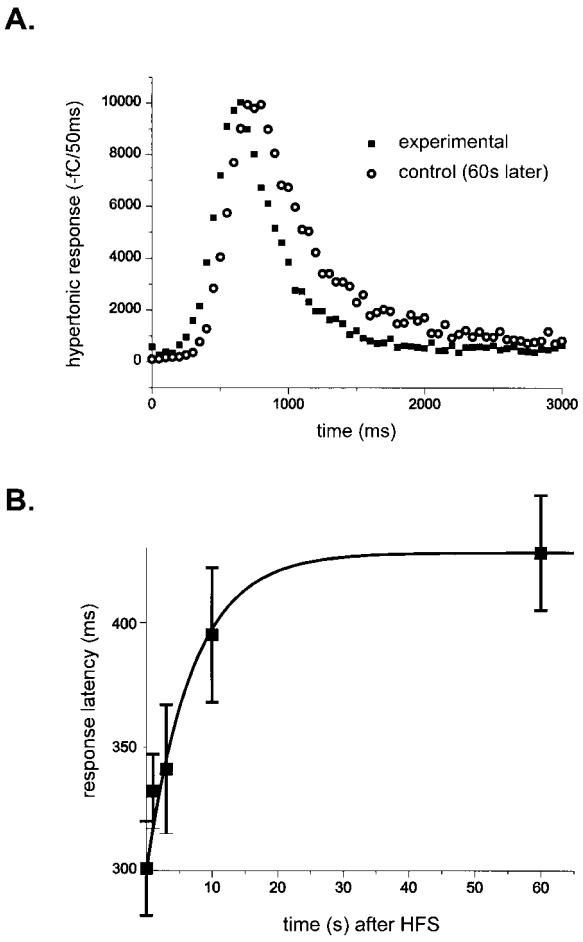

Figure 4. Latency of Response to Hyperosmotic Solution Is Decreased after the Induction of Augmentation

(A) Example response to hypertonic solution immediately after high-frequency stimulation (squares) and 60 s later (circles). Data are plotted as the total charge transfer during successive 50 ms bins after the onset of the hypertonic solution presentation (each point is the average of three trials on a single cell).

(B) The slope of the middle 90% of the rising phase of each hypertonic response reported in Figure 2A was calculated and extrapolated back to zero, and the osmotic latency was quantified as the x intercept. The latency of the response to hypertonic solution is plotted versus time after the high-frequency stimulation (average ± SEM; three cells, at least eight trials per data point) and is overlaid with a single exponential curve with a time constant of 7 s (solid line).

must lower that barrier in some way. Because vesicular fusion is a mechanical process, we favor a mechanical mode of action such as the physical deformation of the active zone that must occur as the synaptic terminals extrude water and lose volume. When hypertonic solution is applied rapidly, the local tonicity increases relatively slowly because of slow steps in mixing the applied solution in the immediate vicinity of the synaptic membrane. The membrane deformations themselves occur with additional delays because of the time it takes the water fluxes across the membranes to reach their steady rates. As these processes progress, the energy barrier for exocytosis is continuously lowered and the release rate increases, as illustrated in Figure 4A. Qualitatively, we can view the sigmoid relation between tonicity and release rate (Blioch et al., 1968; Hubbard et al., 1968; Rosenmund and Stevens, 1996) as being traced out as the local tonicity increases to its final value. The actual response is also shaped by depletion that occurs with release, an effect that we ignore here for simplicity.

What happens when augmentation is present? If augmentation is an increased propensity for vesicle fusion—that is, a lowered energy barrier for exocytosis—the release rate should be increased as the change in energy barrier from the hypertonic solution sums with that resulting from augmentation. The response to hypertonic solution should again trace out the sigmoid relation between tonicity and release rate, but the response should be shifted to earlier times with an increased release associated with each point on the curve.

Manipulations that induce augmentation do, as expected, cause a concurrent decrease in the time it takes for hypertonic solution to act (Figure 4). In 37 experiments from five cells, autapses always responded more quickly to the application of hypertonic solutions that were applied within 10 s of the induction of augmentation than to control applications 30 or 60 s later.

We quantified the latent period decrease by estimating the time between the start of hypertonic solution application and the onset of the synaptic response. A straight line was fitted to the middle 90% of the rising phase of the response, and the latency was defined by extrapolating the best fit back to the time of zero response. The time it takes for hypertonic solution to reach the synapses from the puffer pipette varies somewhat between experimental configurations, causing a certain amount of cell-to-cell variability in the resting latency. Nevertheless, after intense electrical stimulation, the response latency was reduced by an average of about 120 ms immediately after the induction of augmentation, and it relaxed to its nonaugmented value with the same 7 s time constant that characterized the decay of augmentation (Figure 4B). We conclude that augmentation results from a decrease in the energy barrier that must be overcome for vesicle fusion to occur.

Intracellular Calcium Chelation Blocks the Decrease in the Osmotic Response Latency

The finding that the induction of augmentation causes an increase in the rate of release from the readily releasable pool, whether that release is triggered by action potentials or by osmotic shock, and the observation that both effects decay away with the same time course, suggest that the same mechanism underlies the potentiation evident in both assays of transmitter release. Treatments that block augmentation should thus also temper the decrease in hypertonic challenge response latency. When introduced into presynaptic terminals, EGTA has been shown to diminish the accumulation of augmentation by chelating the residual calcium that normally triggers synaptic enhancement (Regehr et al., 1994). The EGTA, then, should block the decrease in the osmotic latency as well.

Experiments similar to the ones described above were conducted on isolated solitary neurons before and after

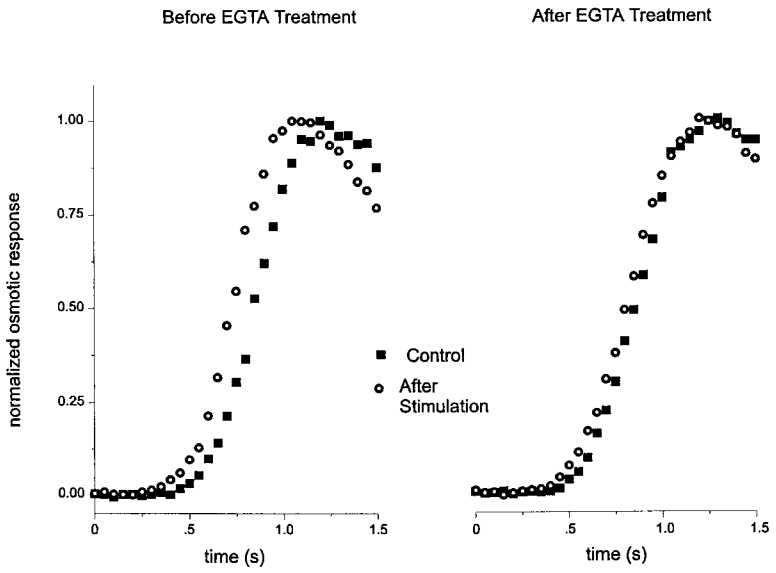

Figure 5. EGTA Blocks the Activity-Dependent Decrease in the Latency of the Response to Hypertonic Challenge

Augmentation was induced in autapses with a high-frequency train of action potentials (10 s at 9 Hz). The cells were then presented with hypertonic solution twice—once starting 1 s after the cessation of action potential stimulation and again 30 s after the end of the first one—before and after treatment of the preparation with EGTA-AM (10 μM, 5 min). Plotted are the average responses to hypertonic treatment normalized by the peak to compare rise times and response latencies (average from two cells, at least five trials each).

the application of EGTA-AM. Cells were patch clamped and augmentation was induced with a high-frequency stimulation (10 s at 9 Hz). The autapses were then presented with hypertonic solution twice. The first presentation was started 1 s after the cessation of action potential stimulation, and the second one began 30 s after the end of the first one. The basic experiment was repeated several times for each cell, and then 10 μM EGTA-AM was bath applied for 5 min. After washing the EGTA-AM out of the recording chamber, the experiment was repeated several more times. The hypertonic challenge response latency for each trial was estimated as described above.

As expected, the EGTA treatment did not alter the delay between the beginning of the hypertonic challenges and the onset of transmitter release under rest conditions, but it did inhibit the action potential–induced decrease in the latency to a step change in tonicity (Figure 5). Before the application of EGTA-AM, the high-frequency trains of action potentials reduced the response latency by an average of 10 ms (± 11 ms SEM; five trials from two cells) and afterward only by 54 ms (± 13 ms SEM; six trials). This difference was significant ($p < 0.02$, two-sample t test for unequal variances).

Augmentation Speeds Onset of Short-Term Depression

If augmentation is indeed an increase in the efficiency with which action potentials trigger the exocytosis of readily releasable vesicles, the available pool should deplete more rapidly at augmented synapses than at control ones. To test this prediction, we have induced augmentation (with 250 μM Ca^{2+} and 2 mM Mg^{2+} in the extracellular bath) as described above for pairs of cells. We then used 10 Hz stimulation to deplete the readily releasable pool after a delay of 1 s during which we switched the extracellular solution to one containing 2.6 mM Ca^{2+} and 1.3 mM Mg^{2+}. As shown in Figure 6, short-term depression occurs more rapidly when augmentation is present, probably reflecting an increase in the rate of readily releasable pool depletion. This observation provides further support for our conclusion that augmentation increases the fraction of the available vesicles that are released by a nerve impulse.

Each action potential of the 10 Hz stimulation used to produce depletion would cause considerable calcium influx, and this would tend to swamp the residual calcium that produces the augmentation induced under low extracellular calcium conditions (Tank et al., 1995). The effect of augmentation on the depletion rate would thus be expected to be underestimated by this method.

Although all the cells that we studied displayed robust enhancement in response to the high-frequency train of action potentials in low calcium, a few of them were not

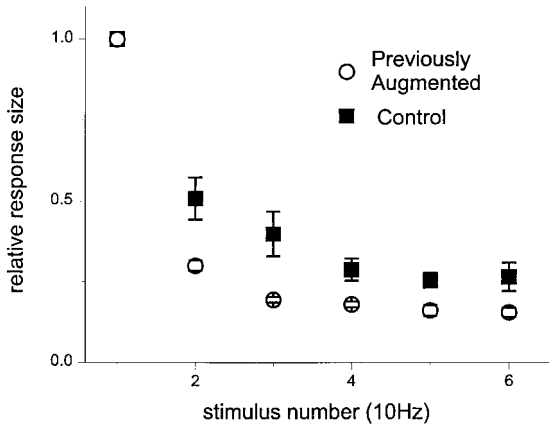

Figure 6. Successive Synaptic Responses to High-Frequency Stimulation (under Normal Extracellular Divalent Ion Conditions) Depress More Rapidly after the Induction of Augmentation

The amplitude of the postsynaptic responses (normalized to the first response) is plotted as a function of the stimulus number for a 10 Hz stimulus train. Open circles represent depression after the synapses had been first augmented under low calcium conditions, and filled squares are for the control condition (average ± SEM; five cells, 15 trials).

augmented when the extracellular solution was exchanged to include normal calcium levels. This may be because the resting fusion efficiency of a subset of the readily releasable vesicle population might be extremely high under normal conditions (Mennerick and Zorumski, 1995; Cummings et al., 1996), masking any further potentiation. Since these synapses were not augmented for these experiments, we excluded them from our analysis.

Discussion

The readily releasable pool provides the quanta that make up the postsynaptic response to presynaptic action potentials (Del Castillo and Katz, 1954; Birks and MacIntosh, 1961; Elmqvist and Quastel, 1965; Rosenmund and Stevens, 1996). Since augmentation is an increase in the quantal content of the synaptic response (reviewed by Zucker, 1989), we reasoned that it must be due either to the release of an increased fraction of the readily releasable pool with each action potential, or to the release of the same fraction of an enlarged pool as several authors have suggested (Llinás et al., 1985; Lin and Llinás, 1993; Delaney and Tank, 1994). The size of the pool can now be measured in a calcium-independent way (Rosenmund and Stevens, 1996; Stevens and Wesseling, 1998), and we show here that it is not increased by the type of repetitive electrical activity that increases synaptic strength about 400%. Since the readily releasable pool does not grow in concert with the size of the postsynaptic current, the remaining option is that augmentation results from an increase in the fraction of the readily releasable pool of vesicles that undergoes exocytosis with each action potential, a parameter that we call the "fusion efficiency."

Not Bigger, but Why Smaller?

Conditions that produce 4-fold augmentation at these synapses left the readily releasable pool slightly smaller than it started. This is an expected consequence of the high-frequency stimulation. The extracellular calcium concentration used in these experiments was low enough that the long trains of action potentials did not release enough transmitter to exhaust the readily releasable pool, as they would have if the calcium concentration had been higher (Rosenmund and Stevens, 1996). The high-frequency stimulation clearly did cause the release of some fraction of the pool, however, leaving it partially depleted.

Low-Frequency Stimulation Cannot Be Used to Track the Recovery of the Readily Releasable Pool

Recently, several reports (Dittman and Regehr, 1998; Wang and Kaczmarek, 1998; see also Hubbard, 1963) have claimed that the readily releasable pool of vesicles at some synapses refills with a time course that is qualitatively different from the one described by Stevens and coworkers (Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996; Stevens and Wesseling, 1998) and that this time course can be modulated by varying the rate of nerve stimulation in a range where Stevens and Wesseling (1998) would predict the modulatory mechanism to be saturated. In those reports, the recovery of the synapses from short-term depression induced by rapid stimulation was monitored by measuring the postsynaptic responses to subsequent low-frequency action potential firing. The sizes of the responses were reported to recover like the sum of two exponentials, one with a time constant on the order of 10 ms and the other, a slower one, with a several second time constant. We show here, however, that at the synapses we study, the sizes of the responses to low-frequency stimulation are not a valid way to measure refilling of the readily releasable pool as assumed in the studies cited above. The single responses are instead a more complicated product of the declining augmentation process and the refilling of the readily releasable pool. In fact, the single exponential refilling of the readily releasable pool that we observe, combined with the multiexponential decay of the fusion efficiency after action potential stimulation, would predict the complicated recovery time courses of synaptic responses from depression.

Experimental Procedures

For these experiments, the recovery of the readily releasable pool was estimated with methods similar to those first used by Stevens and Tsujimoto (1995) and Rosenmund and Stevens (1996). Isolated neurons were grown in cell culture as described previously (Furshpan et al., 1976, 1986; Segal and Furshpan, 1990; Bekkers and Stevens, 1991). Twelve millimeter round coverslips were coated with 0.15% agarose (type IIa) and allowed to dry in 24-well cell culture plates. A glass chromatography atomizer (Fisher) was then used to spray a particulate mist of substrate solution containing rat tail collagen (0.25 mg/ml, either bought commercially [CBI], or prepared directly from rat tails as described [Banker and Goslin, 1991]) and poly-D-lysine (0.1 mg/ml [CBI]). Tissue from the CA1–CA3 regions of newborn mouse hippocampi was dissociated as previously described (Bekkers and Stevens, 1991) and 0.5 ml of a cell suspension diluted to around 3×10^4 cells/ml was added to each well. Neurons were grown in high-glucose (20 mM) media containing 10% horse serum for 8 to 14 days before use.

Neurons were patch clamped in perforated whole-cell mode. The extracellular saline solution contained (in mM) 132 NaCl, 2 KCl, 10 glucose, 15 sorbitol, 10 HEPES, 2 $MgCl_2$, and 0.25 or 0.15 $CaCl_2$. D(−)APV (50 μM) was added to block NMDA-type glutamate currents. The patch-clamp electrodes were filled with a solution containing (in mM) 140 K gluconate, 9 NaCl, 0.2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 1 EGTA, 2 MgATP, and 0.2 LiGTP. Amphotericin (0.1 mg/ml, B-solubilized [Sigma]) was added to permeabilize the patch membrane to ions. All salines were adjusted to have a pH around 7.2 and had an osmolarity between 295 and 305 mOsm/kg. Uncontrolled action potentials were evoked presynaptically by transiently depolarizing the cell bodies as described earlier (Bekkers and Stevens, 1991). Stimulation rates reported in the text are accurate to within ±10%. Hypertonic solution (extracellular saline augmented with 50 mM sucrose) and normal divalent-containing solution (extracellular saline with 2.6 mM Ca^{2+}, 1.3 mM Mg^{2+}, and 5% dextran [w/v] to aid visualization) were applied by picospritzing from a glass pipette with a tip diameter of between 2 and 3 μm. A vacuum pipette with a diameter of 10–50 μm was used to clear the hypertonic solution rapidly from the preparation. To accurately estimate the fractional fullness of the readily releasable pool, we corrected the integral of current flow caused by hypertonic solution by subtracting away the amount of steady-state refilling and exocytosis that occurred during hypertonic challenges (Stevens and Wesseling, 1998).

Acknowledgments

We thank James Williams for help with the design of some experiments and for critical reading of this manuscript. We thank Steve

Heinemann for suggestions about the introduction. This work was supported by the Howard Hughes Medical Institute, by National Institutes of Health grant NS 12961 (C. F. S.), and by a predoctoral fellowship from the National Science Foundation (J. F. W.).

Received October 23, 1998; revised December 17, 1998.

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