Distinct Transmitter Release Properties Determine Differences in Short-Term Plasticity at Functional and Silent Synapses

Carolina Cabezas and Washington Buño
Instituto Cajal, Consejo Superior de Investigaciones Científicas, Madrid, Spain

Submitted 13 July 2005; accepted in final form 21 January 2006

Cabezas, Carolina and Washington Buño. Distinct transmitter release properties determine differences in short-term plasticity at functional and silent synapses. J Neurophysiol 95: 3024–3034, 2006. First published January 25, 2006; doi:10.1152/jn.00739.2005. Recent evidence suggests that functional and silent synapses are not only postsynaptically different but also presynaptically distinct. The presynaptic differences may be of functional importance in memory formation because a proposed mechanism for long-term potentiation is the conversion of silent synapses into functional ones. However, there is little direct experimentally evidence of these differences. We have investigated the transmitter release properties of functional and silent Schaffer collateral synapses and show that on the average functional synapses displayed a lower percentage of failures and higher excitatory postsynaptic current (EPSC) amplitudes than silent synapses at +60 mV. Moreover, functional but not silent synapses show paired-pulse facilitation (PPF) at +60 mV and thus presynaptic short-term plasticity will be distinct in the two types of synapse. We examined whether intraterminal endoplasmic reticulum Ca2+ stores influenced the release properties of these synapses. Ryanodine (100 μM) and thapsigargin (1 μM) increased the percentage of failures and decreased both the EPSC amplitude and PPF in functional synapses. Caffeine (10 mM) had the opposite effects. In contrast, silent synapses were insensitive to both ryanodine and caffeine. Hence we have identified differences in the release properties of functional and silent synapses, suggesting that synaptic terminals of functional synapses express regulatory molecular mechanisms that are absent in silent synapses.

INTRODUCTION

Glutamatergic synapses between Schaffer collateral (SC) and CA1 pyramidal neurons are a classic model for the study of synaptic plasticity such as paired-pulse facilitation (PPF) and long-term potentiation (LTP). There are two types of SC-CA1 pyramidal neuron synapses. One type, called functional synapses, shows postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate receptors (AMPA)-mediated currents when the postsynaptic cell is at its resting potential and both AMPAR and N-methyl-D-aspartate receptors (NMDAR)-mediated currents when depolarized. The other synapse type, termed silent synapse (or conditionally silent), only displays postsynaptic NMDAR-mediated currents at depolarized potentials but does not respond at the resting potential (Durand et al. 1996; Isaac et al. 1995; Liao et al. 1995; reviewed in Atwood and Wojtowicz 1999; Isaac 2003; Nicoll 2003). The different synapse types are thought to be of functional importance in information processing leading to memory formation because a proposed mechanisms for LTP is the conversion of silent synapses into functional ones (Goda and Stevens 1996; Hasselmo 1999; Poncer and Malinow 2001; reviewed in Atwood and Wojtowicz 1999; Isaac 2003; Nicoll 2003).

Cholinergic agonists inhibit transmitter release at the presynaptic terminals of functional synapses, but they do not affect silent synapses (de Sevilla et al. 2002), suggesting that functional and silent synapses are both postsynaptically different. However, the molecular mechanisms underlying the presynaptic functional differences between both glutamatergic types of synapse remain elusive.

In our previous publication, we concentrated our analysis on silent synapses and on a group of functional synapses that showed changes in the percentage of failures but no modifications of the excitatory postsynaptic current (EPSC) amplitude (i.e., averages excluding failures; see following text) during PPF, a presynaptic form of short-term plasticity (de Sevilla et al. 2002; Kamiya and Zucker 1994; Katz and Miledi 1968; Martin and Buño 2003; Zucker and Regehr 2002). The effects of carbachol chloride (CCh), which decreases the probability of release and manipulations that increase the probability of release, did not modify the EPSC amplitude in silent and in those functional synapses (de Sevilla et al. 2002). However, in that same publication we noted that a group (=50%) of functional SC synapses showed both a decrease of the percentage of failures and an increase of the amplitude of the second EPSC (R2) during paired-pulse stimulation, and that the presynaptic inhibition by CCh could increase the percentage of failures and decrease the EPSC amplitude (de Sevilla et al. 2002).

To establish the contribution of the different types of synapses in synaptic plasticity, information handling, and maturation, it is crucial to understand the differences between them and the link connecting their pre- and postsynaptic properties (reviewed in Dumas 2005). Therefore we centered the present analysis on the differences in release properties and paired-pulse plasticity between silent and functional synapses, including those functional synapses that showed larger EPSC amplitudes of R2 as compared with the first EPSC (R1) during PPF. Using “minimal stimulation,” which activates one or very few synapses (Raastad 1995), we analyzed the differences in the release properties of silent and functional SC synapses. Functional synapses exhibited lower percentage of failures and higher EPSC amplitude than silent synapses and functional synapses exhibited PPF. In contrast, PPF was absent in silent synapses. We analyzed if these discrepancies could be caused by differences in the regulation of intraterminal endoplasmic...
reticulum (ER) Ca\(^{2+}\) stores, and we found that ryanodine (100 μM) and thapsigargin (1 μM) increased the percentage of failures and decreased both the EPSC amplitude and PPF in functional synapses. Caffeine (10 mM) had the opposite effects in functional synapses. In contrast, silent synapses were insensitive to these manipulations, suggesting that functional and silent synapses are both post- and presynaptically different. These differences bear on the functional importance of silent synapses in synaptic plasticity and maturation because they suggest that different molecular mechanisms control transmitter release in both synapse types.

**METHODS**

Animal care procedures, surgery, and slice preparation have been described previously (de Sevilla et al. 2002). All the procedures carried out in this study conformed to the International Guidelines on the ethical use of animals with every effort being made to minimize the suffering and number of animals used.

**Preparation and recordings**

Hippocampal slices were obtained from young Wistar rats (12- to 16-day-old) after they were decapitated, and the brain was removed and submerged in cold (≈4°C) artificial cerebrospinal fluid (ACSF; see following text). Slices (350 – 400 μm) were cut with a vibratome (Pelco 101, St. Louis, MO) and maintained at pH 7.3 by bubbling with 95% O\(_2\)-5% CO\(_2\) (1 h at room temperature of 22–25°C). Slices were transferred to a recording chamber on an upright microscope (Olympus BX50WI, Olympus Optical, Tokyo) equipped with infrared and differential interference contrast imaging devices and with a ×40 water-immersion objective. Slices were maintained at room temperature and superfused at a rate of 1–10 ml/min with gassed ACSF. Patch-clamp recordings from CA1 pyramidal neurons in the whole cell voltage-clamp configuration were performed with 3–7 MΩ fire-polished pipettes connected to a PC-ONE amplifier (Dagan Corporation, Minneapolis, MN). Fast and slow capacitances were neutralized and series resistance was compensated (≈80%). Patch recordings were rejected when the access resistance (7–15 MΩ) increased >20% during the experiment. To minimize the contribution of postsynaptically mediated plasticity, control recordings of SC EPSCs were obtained >20 min after accessing the intracellular compartment (Malinow and Tsien 1990; Martin and Buño 2003).

**Solutions**

The ACSF contained (in mM) 119 NaCl, 2.5 KCl, 1.0 KH\(_2\)PO\(_4\), 1.3 MgSO\(_4\), 26.2 NaHCO\(_3\), 2.5 CaCl\(_2\), 30 sucrose, 0.05 picrotoxin, 0.01 glycine, and 11 glucose at pH 7.3, and the internal pipette solution contained (in mM) 107.5 Cs-glucuronate, 8 NaCl, 0.2 EGTA, 20 HEPES, 10 TEA-Cl, 4 Mg-ATP, and 0.3 GTP at pH 7.3. Caffeine, CCh, and d-2-amino-5-phosphonovaleric acid (APV) were dissolved in water, whereas ryanodine and thapsigargin were dissolved in DMSO (0.01%). DMSO at the concentrations used had no effects on synaptic responses or postsynaptic conductances (n = 3). Ryanodine (100 and 50 μM), thapsigargin (1 μM), CCh (5 μM), and APV (50 μM) solutions were superfused, whereas caffeine was added directly to the chamber with an automatic calibrated microsyringe through a pipette (tip diameter: 400 μm) positioned with a mechanical micro-manipulator close to the recording electrode tip (Martin and Buño 2003). A single volume of 100 μl of the caffeine solution was delivered (total delivery time was in ≈1 s). Chemicals were obtained from Sigma-Aldrich Quimica (Madrid, Spain) and Tocris (Avonmouth, UK).

**Synaptic stimulation**

Minimal bipolar stimulation of SCs was achieved using two silver chloride electrodes connected to a digital stimulator (Cibertec, Madrid, Spain), placed in the compartments of a pipette pulled as a patch electrode using theta capillaries (Ø of the tip ≈3–5 μm; WPI, Sarasota, FL) and filled with ACSF. The pipette was moved in the stratum radiatum, close to the apical dendrite (≈100 μm from the soma) of the cell recorded, and fixed when a single afferent was stimulated. There were no differences in the location of stimulation electrodes over the dendrites for functional or silent synapses. Only one afferent was analyzed in any neuron recorded. Stimulation was achieved with single pulses (300 μs duration) at 1.0 s\(^{-1}\) or with pulse pairs at intervals of 50 ms (except when otherwise indicated) and repeated at 0.5 s\(^{-1}\) and in a few cases (n = 6) at 0.1 s\(^{-1}\). No differences in the behavior of silent and functional synapses were found between results obtained at the different stimulation rates. The cell was depolarized to +60 mV to detect silent synapses and was then maintained at −60 mV during ≥5 min before analyzing the properties of silent synapses at +60 mV. The threshold stimulus intensity to evoke an EPSC was determined for functional (at −60 mV) or silent (at +60 mV) synapses and stimulus-response relationships were constructed with peak values of EPSCs averages (100 successive trials including failures) obtained at increasing stimulation intensities. Peak amplitudes of averaged EPSCs were stable until a jump in EPSC amplitude was recorded (suggesting the recruitment of additional afferents); this usually occurred at intensities between 150 and 300% of the initial threshold intensity. The stimulation intensity was then lowered to 25%-50% above the initial threshold and EPSCs carefully checked for changes in either percentage of failures or amplitude throughout the experiment. These procedures minimized the possible activation of more than one afferent and of failures in the activation of the stimulated axon. Synapses that did not show amplitude stability of averaged EPSCs to stimulation during the time of recording were rejected. We found no significant differences in the threshold behaviors of functional and silent synapses, indicating no differences on the axon excitability of the two types of synapses. The experiment was started by delivering 100 successive stimulations (single or paired-pulses). When a putative silent synapse had been analyzed, 100 stimuli were delivered at −60 mV to verify that the synapse was silent and that there had been no changes in the properties of the synapse. We considered a synapse as silent when it responded exclusively at +60 mV and did not respond at −60 mV even with paired-pulse protocols. Therefore we excluded a small proportion of synapses that at −60 mV did not respond to the first stimulus but did respond to the second pulse at very low probability (i.e., “mute synapses”) (Hanse and Gustafsson 2001a; reviewed in Voronin and Cherubini 2004). Essentially identical procedures were used when a functional synapse was detected but stimulation was first at −60 mV and then at +60 mV (100 trials each). It should be emphasized that stimulation intensity (≈10–50 mA) was not modified during any of the experiments included here. However, at the end of the experiment, stimulation intensity was again varied to determine if changes in the threshold or in the amplitude stability of unitary EPSCs, of silent or functional synapses, had occurred during the experiment and rejected when such changes were detected. This test also indicated the absence of long-term plasticity such as LTP and long-term depression (LTD). In many cases (35/62), the effects of adding CCh (5 μM) to the ACSF was also tested at the end of the experiment because it has no effect on silent synapses but inhibits functional synapses (de Sevilla et al. 2002), thus providing a secure “fingerprint” of the synapse type that had been analyzed. These tests were used to reduce the possibility of stimulating more than one SC fiber (Raastad 1995), and synapses that failed to meet these criteria were excluded from this analysis.
Data acquisition and analysis

Data were low-pass filtered at 1.0 kHz and sampled at 10.0 kHz through a Digidata 1200B interface (Axon Instruments, Foster City, CA) and a Pentium-based computer. The pClamp programs (Axon Instruments) were used to generate stimulus-timing signals and transmembrane current command pulses and to record and analyze data. For the analysis of the failures, we used cells that showed EPSCs with an amplitude separation from noise that allowed visual discrimination between failure and success. Independent estimations of failures were made by two scientists who were not involved in this work and who did not know what to expect from the results, and there were no statistically significant differences between their estimations and the measurements made by one of us. In addition, the selected failures were averaged and the visual selection was rejected if the averages contained poststimulus deflections as estimated by statistically significant ($P < 0.05$) differences in the amplitude distribution of records at the baseline and at poststimulus delays where the peak of successful responses occurred. During stimulation at $-60$ mV, the EPSC amplitude was defined as the difference between the peak of the averaged EPSC and the preceding baseline. With stimulation at $+60$ mV, to compare the synaptic potency of functional with silent synapses, the peak amplitude of the NMDA component was estimated (see RESULTS) and during paired-pulse stimulation the peak amplitude of the first EPSC of a pair R1 was estimated as at $-60$ mV, whereas the peak amplitude of the second EPSC R2 was the difference between the amplitude of the peak of NMDA component of the averaged EPSC and the exponential fits of the decaying phase of the preceding first EPSC R1 (Fig. 2A). The noise-free coefficient of variation ($CV_{NF}$) of the synaptic responses was calculated both for all 100 stimulations including failures and when failures were eliminated, with the formalism, $CV_{NF} = (\sqrt{d_{EPSC}^2 - d_{noise}^2})/m$, where $d_{EPSC}$ and $d_{noise}$ are the variance of the baseline and the peak EPSC amplitude and $m$ is the mean EPSC peak amplitude, respectively (de Sevilla et al. 2002). The inverse of the square of the $CV_{NF}$ ($1/CV_{NF}^2$) was used to estimate differences in release properties between two conditions where changes in release properties were expected because the methodology provides an estimation of presynaptic function (Debanne et al. 1995, 1996; Faber and Korn 1991; Foster and McNaughton 1991; Voronin et al. 1999). The $CV_{NF}$ of responses excluding failures was used to estimate the variance of successful release as an indicator of the variability of quantal responses. Results were expressed as means ± SE. Data were compared using the Student’s paired-or unpaired-t-test as appropriate ($P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***)). No age-related differences were found in the cells sampled.

RESULTS

Release properties are different in functional and silent synapses

Functional SC synapses produced glutamatergic EPSCs at both $-60$ and $+60$ mV ($n = 36/62$ or 58%; functional synapse, Fig. 1A). In contrast, silent synapses only displayed EPSCs at $+60$ mV ($n = 26/62$ or 42%; silent synapse, Fig. 1B). The EPSC rise time (estimated from fits to single-exponential functions) was faster in functional than in silent synapses and reached $\tau = 2.7 ± 0.3$ ms ($n = 36$, $\tau_{max} = 3.0$ ms) in functional synapses compared with $\tau = 3.8 ± 0.2$ ms ($n = 26$, $\tau_{min} = 3.2$ ms) in silent synapses ($P < 0.01$; both at $+60$ mV; Fig. 1A and B). These measurements confirmed that the faster AMPA components that evoked EPSC in functional synapses were absent in silent synapses that did not conduct at $-60$ mV because of the voltage-dependent Mg$^{2+}$ block of NMDA receptor channels.

We compared the percentage of failures of functional and silent synapses at $+60$ mV, and it was significantly larger in silent ($73.5 ± 18.2$; $n = 26$) than in functional synapses ($58.3 ± 2.7$; $P < 0.001$; $n = 36$, Fig. 1C). We also compared the “synaptic potency” at $+60$ mV (i.e., the average peak amplitude of EPSCs when failures are excluded) (Stevens and Wang 1994). Functional synapses show EPSCs with both an early AMPA and a late NMDA component, whereas silent synapses only display an NMDA EPSC. Therefore to compare the amplitude of the NMDA component in functional synapses by measuring at delays of 30 ms from the stimulation artifact, where at $+60$ mV the AMPA component has terminated, and there is only an NMDA component. We found that the synaptic potency of the NMDA component (at $+60$ mV, measured at 30 ms) was higher in functional ($20.4 ± 1.5$ pA; $n = 36$) than in silent synapses ($15.2 ± 1.3$ pA; $P < 0.05$; $n = 26$, Fig. 1D). We also checked that the isolated AMPA component had terminated within 30 ms in seven functional synapses where averaged EPSCs evoked by 100 successive stimuli were recorded at $+60$ mV both in control ACSF (i.e., the “compound”, AMPA + NMDA EPSC) and after isolating the AMPA component by blocking the NMDA responses with APV (50 μM). The compound EPSC peaked at delays of 12.5 ± 0.6 ms with a mean peak amplitude of 26.0 ± 2.7 pA ($n = 7$; data not shown). These
values were not statistically different from those of the other functional synapses analyzed (28.4 ± 1.5 pA; 11.9 ± 1.3 ms; P > 0.05; n = 36). Therefore these 7 functional synapses are representative of the sample of 36 functional synapses analyzed here. In these synapses, the isolated AMPA component peaked at 3.0 ± 0.4 ms and ended at delays of 27.5 ± 0.8 ms.

Using the same amplitude estimation procedure, we calculated the CVNF of all 100 stimuli and only of successful NMDA responses for both types of synapses, which were 1.02 ± 0.1 and 0.52 ± 0.03 for functional and 2.3 ± 0.7 and 0.29 ± 0.03 for silent synapses, respectively (P < 0.001 in both cases; n = 36 and n = 26, respectively). The CVNF of all 100 trials was significantly higher for silent than functional synapses, consistent with the higher percentage of failures of the former. In contrast, the CVNF of successful responses was higher in functional than silent synapses, suggesting lower amplitude variability of the quantal responses in silent synapses.

Taken together, these results point to the existence of different presynaptic transmitter release properties in these two types of synapse and transform the notion that silent synapses are exclusively presynaptic or postsynaptic.

Functional synapses exhibit PPF; in contrast, silent synapses lack PPF

To verify these differences, we analyzed the PPF, a presynaptic form of short-term plasticity present in our sample of functional synapses. With conventional stimulation, PPF is characterized by an increased second “compound” EPSC when it is elicited shortly after an initial EPSC (Creager et al. 1980; de Sevilla et al. 2002). With “minimal stimulation,” PPF may not be expressed in response to single paired pulses due to the probabilistic nature of release; however, it can be observed in averages when the percentage of failures of the second response R2 is lower than the first R1 (Creager et al. 1980; de Sevilla et al. 2002; Dobrunz and Stevens 1997; Hanse and Gustafsson 2001a–c; Zucker and Regehr 2002).

In functional synapses, the averages of EPSPs evoked by stimulation with pulse pairs (50-ms delay), including the failures, showed higher mean peak amplitudes of R2 than R1, both at +60 mV (R2 = 15.9 ± 1.4 pA and R1 = 11.1 ± 1.0 pA; P < 0.001; n = 36) and at −60 mV (R2 = −11.1 ± 0.9 pA and R1 = −6.8 ± 0.5 pA; P < 0.001; n = 36). This was consistent with a PPF being present (Fig. 2A) and implied that the synaptic efficacy of R2 was higher than that of R1 (Fig. 2B). We calculated a PPF index [(R2 − R1)/R1] (de Sevilla et al. 2002; Martín and Bueno 2003), which was 0.5 ± 0.1 at +60 mV and 0.7 ± 0.1 at −60 mV (P > 0.05; n = 36), indicating that there was no significant difference at either membrane potential (Fig. 2C).

During PPF, the percentage of failures of R1 was higher than at R2. At +60 mV, the percentage of failures of R1 was 58.3 ± 2.7%, and for R2, it was 48.1 ± 2.8% (P < 0.001; n = 36), whereas at −60 mV, R1 failed 61.5 ± 2.1% and R2 45.2 ± 3.2% (P < 0.001; n = 36). Hence, the percentage of failures of R1 and R2 were essentially identical at −60 and +60 mV (P > 0.05; n = 36; Fig. 2E), supporting the assumption that the differences in the percentage of failures between R1 and R2 resulted from a presynaptic influence and minimizing the possibility that we were stimulating more than one type of synapse. In addition, the 1/CV^2 calculated for all 100 stimulations was higher for R2 than R1 (10.3 ± 1.6 and 1.5 ± 0.1, respectively, P < 0.001; n = 36), consistent with a presynaptic mediated effect.

On the average in functional synapses, the synaptic potency of R2 was higher than that of R1 at both −60 and +60 mV during PPF (Fig. 2, D and F). At +60 mV, the synaptic potency was 28.4 ± 1.5 pA for R1 and 32.7 ± 1.9 pA for R2 (P < 0.001; n = 36) and at −60 mV, it was −16.0 ± 0.9 pA for R1 and −19.5 ± 1.2 pA for R2 (P < 0.001; n = 36). We also calculated a synaptic potency index [(P2 − P1)/P1] where P1 and P2 are the potency of the first and second EPSC, respectively. A potency index of 0 indicates that on the average the peak amplitude of the R2 should be identical to that of the R1 EPSC, a result consistent with the same number of quanta being released by the first and second action potentials. However, the potency index was >0 for the greater part of the functional synapses considered (P < 0.001; n = 26), a result probably caused by an increased release probability of R2 relative to R1 combined with a greater number of synaptic contacts per afferent (Hsia et al. 1998; reviewed in Dumas 2005) that was either caused by activation of a single terminal with multiple release sites or by the recruitment of several terminals. In this scenario, the number of quanta released by a
single action potential reaching the terminal will be given by the number of release sites formed by the SC afferent with the postsynaptic CA1 neuron, which is invariant in our conditions, and the release probability at each release site, which will vary during paired pulse stimulation. In addition, there was a group of functional synapses \((n = 10)\) in which the potency index was not statistically different from 0 (cf., de Sevilla et al., 2002). In all of the functional synapses, the potency index was practically identical at \(-60\) and \(+60\) mV \((P > 0.05; n = 36)\) and the \(1/C\nu^2\) calculated for successful responses was higher for R2 than R1 \((8.5 \pm 1.3\) and \(5.4 \pm 0.7\), respectively; \(P > 0.05\); same synapses), supporting the assumption that the differences in synaptic potency of R1 and R2 most likely resulted from a presynaptic change. In these studies, the voltage insensitivity of the above-described behaviors minimizes the possible contribution of recruitment of silent synapses and of “spillover” (Choi et al. 2000; Maggi et al. 2003).

To test that the higher amplitude of R2 was not due to a postsynaptic effect, we calculated the synaptic potency of R2 EPSCs both when the R1 response failed and succeeded. When R1 fails, the action potential that reaches the terminal is unable to elicit release, and there is no possible postsynaptic action of the first stimulation that could modify the R2 EPSC. We found that the synaptic potency of R2 was similar when calculated for responses with or lacking an R1 EPSC \((P > 0.05; n = 36; \text{Figs. 3A and 4B2})\). The cumulative distributions of the R2 EPSC peak amplitudes were also practically identical in the presence or absence of R1 EPSCs \((P > 0.05, n = 36; \text{Fig. 3B, 1 and 2})\).

Therefore in our sample these results imply that the increased synaptic potency of R2 associated with PPF depends on presynaptic release mechanisms and minimizes a possible postsynaptic contribution.

The EPSC rise time and the time to peak were similar \((P > 0.05; n = 36)\) at both membrane potentials \((-60\) and \(+60\) mV) for these functional synapses. In addition, the waveform of the average of R1 and R2 EPSCs (Fig. 3C) were also similar, a result that reduces the possible stimulation of several presynaptic fibers that would result in a slower rise time and a wider duration of R2 EPSCs due to the unsynchronized release caused by the simultaneous activation of additional fibers with dissimilar conduction velocities. The same was true for EPSCs evoked by single pulses where rise times did not correlate with EPSC amplitude \((P > 0.05)\).

Superfusion with CCh (5 \(\mu\)M) increased the PPF, increased the percentage of failures, and reduced the synaptic potency both of R1 and R2 in this type of functional synapse (the synaptic potency of R1 decreased 21.7 \(\pm\) 2.7\% and that of R2 31.7 \(\pm\) 3.5\%; \(P < 0.01\); \(n = 7\); data not shown). This result is consistent with a reduction of the probability of release in synapses that have several contacts per afferent.

In the same type of analysis on silent synapses, the mean peak amplitudes of R1 and R2 EPSCs were similar \((R1 = 7.1 \pm 0.8\) pA and R1 = 7.0 \(\pm\) 0.8 pA; \(P > 0.05; n = 26; \text{Fig. 4A})\), implying that there was no PPF (the mean PPF index was 0.01 \(\pm\) 0.04; \(\text{Fig. 4, B and C})\). In addition, we found no difference in the percentage of failures of R1 \((73.5 \pm 1.8\%)\) and R2 \((72.5 \pm 2.2\%\); \(P > 0.05; n = 26\); \text{Fig. 4E}) and the synaptic potency of R1 \((22.6 \pm 1.2\) pA) and R2 \((24.2 \pm 1.3\) pA; \(P > 0.05; n = 26\)) were also similar (\(\text{Fig. 4, D and F})\). Moreover, the \(1/C\nu^2\) calculated for all 100 stimulations was essentially identical for R2 and R1 \((0.63 \pm 0.1\) and 0.74 \(\pm\) 0.08, respectively), and the same was true for \(1/C\nu^2\) calculated for successful responses that was 26.1 \(\pm\) 7.0 for R1 and 40.8 \(\pm\) 15.3 for R2, respectively \((P > 0.05; \text{same cells})\).

Therefore consistent with the essentially identical percentage of failures, amplitude, and \(1/C\nu^2\) of R1 and R2, PPF was absent. In addition, both the PPF and the potency indexes were practically zero for all silent synapses \((P > 0.05; n = 26)\). We also analyzed the effects of paired pulses at different intervals between 20 and 200 ms, and PPF was absent in all the silent synapses analyzed \((n = 4; \text{data not shown})\). Moreover, as described previously (de Sevilla et al. 2002), CCh did not affect silent synapses \((P > 0.05; n = 7; \text{data not shown})\).

Taken together, these results suggest that at functional synapses the PPF arises from the combined effects of a lower percentage of failures and an increased release of transmitter at R2 in comparison with R1 (Debanne et al. 1996). The decreased percentage of failures of R2 with respect to R1 is thought to result from a Ca\(^{2+}\) signal that remains in the terminal after the first action potential and accumulates with the Ca\(^{2+}\) inflow during the second action potential (Dobrunz and Stevens 1997; Kamiya and Zucker 1994; Katz and Miledi 1968; Zucker and Regehr 2002). The increased release probability of R2 as compared with R1 combined with more than one
synaptic contact per afferent could explain the increased release at R2 relative to R1 (see preceding text) (Debanne et al. 1996). In contrast, the percentage of failures and the synaptic potency of R1 and R2 were similar and PPF was absent in all silent synapses, suggesting that the activity-dependent presynaptic control during PPF is not engaged in silent synapses.

It is noteworthy that in a group of functional synapses that showed PPF it was not paralleled by an increased synaptic potency of R2 relative to R1. In those functional synapses, PPF was exclusively caused by a decreased proportion of failures of R2 as compared with R1 (n = 10). The synaptic potency was also unchanged by other manipulations that modified release probability in those functional synapses (de Sevilla et al. 2002). Therefore these functional synapses probably have a single synaptic contact and release site per afferent. Consequently, functional synapses are not homogeneous in their release properties. Although the differences in release properties between functional synapses may be of physiological relevance, their investigation would require further analysis that should be treated independently and is out of the scope of the present work that centers on the global differences between the properties of functional and silent synapses.

The differences in release properties between silent and functional synapses suggest that the amount of transmitter released by an action potential reaching the terminal is a more finely regulated process in functional than in silent synapses. Therefore a key conclusion from the preceding results is that presynaptic terminals of both types of synapse are functionally different.

Transmitter release is regulated by presynaptic ER Ca\(^{2+}\) stores in functional but not in silent synapses

The contribution of ER Ca\(^{2+}\) stores and Ca\(^{2+}\)-induced Ca\(^{2+}\)-release (CICR) to neurotransmitter release in central synapses is a matter of debate (see DISCUSSION). In hippocampal synapses, CICR may contribute to PPF by amplifying the Ca\(^{2+}\) signal induced by the arrival of the second action potential (Empagne et al. 2001), thus the differences in PPF of silent and functional synapses could reside in the properties of CICR at the corresponding terminals or in the control of the intraterminal Ca\(^{2+}\) concentration by the regulation of Ca\(^{2+}\) sequestration and release from the ER (Galante and Marty 2003). Therefore we tested the effects interfering with CICR and the regulation of ER Ca\(^{2+}\) stores.

Bath-applied ryanodine (100 \(\mu\)M), that blocks ryanodine receptors, increased the percentage of failures and reduced the synaptic potency and PPF in functional synapses in a gradual manner (Fig. 5, A–D). The effect of ryanodine reached a maximum after \(~\)25–30 min and was identical at \(-60\) and \(+60\) mV \((P > 0.05)\); only the data at \(+60\) mV will be shown for simplicity). The percentage of failures increased for R1 32\% (from 55.4 \(\pm\) 6.9 to 73.2 \(\pm\) 4.7; \(P < 0.05\); n = 6) and for R2 56\% (from 45.2 \(\pm\) 8.5 to 70.6 \(\pm\) 5.4; \(P < 0.05\); n = 6), whereas the synaptic potency decreased 21\% for R1 (from 35.8 \(\pm\) 2.2 to 28.1 \(\pm\) 1.1; \(P < 0.01\); n = 6) and 24\% for R2 (from 41.4 \(\pm\) 2.4 to 31.2 \(\pm\) 0.6; \(P < 0.05\); n = 6).

Ryanodine also reduced the PPF index from 0.44 \(\pm\) 0.1 to 0.16 \(\pm\) 0.05 \((P < 0.05\), same cells\)). In this case, a reduction of the release probability caused a decreased PPF, thus contradicting the usually accepted inverse relationship between release probability and PPF (Dobrnz 2002; Dobrnz and Stevens 1997; but see Hanse and Gustafsson 2002). However, a similar unexpected effect of ryanodine on PPF has been interpreted to indicate that CICR amplifies the Ca\(^{2+}\) signal induced by the arrival of the first action potential at the presynaptic terminals, which adds with the Ca\(^{2+}\) inflow activated by the second action potential (Empagne et al. 2002). The effects of ryanodine were identical at \(-60\) and \(+60\) mV, suggesting a presynaptic site of action and minimizing postsynaptically mediated modifications. A lower concentration of ryanodine (50 \(\mu\)M) showed a tendency to similar effects but changes were not statistically significant (data not shown; n = 6). The effects of ryanodine on functional synapses was reduced (\(~\)20\%; n = 6) following prolonged \(~\)50-min washout.

To verify that the regulation of ER Ca\(^{2+}\) stores or CICR were involved in transmitter release by functional synapses, we applied thapsigargin to deplete calcium stores by inhibiting Ca\(^{2+}\) uptake by the Ca\(^{2+}\)-ATPase. Initially, superfusion with thapsigargin (1 \(\mu\)M, \(~\)15 min of superfusion) decreased the percentage of failures and increased the synaptic potency and PPF, consistent with the initial transient increase of intraterminal Ca\(^{2+}\) due to depletion of the presynaptic ER stores (Clementi et al. 1992). However, after this initial period thap-
that can be attributed to the caffeine–induced persistent potentiation of glutamate release in SC synapses (Martín and Bueno 2003).

In contrast, ryanodine did not significantly modify the percentage of failures of either R1 or R2 EPSCs in silent synapses (from 76.4 ± 1.8 to 80.0 ± 2.1; P > 0.05; n = 8 for R1, and from 75.6 ± 3.0 to 81.3 ± 2.5; P > 0.05; n = 8 for R2; Fig. 5G). The synaptic potency was also unaffected by ryanodine (from 21.1 ± 1.7 to 18.5 ± 1.4, for R1; and from 23.1 ± 2.1 to 18.7 ± 1.5, for R2; P > 0.05; n = 8 in both cases, Fig. 5G). In addition, silent synapses were also insensitive to caffeine because the percentage of failures of neither R1 nor R2 EPSCs changed (from 70.3 ± 4.9 to 64.0 ± 8.7, for R1; and from 69.6 ± 3.4 to 68.3 ± 7.4, for R2; P > 0.05; n = 7 in both cases), and exposure to caffeine did not modify the synaptic potency of silent synapses (from 24.8 ± 3.1 to 27.2 ± 3.2, for R1; and from 27.6 ± 3.4 to 26.8 ± 2.9, for R2; P > 0.05; n = 7 in both cases; Fig. 5F). Moreover, neither ryanodine nor caffeine modified the PPF index of silent synapses (P > 0.05; n = 8 and 7 respectively; Fig. 5H).

**Discussion**

**Release properties are different in functional and silent synapses**

We show that presynaptic terminals of functional and silent SC-CA1 pyramidal neuron synapses express different transmitter release properties that are regulated by distinct presynaptic mechanisms. First the percentage of failures is higher and...
the synaptic potency is lower in silent than functional synapses. Second, PPF is detected in functional synapses but is absent in silent synapses. Third, release is regulated by presynaptic ER Ca$^{2+}$ stores in functional synapses, regulatory mechanisms that are not active in silent synapses. Finally, manipulations that modify release probability, such as PPF and caffeine (present results) or increasing the extracellular Ca$^{2+}$/Mg$^{2+}$ ratio or CCh (de Sevilla et al. 2002), do not convert one type of synapse into the other, suggesting that the type of synapse depends primarily on the nature of the postsynaptic glutamatergic receptors and on the voltage-dependent Mg$^{2+}$-block of NMDA channels (Isaac et al. 1995; Liao et al. 1995).

It should be emphasized that artifacts caused by the possible simultaneous stimulation of more than one SC fiber or of failures in the activation of SC fibers are unlikely because we have performed several tests and controls to minimize these possibilities.

Schaffer collateral synapses are thought to have a single release site in neonatal animals (Bolshakov and Siegelbaum 1995; Dobrunz and Stevens 1997; Hanse and Gustafsson 2001a,c, 2002) but may show a greater number of synaptic contacts and release sites per afferent, later (>1 wk) during development (Hsia et al. 1998; Kullmann and Nicoll 1992; Larkman et al. 1992; Liao et al. 1995; Sorra and Harris 1993) and as a result of presynaptic changes with LTP (Bolshakov et al. 1997; Malinow 1991; Malinow and Tsien 1990; Voronin et al. 1999). Moreover, in neonatal rats (<1 wk), the AMPA/NMDA ratio is low (≈0.2), suggesting that most synapses are silent (Hsia et al. 1998). Those synapses lack PPF, a result that agrees with our present findings. However, the reason underlying this neonatal behavior is uncertain because the absence of PPF has been related either with a high release probability in these synapses (Bolshakov and Siegelbaum 1995), which was not the case in our experiments, or to other presynaptic mechanisms (Hsia et al. 1998). The discrepancy between our results and those of Bolshakov and Siegelbaum (1995) could indicate that silent synapses undergo changes related to maturation in our older age sample, as occurs in thalamocortical synapses (Yanagisawa et al. 2004) and in hippocampal synapses around the second and third postnatal week (reviewed in Dumas 2005).

Fluctuations in synaptic potency in SC synapses during PPF have also been found using Ca$^{2+}$ imaging techniques that guarantee recordings from a single spine (Oertner et al. 2002; Reid et al. 2004) and with paired recordings of monosynaptically connected CA3–CA1 pyramidal neurons that ensure the activation of a single axon (Chen et al. 2004; Debanne et al. 1996; Foster and McNaughton 1991; Malinow 1991; Malinow and Tsien 1990).

The differences in synaptic potency of the NMDA component between silent and functional synapses deserve attention and could reflect: presynaptic differences in the number of release sites in individual buttons or of the number of synapses made by single SC fibers (Hsia et al. 1998; Kullmann and Nicoll 1992; Larkman et al. 1992; Liao et al. 1995; Sorra and Harris 1993); distinct presynaptic Ca$^{2+}$ channel types (Iwasaki et al. 2000; Scheuer et al. 2004); different intraterminal second-messenger cascades; a postsynaptic divergence linked to changes in the number of NMDA receptors present in the corresponding spine; and to differences in the type of NMDA receptor expressed in both synapse types (Kirson et al. 1999; Yanagisawa et al. 2004; reviewed in Dumas 2005).

In addition, we show that the synaptic potency of R2 EPSCs was similar when calculated for responses with or lacking an R1 EPSC and that the cumulative distributions of the R2 EPSC peak amplitudes were also practically identical in the presence or absence of R1 EPSCs. We interpreted this result to indicate the absence of a postsynaptic mediated effect in the regulation of synaptic potency during PPF. This result contradicts reports that show that when there is a R1 EPSC the reduction of the ready releasable pool (RRP) of vesicles leaves less releasable vesicles when the second action potential reaches the terminal thus reducing the probability of release during R2 (Debanne et al. 1996; Dobrunz and Stevens 1997; Stevens and Wang 1994). This discrepancy may indicate that release does not critically affect the size of the RRP in our experimental conditions. A possibility that may explain the disagreement is that activation of a single SC afferent may recruit several release sites with low release probability at each site that would not appreciably deplete the RRP at each site. Indeed, the relationship between release and the RRP is a matter of debate and both a decrease and no change of R2 as a result of R1 has been reported (Debanne et al. 1996; Dobrunz and Stevens 1997; Hanse and Gustafson 2001a—c, 2002; Stevens and Wang 1994). Although saturation of postsynaptic receptors could also explain the argument, saturation is unlikely in our sample because the synaptic potency increased during the PPF and the increase was similar for AMPA and NMDA EPSC components that have very different sensitivity to released glutamate.

It is significant that other presynaptic mechanisms may control synaptic potency. Indeed, such changes may involve either a regulation in the “size” of the vesicles (i.e., the amount of transmitter each vesicle holds) (Atwood and Wojtowicz 1999) or the amount of transmitter released by a single vesicle may be controlled in an activity-dependent manner by the mechanism known as “kiss and run” (Aravanis et al. 2003; Becherer et al. 2003; Chen et al. 2004; Gandhi and Stevens 2003; Stevens and Williams 2000). However, present results do not enable discrimination among the possible mechanisms. In addition, to what extent a single release site releases at most one or several vesicles at a time is a matter of debate (Auger and Marty 2000; Wadike and Jahr 2001).
Presynaptic ER Ca\(^{2+}\) stores regulate release in functional but not in silent synapses

The contribution of ER Ca\(^{2+}\) stores and Ca\(^{2+}\)-induced Ca\(^{2+}\)-release (CICR) to neurotransmitter release in central synapses is a matter of debate, and positive (Emptage et al. 2001; Galante and Marty 2003; Llanò et al. 2000; Martín and Buño 2003) and negative (Carter et al. 2002) results have been reported. In addition, contradictory results on the contribution of CICR in transmitter release have also been reported for SC-CA1 pyramidal neuron contacts where either no effect of ryanodine was observed at low concentrations or—as reported in SC-CA1 pyramidal neuron contacts where either no effect of ryanodine was observed at low concentrations or—as reported here—a decrease of the EPSC was recorded with high concentrations (100 \(\mu\)M) of ryanodine (Carter et al. 2002). Consistent with our results, the inhibitory effect of ryanodine (and thapsigargin) on the EPSC had been reported previously in inhibitory cerebellar synapses and interpreted as caused by a decreased Ca\(^{2+}\) sensitivity of the mechanism of vesicle exocytosis possibly produced by a local reduction of the resting Ca\(^{2+}\) concentration (Galante and Marty 2003).

The data available in the literature were obtained with “conventional” stimulation or by analyzing mEPSCs and miniature excitatory postsynaptic potentials (mEPSPs) and with the postsynaptic neuron held at the resting membrane potential. In that situation, only non-NMDA EPSP components of functional synapses are recorded and silent synapses are not because of the voltage-dependent Mg\(^{2+}\) block of NMDA receptors. However, to our knowledge no attempt has been made to separately analyze CICR in functional and silent SC synapses, which may show differences in the regulation of ER Ca\(^{2+}\) stores. In addition, the differences in the effects of ryanodine and the negative results should be taken with care because the metabolic state of the neuron may have profound consequences on the function of intracellular Ca\(^{2+}\) stores (reviewed in Collin et al. 2005).

Ryanodine (50–100 \(\mu\)M) blocks ryanodine receptors and inhibits CICR. In contrast, caffeine at mM concentrations increases the Ca\(^{2+}\) sensitivity of ryanodine receptors, thus amplifying CICR (Galante and Marty 2003; Llanò et al. 2000; Martín and Buño 2003). Caffeine also increases Ca\(^{2+}\) influx through presynaptic N-type channels by inhibiting presynaptic adenosine receptors, thus relieving the adenosine-mediated block of N-type channels (Martín and Buño 2003; Qian and Saggau 1997). Therefore caffeine effects are complicated because at millimolar concentrations, it increases the intraterminal Ca\(^{2+}\) both by raising the influx of Ca\(^{2+}\) following an increase in CICR. However, we had previously shown that SC NMDA and non-NMDA EPSC components were blocked to a similar degree by adenosine (de Sevilla et al. 2002), reducing the possibility of a contribution of differences in the regulation of Ca\(^{2+}\) influx by a divergence in the caffeine sensitivity of presynaptic adenosine receptors. Therefore the dissimilar effects of caffeine on silent and functional synapses described in the results are most likely due to a specific effect on the ER Ca\(^{2+}\) stores and the CICR mechanism.

Others have shown that blockade of CICR can effectively reduce the Ca\(^{2+}\) signal in the synaptic terminals of SC synapses evoked by the arrival of both action potentials during paired-pulse stimulation (Emptage et al. 2001). These same authors demonstrated that although ryanodine decreased intraterminal Ca\(^{2+}\) on the first stimulus, it did not affect transmitter release presumably because CICR did not occur fast enough. However, on arrival of the second stimulus, ryanodine both reduced Ca\(^{2+}\) signal and decreased release. In view of these results, the authors argue that in normal conditions, the Ca\(^{2+}\) signal from the CICR caused by the first stimulus persists long enough to increase PPF by adding with the Ca\(^{2+}\) influx caused by the second action potential or by sensitizing the release apparatus to a subsequent stimulus. However, the effects of ryanodine and thapsigargin reported here may not only reflect the consequences of blocking CICR. Rather, the drugs could empty the Ca\(^{2+}\) stores in the ER, ultimately leading to a reduction in presynaptic cytoplasmic Ca\(^{2+}\), which would explain the effect on release by single action potentials (Galante and Marty 2003). This could also explain the reduced facilitation, although a block of CICR may also be involved. Likewise the effects of 10 mM caffeine on release by one spike are almost certainly due to an increase in resting presynaptic Ca\(^{2+}\), which would be expected if ER Ca\(^{2+}\) stores are present. If release to the first spike is saturated, this would reduce facilitation.

We show that both ryanodine and thapsigargin increased the percentage of failures and decreased both the synaptic potency and PPF in functional synapses. In addition, we show that caffeine decreased the percentage of failures, increased the synaptic potency, and reduced PPF in this group of functional synapses. In contrast, the drugs are inactive in silent synapses.

We should like to emphasize that whatever the mechanism of action of ryanodine, thapsigargin, and caffeine on these functional synapses, the key point is that those effects were absent in silent synapses, suggesting that regulation of presynaptic Ca\(^{2+}\) by ER stores plays a role in controlling release in functional but not in silent synapses.

Different presynaptic Ca\(^{2+}\) channel types or dissimilarities in the density of presynaptic Ca\(^{2+}\) channels (Iwasaki et al. 2000; Scheuber et al. 2004; Wu and Saggau 1994) may contribute to the mechanisms that differentiate both kinds of synapse, without ruling out the contribution of CICR. However, the analysis of those differences was out of the scope of the present work and will be carried out in the future.

Final considerations

The concept that silent synapses lack AMPA receptors is actively debated and alternative mechanisms have been proposed to explain silent synapses. It has been argued that silent synapses may contain both AMPA and NMDA receptors but do not conduct due to having a release probability close to zero (“mute synapses”) (Hanse and Gustafsson 2001a; reviewed in Voronin and Cherubini 2004), because the concentration of glutamate in the cleft activates their NMDA receptors yet is insufficient to stimulate AMPA receptors (Choi et al. 2000), or because of “spillover,” where glutamate diffusing out of the synaptic cleft into neighboring synapses is sufficient to activate the postsynaptic high affinity NMDA receptors but not the less sensitive AMPA receptors (Kullmann and Asztely 1998). Although our results are consistent with the classical scenario where silent synapses lack AMPA receptors and hence are “conditionally silent” or “deaf,” they also suggest presynaptic differences in both synapses types and hold up the notion that these silent synapses are both pre- and postsynaptically different from functional ones.
It is likely that the mechanisms of synaptic plasticity are not exclusively pre- or postsynaptic but rather involve a balance and integration of reciprocal influences on the development and maturation of synaptic machinery on both sides of the synaptic cleft. Therefore a possible functional consequence of our results is that the conversion of silent to functional synapses with maturation and LTP must be associated with modifications of the presynaptic terminals as determined both with electrophysiological and imaging techniques (Bolshakov et al. 1997; Malinow 1991; Palmer et al. 2004; Stanton et al. 2005; Voronin et al. 1999; reviewed in Dumas 2005). The link connecting pre- and postsynaptic properties may need an unknown signal that modifies the terminal and that could either arise in the pre- or postsynaptic neuron or in a third element that could be the surrounding atrocities.

ACKNOWLEDGMENTS

Many thanks are due to Drs. Alfonso Araque and Pablo E. Castillo and David Fernández de Sevilla for helpful discussions and comments on an early version of the manuscript. Many thanks are due to Dr. Jean C. Poncer for the extremely helpful discussion and suggestions on a final version of the manuscript. We also thank M. Sefton for correcting the English and for correcting extremely helpful discussion and suggestions on a final version of the manuscript.

GRANTS

This work was supported by the Dirección General de Investigación Científica y Tecnológica, Ministerio de Ciencia y Tecnología (MCyT), Spain (BFI2002-01107) grant to W. Buito. C. Cabezas is a doctoral fellow supported by MCyT.

REFERENCES


Foster TC and McNaughton BL. Long-term enhancement of CA1 synaptic transmission is due to increased quantal size, not quantal content. Hippocampus. 1: 79–91, 1991.


J Neurophysiol. Vol. 95 • May 2006 • www.jn.org


