An increase in calcium influx contributes to post-tetanic potentiation at the rat calyx of Held synapse

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ABSTRACT

We studied the contribution of a change in presynaptic calcium influx to post-tetanic potentiation (PTP) in the calyx of Held synapse, an axosomatic synapse in the auditory brainstem. We made whole-cell patch-clamp recordings of a principal cell following loading of the presynaptic terminal with a calcium dye. After induction of PTP by a high-frequency train of afferent stimuli, the Fluo-4 fluorescence transients evoked by an action potential became on average 15 ± 4% larger (n=7). Model predictions did not match the fluorescence transients evoked by trains of brief calcium currents unless the endogenous calcium buffer had low affinity for calcium, making a contribution of saturation of the endogenous buffer to the synaptic potentiation we observed in the present experiments less likely. Our data therefore suggest that the increase of release probability during PTP at the calyx of Held synapse is largely due to an increase in the calcium influx per action potential.
The calyx of Held synapse, a fast relay in the auditory brainstem, displays several forms of short-term synaptic plasticity. Depending on the stimulus conditions, the response to a second stimulus may be increased (paired-pulse facilitation, PPF; Forsythe and Barnes-Davies 1993), or decreased (short-term depression, STD; Borst et al. 1995). In addition, following a high-frequency train of stimuli, both a several minute-long decrease (post-tetanic depression, PTD; Forsythe et al. 1998) or increase (post-tetanic potentiation, PTP; Habets and Borst 2005; Korogod et al. 2005) in the synaptic responses may occur. The accessibility of this synapse to patch-clamp recordings has allowed great progress in identifying different underlying mechanisms that contribute to these forms of short-term plasticity (reviewed in von Gersdorff and Borst 2002). The four different forms of short-term plasticity that occur at the calyx have in common that they are largely presynaptic phenomena. A change in the output of a synapse can be due to an increase in the size of the readily-releasable pool (RRP), which is typically defined as the pool of vesicles that can be released by a very large stimulus or to a change in the release probability of vesicles in the readily-releasable pool. PTP is accompanied by a large increase in the release probability (Habets and Borst 2005; Korogod et al. 2005). In addition, we also observed a small increase in the RRP (Habets and Borst 2005). The PTP decayed with a similar time course as the presynaptic calcium increase. However, based on the measured affinity of the phasic calcium sensor (Bollmann et al. 2000; Lou et al. 2005; Schneggenburger and Neher 2000), by itself this increase in presynaptic calcium would be insufficient to cause a substantial increase in the activation of the phasic calcium sensor. In this paper we further explore the mechanisms that underlie the increase in release probability during PTP at the calyx of Held synapse. We focus on possible changes in the calcium influx for several reasons. Firstly, small changes in calcium influx lead to large changes in
release. Release typically is proportional to the third or fourth power of the calcium influx (reviewed in Schneggenburger and Neher 2005). Therefore, even relatively small changes in calcium influx would make substantial contribution to PTP. Secondly, the calcium currents at the calyx of Held facilitate calcium-dependently and this increase may contribute to PPF (Borst and Sakmann 1998b; Cuttle et al. 1998; Inchauspe et al. 2004; Ishikawa et al. 2005). Since PTP cannot be observed while the terminals are in whole-cell patch-clamp configuration (Habets and Borst 2005; Korogod et al. 2005), we used a fluorometric method to investigate a possible contribution of changes in calcium influx to PTP.
MATERIALS AND METHODS

Preparation of slices

Preparation of slices and electrophysiological measurements were done as described previously (Habets and Borst 2005). Animal procedures were in accordance with guidelines provided by the animal committee of the Erasmus MC. In brief, seven to ten day old Wistar rats were decapitated without prior anesthesia. The brainstem was dissected and immersed in ice-cold saline containing (in mM): 125 NaCl, 2.5 KCl, 3 MgSO₄, 0.1 CaCl₂, 1.25 NaH₂PO₄, 0.4 ascorbic acid, 3 myo-inositol, 2 pyruvic acid, 25 D-glucose, 25 NaHCO₃ (Merck); pH 7.4. Transverse slices of 200 µm thickness were cut with a vibratome (Vibratome, St. Louis, MO).

Electrophysiological recordings

Normal Ringer solution had the same composition as the solution that was used for slicing, except that the concentration of CaCl₂ and MgSO₄ were 2 and 1 mM, respectively. Neurons were visualized with an upright microscope (BX-50; Olympus, Tokyo, Japan), equipped with infrared differential interference contrast optics. Axons originating from the cochlear nucleus were stimulated (0.5 ms, 0.03-0.5 mA) in the midline by a bipolar electrode (Frederic Hear & Co, Bowdoinham, ME). Test frequency was increased compared to earlier experiments (0.5 vs. 0.1 Hz; Habets and Borst 2005) to allow collection of more fluorescence data in the same time period. At this frequency there was already some synaptic depression (von Gersdorff et al. 1997). PTP was elicited by a 5 min, 20 Hz tetanus. Cells were selected when extracellular recordings indicated postsynaptic action potential firing (Borst et al. 1995). Electrophysiological recordings were made at room temperature with an
Axopatch 200B amplifier (Axon Instruments, Union City, CA). Pipette solutions contained (in mM): 125 K-gluconate, 20 KCl, 10 Na$_2$-phosphocreatine, 4 MgATP, 0.3 Na$_2$GTP, 10 HEPES (Sigma) and 0.05-0.2 calcium sensitive dye (Molecular Probes, Eugene, OR) or 0.5 EGTA for pre- or postsynaptic recordings, respectively. Calcium currents were pharmacologically isolated by substituting 20 mM TEA-Cl (Fluka, Buchs, Switzerland) for 20 mM NaCl and adding 1 µM tetrodotoxin (Alomone labs, Jerusalem, Israel) and 0.1 mM 3,4-diaminopyridine to the Ringer solution. In these experiments, the internal solution contained (in mM) 125 Cs-CH$_3$SO$_3$, 20 CsCl, 10 Na$_2$-phosphocreatine, 4 MgATP, 0.3 Na$_2$GTP, 10 HEPES (Sigma). In some experiments Cs-CH$_3$SO$_3$ was replaced with Cs-Gluconate. Series resistance was 8-30 MΩ (compensated 80-98%, prediction was set to 80%). Leak subtraction was done with the P/8 method. Holding potential in voltage clamp experiments was -80 mV. Potentials were corrected for a -11 mV junction potential. Postsynaptic series resistance (<15 MΩ) was electronically compensated by 80-98% with a lag of 5 µs. Signals were low-pass (10 kHz) filtered with a 4-pole Bessel filter. Only cells with a membrane resistance higher than 100 MΩ were accepted for analysis. Signals were sampled at 20-50 kHz with a Digidata 1320A (Axon Instruments). Data acquisition and analysis was done with pClamp 8 (Axon Instruments) or Igor 5 (Wavemetrics, Lake Oswego, OR).

**Imaging**

Terminals were pre-filled with fura-2, Fluo-4, rhod-dextran or Oregon Green BAPTA-5N (OGB-5N) for 10 min via the patch pipette. Only cells in which a GΩ outside-out patch formed after retraction were selected for analysis. The tissue was illuminated through a 40X objective (NA 0.8, Olympus, Tokyo, Japan) by a
monochromator (Polychrome IV; 8 nm bandwidth, TILL Photonics, Martinsried, Germany). Excitation intensity was about 0.1 mW, when measured under the objective. Emission light was filtered through an appropriate bandpass filter and detected with a cooled photomultiplier tube (PMT; H7422-40, Hamamatsu, Hamamatsu City, Japan). Excess background fluorescence was removed by a 1 mm pinhole at the image plane of the microscope. PMT signals were amplified and low-pass filtered (2 kHz) with an 8-pole Bessel filter (Model 3382, Krohn-Hite, Brockton, MA) before digitization (Digidata 1320A, Axon Instruments). For fura-2, calcium concentrations were calculated as described in Habets and Borst (2005). For non-ratiometric dyes, responses evoked by an action potential ($\Delta F_{AP}$) are given as a percentage of the basal fluorescence of the terminal, which was calculated as the difference between the fluorescence in the absence of stimuli and the fluorescence from a nearby region. In the case of PTP experiments, responses are expressed relative to the basal fluorescence before the tetanus ($\Delta F_{AP}/F_0$).

Data analysis

PTP

The amount of PTP was calculated as the percentage increase of the average amplitude of the first ten EPSCs after tetanic stimulation relative to the average amplitude of the last ten EPSCs before the tetanus.

APW trains

To test the relation between calcium influx and fluorescence changes, terminals were voltage clamped using a train of 10 action potential waveforms (APWs) at 100 Hz, as described earlier (Borst and Helmchen 1998). Since clearance will start as soon as the
influx starts, we corrected the amplitude of fluorescence changes for clearance during the rising phase assuming a similar time course for clearance as after the rising phase. In experiments in which terminals were loaded with a single dye, decay of the fluorescence between stimuli was fitted with a single exponential function. Its time constant was set to the time constant of a fit of the decay at the end of the train and its offset (value at $t=\infty$) was set to the baseline level before the first stimulus. To calculate the amplitude of the fluorescence change triggered by an APW, the fit that described the decay of the calcium transient elicited by the preceding APW was extrapolated to the time point where the increase of the fluorescence due to the actual APW was half-maximal. The fit of the decay following the actual APW was back-extrapolated, again to the time point where the increase of the fluorescence due to the actual APW was half-maximal. The amplitude was then taken as the difference between both fits at that point. In experiments in which two dyes were loaded, decays were fit with a double exponential function and fits were extrapolated to the peak value rather than the mid-point of the rising phase. Slow calcium-activated currents were subtracted before integration of the calcium currents.

**Simulations**

To assess the influence of the kinetics of the dye and the endogenous calcium buffer on the measured fluorescence transients, these transients were simulated using a single compartment model (Helmchen et al. 1997). This model assumes spatial equilibrium at all time points. Even though diffusion of $\text{Ca}^{2+}$ is disregarded, in the absence of significant buffer depletion, a single-compartment model may accurately describe the effect of the kinetics of calcium dyes on volume-averaged calcium
transients at the calyx of Held (Helmchen et al. 1997; Meinrenken et al. 2003). We refer to (Meinrenken et al. 2003) for a discussion of its limitations. Standard equations for buffering were solved numerically, by forward Euler finite difference using an adaptive step size. Calcium influx during an action potential was either provided by the response of a two-state Hodgkin Huxley model of calyceal calcium currents to a previously recorded action potential (Borst and Sakmann 1998a), or the simultaneously measured calcium influx in presynaptic voltage clamp recordings was used, after filtering, truncation of outward currents and subtraction of slow calcium-activated currents. In the case of the modeled calcium influx, total Ca\(^{2+}\) influx during an action potential was 0.91 pC, leading to an increase of the total calcium concentration to 12 µM in the calyx volume of 0.4 pl (Helmchen et al. 1997). The standard model solution contained: free Ca\(^{2+}\) at a starting concentration of 50 nM; endogenous buffer concentration 1.3 mM, forward Ca\(^{2+}\) binding rate 5 \times 10^8 per Msec (Klingauf and Neher 1997), off rate 16000 s\(^{-1}\) (calcium-binding ratio 40, calculated as described in Helmchen et al. 1997); no ATP and 50-200 µM of fura-2, on-rate 4 \times 10^8 per Msec, off rate 103 s\(^{-1}\); Fluo-4, on-rate 7.1 \times 10^8 per Msec, off rate 369 s\(^{-1}\) (Naraghi 1997) or Oregon Green BAPTA-5N (OGB-5N), on-rate 2.5 \times 10^8 per Msec, off rate 8000 s\(^{-1}\) (Faas et al. 2005). Removal of Ca\(^{2+}\) from the cytoplasm was modelled as a linear, non-saturable clearance mechanism. To match the experimentally observed decays, its rate constant was set to 800 (Figure 1) or 400 s\(^{-1}\) (Figure 4). In the simulations shown in Figure 1, a concentration of 50 µM was assumed, to account for loss of dye into the axon during the PTP experiments.

Simulated fluorescence transients were resampled to 50 kHz and digitally filtered with a binomial (Gaussian) filter to 2 kHz before analysis. To be able to compare the onset of simulated and measured responses in current clamp recordings, the measured
prespike was aligned with the inverted first derivative of the simulated action potential after correcting for the delay introduced by the Bessel filtering.

Data are given as mean ± standard error of the mean (SEM). Statistical comparisons were done using Student’s $t$-test.
RESULTS

Rapid calcium transients at the calyx of Held synapse

PTP at the calyx of Held synapse rapidly washes out during presynaptic whole-cell recordings (Habets and Borst 2005; Korogod et al. 2005). To prevent washout of PTP we therefore switched to fluorometric methods and pre-loaded dyes into the terminal during whole-cell recordings. Dyes were preloaded for 10 min to a final concentration of 200 µM (Figure 1A), after which the presynaptic pipette was withdrawn. Release was estimated from the size of the glutamatergic EPSCs in postsynaptic whole-cell recordings. We optimized fluorometric measurements by using dyes with a relatively low basal fluorescence and by using a cooled, high-quantum-efficiency PMT for detection. As a result, not only the amplitude of the fluorescence transient that was triggered by an action potential ($\Delta F_{AP}$) could be accurately measured, but its rise time could also be resolved. The time course of the transients differed between dyes. Rise times of transients in the presence of OGB-5N, a low affinity dye ($K_d$ 32 µM), were 0.32 ± 0.03 ms. Their decay was well approximated by a single exponential function with a time constant of 57 ± 8 ms (n=5; Figure 1B). In the presence of Fluo-4, a high affinity calcium dye ($K_d$ 350 nM), 20-80% rise times were 0.68 ± 0.06 ms and decay time constants were 295 ± 42 ms (n=7; Figure 1C). Even in the absence of a presynaptic whole-cell recording, it was still possible to get information about the timing of the presynaptic action potential using the prespike in the postsynaptic recordings. The prespike is the capacitatively coupled presynaptic action potential (Forsythe 1994). Its positive peak corresponds to the time when the speed of repolarization is maximal. For both dyes, the rise started around this time, which is shortly after the onset of the presynaptic calcium current (Borst and Sakmann 1998a).
Single compartment model

The $\Delta F_{AP}$ transients shown in Figure 1 were compared with the predicted transients for a single compartment model that featured apart from the calcium dye, an endogenous calcium buffer, calcium influx and a linear clearance mechanism (see Methods for details). As long as the off-rate of the endogenous buffer was high (>5000 s$^{-1}$), the time course of the simulated and measured responses overlaid well for both the OGB-5N and the Fluo-4 transients (Figure 1D). If the off-rate of the endogenous buffer was lowered to 5000 s$^{-1}$ or less, while adjusting its concentration to keep the endogenous binding ratio constant, the endogenous buffer was no longer able to ‘track’ the calcium influx and a prominent overshoot in the simulated volume-averaged calcium concentration at the end of the repolarization phase became apparent in the simulations (Figure 1D). This component resulted in a bi-phasic decay of the OGB-5N transients. At 5000 s$^{-1}$, the fast component was already sufficiently large (>5% of the peak amplitude) to be well above the detection threshold in the measured transients. Since such a component was not present, this suggests that the endogenous buffer has a high unbinding rate for Ca$^{2+}$. This high off-rate implies that its $K_d$, which is the ratio of off-rate and on-rate, has to be large as well. Even if its on-rate were close to the diffusion limit, ~$1.10^9$ per Msec, its $K_d$ would have to be >5 µM. At lower on-rates, the $K_d$ would have to be correspondingly larger. These simulations therefore suggest that the endogenous buffer has a low affinity for calcium, enabling it to rapidly follow changes in the calcium concentration. The lack of a large overshoot in the volume-averaged calcium concentration, in combination with the rapid kinetics means that the rise phase of the OGB-5N transients largely reflects the integral of the calcium currents (Sabatini and Regehr 1998). This is not
the case for the Fluo-4 transients. Fluo-4 has a much smaller off-rate than OGB-5N and as a result, the rising phase of the Fluo-4 transients probably largely reflects the transfer of calcium ions between the endogenous buffer and Fluo-4 (Sabatini and Regehr 1998). The around 5-fold slower decay can be explained by the much larger calcium binding ratio of this high-affinity dye (Helmchen et al. 1997). Because of the inverse relation between total binding ratio (i.e. the sum of the contributions of the endogenous and the exogenous buffers) and the time constant of the decay of the fluorescence transients (Helmchen et al. 1997; Neher 1995), this indicates that on average Fluo-4 captures at least 80% of inflowing calcium ions.

In summary, the simulations suggest that the large majority of the endogenous calcium buffer of the calyx of Held has low affinity for Ca\textsuperscript{2+} (K\textsubscript{d} >5 µM) and that at the concentration used in the PTP experiments, Fluo-4 will capture most inflowing calcium ions.

**Calcium transients during PTP**

We used the fluorometric signals to test for a change in the calcium transients after the induction of PTP. Unfortunately, it was no longer possible to induce PTP after prolonged calcium imaging. Probably, this was due to a phototoxic effect on the terminal. Only when light exposure was restricted to a short baseline period before the tetanus was it possible to induce PTP. Following a 5 min, 20 Hz tetanus, the EPSCs in the terminals that had been preloaded with Fluo-4 increased by 98 ± 22% (n=7), similar to intact terminals (Habets and Borst 2005). The PTP was accompanied by a clear increase in the amplitude of ΔF\textsubscript{AP} (Figure 2A). The amount of PTP and the increase in ΔF\textsubscript{AP} were correlated (Figure 2F). On average the fluorescence transient increased by 15 ± 4% (n=7). There was a small increase (0.24 ± 0.11 ms) in the time
to onset of $\Delta F_{AP}$. A clear example is shown in Figure 2B. This increase correlated well ($r=0.99$) with an increase in the delay of the EPSCs in the same experiments (Figure 2C), indicating that the increased delay following PTP induction was due to an increased delay until presynaptic calcium channels opened, rather than changes downstream of Ca$^{2+}$ entry. Apart from the small increase in the delay of $\Delta F_{AP}$, its kinetics were very similar after the tetanus: both its rise time ($p=0.75$; paired t-test) and its decay ($p=0.24$) did not change significantly (Figure 2C). The lack of a change in the kinetics of the fluorescence transients argues against a change in clearance after the induction of PTP. In most experiments, the decay of PTP matched the decay of the increase in $\Delta F_{AP}$ (Figure 2D-2E). In the experiment shown in Figure 2D the decay of PTP also matched the increase in basal fluorescence, as previously observed (Habets and Borst 2005; Korogod et al. 2005). However, in most experiments this relation could not be reliably assessed, since basal fluorescence was generally not stable during the control period, probably due to washout of extracellular dye.

Surprisingly, in the presence of the low-affinity dyes rhod-dextran or OGB-5N, in only one out of six synapses a large increase in the size of the EPSC was observed. This was accompanied by a clear increase in $\Delta F_{AP}$. In the other five synapses, EPSCs increased by only 29 ± 3% and $\Delta F_{AP}$ changed little after the tetanus (-1 ± 4%).

**Relation between calcium influx and fluorescence signal**

The increase in the Fluo-4 transients following induction of PTP could be due to an increase in calcium influx, an increase in the fraction of inflowing calcium ions that are captured by the dye, or a combination of the two. A possible increase in the captured fraction of inflowing calcium ions could be due to a decrease in the calcium clearance or due to a decrease in competing endogenous buffers. Our earlier
conclusion that the endogenous buffer most likely has low affinity and that Fluo-4 captures most of the calcium ions at the concentration used in the PTP experiments suggests that an effect on calcium influx is more likely than a selective depletion of endogenous buffers. To test the relation between the size of the calcium influx and the calcium signals at different presynaptic Ca$^{2+}$ levels, we voltage clamped the presynaptic terminals with trains of ten action potential waveforms (APWs) at 100 Hz after pharmacologically isolating the calcium currents. This allowed us to directly compare the calcium influx per action potential with the fluorescence signals. Both in the presence of Fluo-4 and in the presence of OGB-5N, the calcium currents facilitated during the train (Figure 3A-3B). In the presence of Fluo-4, the influx per APW increased by around 20% at the end of the train, in agreement with earlier results (Borst and Sakmann 1998b). In the presence of OGB-5N, the facilitation was more transient (Figure 3D), which is in line with the lack of an increase in $\Delta F_{\text{AP}}$ following a tetanus in most of the PTP experiments in which OGB-5N was used. The absence of calcium channel facilitation during long trains in the presence of OGB-5N may have contributed to the reduced PTP in the experiments described above. The train of APWs led to clearly resolvable fluorescent transients ($\Delta F_{\text{APW}}$), consisting of a rapid rising phase followed by an exponential decay (Figure 3A-3B). The time constant of this decay changed little during the train, both for terminals filled with OGB-5N (200 µM) and with Fluo-4 (100 µM). The amplitude of the fluorescence increase following the first and the last APW were similar for the OGB-5N transients (Figure 3A, inset), but the last APW evoked a smaller fluorescence transient than the first APW in the terminals filled with Fluo-4 (Figure 3B, inset), despite the larger calcium influx (Figure 3D). To take into account the changes in the calcium influx during the train, we calculated the ratio between fluorescence change and calcium
influx ($\Delta F/\Delta Q$). In the presence of the low affinity dye OGB-5N, $\Delta F/\Delta Q$ largely remained the same, with a small increase during the first APWs and a small decrease towards the end of the train (Figure 3D). In contrast, in the presence of the high-affinity dye Fluo-4, $\Delta F/\Delta Q$ gradually decreased during the train, as $\text{Ca}^{2+}$ accumulated. These experiments provide more evidence for our earlier conclusion that the large majority of the endogenous buffer has low affinity for $\text{Ca}^{2+}$. As a result the increase in the calcium concentration during the train leads to little saturation of both the endogenous buffer and OGB-5N, resulting in little changes in $\Delta F/\Delta Q$. At the same time the gradual decrease in the availability of Fluo-4 leads to a gradual decrease in $\Delta F/\Delta Q$.

Although these experiments are in agreement with our earlier conclusion that the endogenous buffer has low affinity for $\text{Ca}^{2+}$, they do not provide positive evidence that we would be able to detect the presence of a low concentration of an endogenous high-affinity calcium buffer. We therefore repeated these experiments in the presence of both OGB-5N (200 µM) and fura-2 (50 µM). The decay of the OGB-5N transients now clearly became bi-phasic (Figure 3C). If the decay of the response to a single APW was fitted with two exponential functions, the fast time constant ranged between 1-2 ms, whereas the slow time constant was $>100$ ms ($n=4$; not shown).

Because of the different spectral properties of the two dyes, we could also measure the fura-2 fluorescence transients within the same experiment. The presynaptic calcium concentration increased from a basal level of about 100 nM to about 160 nM after the first APW ($n=4$). The rising phase of the fura-2 transients was much slower than of the OGB-5N transients, and it largely matched the fast component in the decay of the OGB-5N transients (Figure 3C). As calcium accumulated to a maximum level of about 1 µM at the end of the train, the relative contribution of the fast phase
decreased from $81 \pm 5\%$ to $6 \pm 3\%$ (n=4) following the last APW. The $\Delta F/\Delta Q$ for OGB-5N showed a clear increase during the train, which we interpret as being due to the saturation of the competing other exogenous calcium buffer fura-2, since $\Delta F/\Delta Q$ of fura-2 became much smaller at the same time.

**Train simulations**

Our interpretation of the fluorescence signals during trains of APWs does not take diffusion or heterogeneities in calcium influx or calcium buffering into account. To test whether the binding kinetics and relative affinities of the different endogenous and exogenous calcium buffers provided a sufficient interpretation of the observed signals we repeated the simulations of the single compartment model for trains of APWs. In these simulations we used the measured calcium currents illustrated in Figure 3 (after filtering, truncation of outward currents and subtraction of slow calcium-activated currents) as inputs and added the exogenous calcium buffers using the same low-affinity endogenous buffer as in Figure 1. During these trains the volume-averaged calcium concentration rose to about 0.6 µM in the presence of a high affinity calcium dye (Fluo-4, or fura-2 in combination with OGB-5N) and about 1.3 µM in the presence of OGB-5N. Both the simulated fluorescence transients (Figure 4A-4C) and the resulting $\Delta F/\Delta Q$ (Figure 4D) were qualitatively similar to the data illustrated in Figure 3. The simulations also confirmed the validity of the interpretation of the experiments in which we added both a high- and a low-affinity dye. The low-affinity dye is able to report how the calcium transfers from the low-affinity buffers to the high-affinity dye. Due to its slow equilibration, the high-affinity dye is not able to report subtle changes in the time course of the calcium influx, as may happen following PTP induction. The contribution of the high-affinity dye
becomes less pronounced as calcium accumulates and the dye saturates. As a result, the low-affinity dye faces less competition and $\Delta F/\Delta Q$ will increase during the train. The experiments and simulations with two different exogenous dyes confirm that even small concentrations ($<50 \, \mu\text{M}$) of a slowly equilibrating calcium buffer (off-rate $<5000 \, \text{s}^{-1}$), would lead to clear deviations from a single exponential decay for the OGB-5N transients, in contrast to what was observed in Figures 1 and 3A.
DISCUSSION

By choosing dyes with low background fluorescence and by using a low-noise photomultiplier, we were able to record the calcium signals that were evoked by an action potential in a single presynaptic terminal at much higher time resolution and with a much better signal-to-noise ratio than in earlier studies of the calyx of Held (e.g. Billups and Forsythe 2002; Helmchen et al. 1997; Schneggenburger et al. 1999). The signal-to noise ratio was also much higher than in our earlier study, in which we used fura-2 (Habets and Borst 2005). This allowed us to compare the calcium transients evoked by an action potential before and after induction of PTP. We show that PTP at the calyx of Held synapse is accompanied by a clear increase in these calcium transients. The most likely cause for this increase is an increase in calcium influx during an action potential. In this Discussion we review the evidence for this conclusion and we discuss the significance of our findings for the mechanism of PTP at the calyx of Held synapse.

Increased calcium transients during PTP

We observed a clear increase in the Fluo-4 transients following PTP induction. On average, the transients increased by around 15%. There are three possible causes for the observed increase in the Fluo-4 signal. It could be due to an increase in the calcium influx, a decrease in calcium clearance or it could be due to a decrease in the endogenous calcium buffers that compete with the Fluo-4. We have several arguments that support our conclusion that the increased Fluo-4 signal was due to increased calcium influx. A decrease in Ca$^{2+}$ clearance is unlikely since the time course of the Fluo-4 signal was not changed after PTP induction. Following the induction of PTP,
the presynaptic calcium concentration is elevated by around 100 nM and the decay of PTP largely matches the decay of this increase (Habets and Borst 2005). This increase in residual calcium could lead to a partial depletion of endogenous calcium buffers. At other terminals, fluorometric evidence for a high-affinity endogenous buffer component has been obtained (Collin et al. 2005; Jackson and Redman 2003; Lin et al. 2005; Sinha et al. 1997) and partial depletion of the endogenous calcium buffer has been proposed to be responsible for most of the short-term facilitation of release at the calyx of Held synapse (Felmy et al. 2003). We therefore analyzed the measured calcium transients in detail, to investigate whether under the conditions in which we observed the increase in calcium transients, we could find fluorometric evidence for a high-affinity endogenous calcium buffer, whose depletion could be partially responsible for the observed increase in the calcium transients.

Analysis of calcium transients suggests low endogenous buffer affinity

A comparison of amplitude and time course of different calcium dyes with model predictions indicated that under our experimental conditions the large majority of the endogenous buffer has low affinity (>5 µM) for Ca\textsuperscript{2+}. The OGB-5N transients that were evoked by a single action potential decayed mono-phasically. Simulations (Figure 1D) and experiments (Figure 3C) suggested that the presence of even small amounts (<50 µM) of a high-affinity endogenous calcium buffer would lead to clear deviations from a mono-exponential decay. In presynaptic voltage clamp experiments we compared calcium influx during action-potential waveform stimuli and the resulting fluorescence changes at different Ca\textsuperscript{2+} concentrations. If the endogenous buffer has low affinity, both the amplitude and the time course of the low-affinity OGB-5N transients are predicted not to change, as long as the calcium clearance
mechanism is linear and not saturated. The low-affinity buffer OGB-5N initially showed a small increase in the fraction of calcium ions that it captured per stimulus, followed by a small decrease as Ca\(^{2+}\) accumulated during the train (Figure 3D). The increase of a few percent for the OGB-5N signal could be due to saturation of a small component of the endogenous buffer with a higher affinity for Ca\(^{2+}\) than the dye. Alternatively, it could be due to small errors in the quantification of the calcium influx, due to incorrect estimate of calcium-activated currents or gating currents, or small errors in the quantification of the fluorescence step, due to incorrect correction for clearance during the rising phase of the transients. Apart from this small increase, both the amplitudes and the time course of the OGB-5N transients closely followed the prediction of the model with a low-affinity endogenous buffer and a linear clearance mechanism, up to a level of at least 1 µM. This concentration was much higher than the increase in residual calcium observed after PTP induction at the calyx of Held (Habets and Borst 2005; Korogod et al. 2005). A linear clearance mechanism has also been observed at the crayfish neuromuscular junction (Tank et al. 1995).

In contrast, in the presence of a low-affinity endogenous buffer, a high-affinity dye like Fluo-4 is expected to show a gradual decrease in the fraction of inflowing calcium ions that it captures during the train, as less and less dye will be available to compete with the endogenous buffer. At other terminals it was not possible to directly measure the calcium influx during trains of action potentials, but nevertheless qualitatively similar results have been obtained, a decrease in signals during the train for high-affinity dyes, but increases or no change for low-affinity dyes (David et al. 1997; Koester and Johnston 2005; Kreitzer and Regehr 2000).

From the analysis of the time course and amplitudes of the calcium transients we therefore conclude that the endogenous calcium buffer in these experiments had a low
affinity for Ca\(^{2+}\). A similar conclusion was also reached along different lines by (Bollmann and Sakmann 2005). At cerebellar terminals (Sabatini and Regehr 1998) or in chromaffin cells (Xu et al. 1997) the endogenous buffer also has low affinity for Ca\(^{2+}\). Our data cannot exclude the washout during dye loading of a high-affinity mobile buffer. The absence of PTP in experiments in which the terminals were loaded with low-affinity dye indicates that calcium buffering is of importance for the induction of PTP. Possibly these buffers interfered with PTP induction by limiting the maximal Ca\(^{2+}\) increase during the tetanus more effectively than the low-affinity buffer fluo-4. Alternatively, the low-affinity buffers shortened the decay time of the PTP to the extent that it was missed in our recordings, which did not start until more than one minute after the tetanus to allow for pool recovery. At later developmental stages, high-affinity calcium binding proteins such as calretinin or parvalbumin may make a larger contribution to calcium binding (Felmy and Schneggenburger 2004; Lohmann and Friauf 1996) and this might explain why longer stimulation is needed to induce PTP in older animals (Korogod et al. 2005).

**Increased calcium influx during PTP**

If the endogenous buffer is low, this means that the increase in the Fluo-4 transients after PTP induction was not due to selective depletion of a high-affinity endogenous calcium buffer. We therefore conclude that this increase must have been due to an increase in calcium influx. Although the Fluo-4 concentrations we used were low (<200 µM) and comparable to the buffer concentrations we used in our previous study in which we focussed on residual calcium (Habets and Borst 2005), due to the low endogenous buffer capacity of the calyx of Held at young ages (Helmchen et al. 1997), the calcium dye nevertheless captured most of the incoming calcium ions
(>80%) in the PTP experiments. This means that in these experiments, the ‘overload’ condition was almost reached, meaning that under these conditions the dye provided a sensitive indication for calcium influx (Neher 1995). Changes in the endogenous buffer will therefore have comparatively little effect on the measured Fluo-4 transients and it is hard to come up with a scenario for which these effects are larger than the effects that residual calcium will have on the availability of Fluo-4 after PTP induction. This means that the increase of 15% has to be viewed as a lower estimate for the increase in calcium influx.

**What caused the increase in calcium influx?**

The increase in calcium influx can be due to a facilitation of the calcium currents (Borst and Sakmann 1998b; Cuttle et al. 1998) or to a change in the action potential shape. We did observe a change in the prespike (Figure 2A-2B), suggesting that a broadening of the action potential may have contributed to the increased calcium influx (Borst and Sakmann 1999). We were not able to address this conclusively, because of the inability to evoke PTP in presynaptic whole-cell recordings and because the Fluo-4 transients were too slow to detect changes in the time course of the calcium influx. Facilitation of calcium currents very likely contributed to the observed changes in the calcium influx. A few action potential waveforms were sufficient to induce a facilitation of the calcium influx that was comparable to the increases that we observed in the Fluo-4 signal after PTP induction (Figure 3). In contrast, in the presence of OGB-5N, a train of action potential waveforms did not give a sustained facilitation of the calcium influx and no increase in the calcium signals following a tetanus was observed in most current clamp experiments that used OGB-5N as the
dye. Since PTP was reduced in the presence of OGB-5N, whereas in the fluo-4 experiments it was comparable in size to the PTP observed in intact terminals, these experiments suggest that the increase of the calcium influx that we observed in the Fluo-4 voltage clamp experiments best matches the situation in the undialysed terminals. Interestingly, an increase of around 15% in calcium signals during action potential trains was also observed in granule cell terminals of the cerebellum (Kreitzer and Regehr 2000).

Our experiments provide further evidence for the key role that the modulation of calcium channels plays in the regulation of short-term plasticity. Facilitation of calcium currents is also important for short-term facilitation of transmitter release at the calyx of Held (Inchauspe et al. 2004; Ishikawa et al. 2005; Taschenberger et al. 2002) and inactivation of calcium currents contributes to synaptic depression (Forsythe et al. 1998; Xu and Wu 2005). The facilitation of calcium currents depends on an interaction with the high-affinity calcium-binding protein neuronal calcium sensor 1 (NCS-1; Tsujimoto et al. 2002) and this protein has also been implicated in short-term facilitation of transmitter release at the neuromuscular junction or the hippocampus (Rivosecchi et al. 1994; Sippy et al. 2003), although as of yet there is no evidence that it exerts its effects via calcium channels at these synapses. The regulation of calcium channels by calcium binding proteins like NCS-1 or other members of this family is complex and they may have both Ca^{2+}-dependent and Ca^{2+}-independent effects on both inactivation and facilitation of calcium channels (Burgoyne et al. 2004; Few et al. 2005). It would be interesting to test to what extent these differential effects could explain why following a long tetanus calcium currents inactivate during presynaptic whole-cell recordings, leading to PTD (Forsythe et al. 1998), whereas the opposite appears to be true when synapses are intact.
The observed increase in calcium influx most likely played a significant role in the increase in release probability following the tetanus. A third power relation between volume-averaged calcium signals and release has been observed in previous experiments at the calyx of Held synapse (Borst and Sakmann 1999; Wu et al. 1999) and combined with the pool size increase of around 30% that we observed in our earlier experiments following PTP induction (Habets and Borst 2005), this would be sufficient to account for most if not all of the PTP (Figure 2F). This conclusion depends on the assumption that even if the time course of the calcium influx would change, the third power relation between influx and release would remain valid. Although this may be true for the changes in the time course of calcium influx due to action potential changes during high-frequency trains (Borst and Sakmann 1999), both lower (Bollmann and Sakmann 2005) and higher (Fedchyshyn and Wang 2005) values for this power relation have also been observed when the time course of the calcium transients were changed. Therefore, our experiments cannot exclude that local buffer saturation or changes downstream of Ca\(^{2+}\) may provide additional contributions, as suggested by other experiments in the same preparation (Awatramani et al. 2005; Felmy et al. 2003; Lou et al. 2005).

In earlier experiments we observed that the potentiation of evoked release decayed similarly to residual calcium, whereas increases in the frequency of spontaneous release decayed more rapidly (Habets and Borst 2005). An attractive feature of the possibility that the increased calcium influx was due to the interaction of a high-affinity calcium binding protein such as NCS-1 with the presynaptic calcium channels is that it provides a simple explanation for this observation, since a regulation of calcium influx by residual calcium will preferentially affect evoked release over
spontaneous release. The more rapid decay of spontaneous release could be due to supralinear effects of a direct activation of the calcium sensor for release by residual calcium (Habets and Borst 2005).
ACKNOWLEDGEMENTS

We thank Kees Donkersloot for help with setting up the photomultiplier measurements.

GRANTS

This work was supported by a Neuro-Bsik grant.

DISCLOSURES

The authors declare to have no conflict of interest.
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FIGURE LEGENDS

Figure 1.
Fluorescent calcium transients at the calyx of Held synapse.
A: Nomarski (left) and fluorescent CCD image (right) of a calyx of Held filled with OGB-5N. B: Top traces: presynaptic OGB-5N fluorescence transients during an action potential, recorded with a photomultiplier tube. Bottom traces: excitatory postsynaptic currents. The right panel shows the same traces as the left panel at higher time resolution to emphasize the relation between the prespike and the presynaptic calcium transient. Responses are the average of 100 traces. Stimulation artifacts have been truncated. Broken line in top traces is the response predicted from the single compartment model scaled to the same peak amplitude. In the simulations, dye concentration was 50 µM, for the other parameters see Methods. C: Same as B except terminal was pre-loaded with Fluo-4. D: Effect of off-rate of the endogenous buffer on fluorescence transients. Lowering the off-rate of the endogenous buffer in the simulations from the control value of 16000 s\(^{-1}\) (broken line) to 10000 s\(^{-1}\) (continuous line), while keeping the on-rate at 5·10\(^8\) per Msec and the endogenous binding ratio at about 40 results in a clear overshoot in the volume-averaged [Ca\(^{2+}\)] (top traces), which is reported as a rapid component in the decay of the simulated OGB-5N transient (lower traces).

Figure 2.
Relation between fluorescence transients and postsynaptic currents during PTP at the calyx of Held synapse.
A: EPSCs (bottom traces; average of 10) before (black) and after (gray) tetanization and accompanying fluorescence transients (top traces) of a terminal pre-loaded with 200 µM Fluo-4. The fluorescence level before the action potential was subtracted to illustrate the difference in amplitude before and after PTP induction. Fluorescence transients are shown as $\Delta F_{\text{AP}}/F_0 *100\%$. B: Responses from a different synapse as in A, which showed a clear increase in delay of both EPSCs and fluorescence transients during PTP. C: Same transients as in B, except scaled to the same peak amplitude and shifted in time until rising phase of EPSCs matched. D: Amplitudes of fluorescence transients evoked by an action potential relative to the basal fluorescence before the tetanus ($\Delta F_{\text{AP}}/F_0$; top traces), basal fluorescence ($F$; middle traces) and amplitude of EPSCs (lower traces) before and after a 5 min, 20 Hz tetanus. Time points are given relative to the end of the tetanus. E: EPSC amplitude plotted against the calcium transient amplitude $\Delta F_{\text{AP}}/F_0$. Solid line is the fit with a power law $\text{EPSC}=A*(\Delta F_{\text{AP}}/F_0)^m$, where the scaling variable $A$ was -3, and $m$ was 2.6. Data in A, D, E are from the same experiment. F: Relation between EPSC amplitude following a tetanus and the amplitude of Fluo-4 transients. Each point represents a different experiment. The solid line is given by the function $\text{EPSC}_{\text{after}}/\text{EPSC}_{\text{before}} =1.3*(\Delta F_{\text{after}}/\Delta F_{\text{before}})^3$, where the factor 1.3 represents the relative pool size following PTP induction and the factor 3 the power relation between calcium signals and EPSC sizes.

Figure 3.
Calibration of fluorescence transients.
A: Example traces of a terminal which was filled with OGB-5N (200 µM) and was voltage clamped with ten action potential waveforms (APW) in the presence of blockers of Na⁺ and K⁺ channels. Top trace shows the calcium currents, bottom trace shows the fluorescence responses (average of 10 responses) of the terminal. Inset shows the first (black) and the last (gray) fluorescence transient overlaid, enlarged to illustrate the difference in time course. B: Same as A, except the terminal contained Fluo-4 (100 µM). C: Same as in A and B, except the terminal contained both OGB-5N (200 µM, middle traces) and fura-2 (50 µM; lower traces). D: Calcium influx per APW (top), fluorescence changes (middle) and the ratio between the two (lower panel) during the train, for terminals filled with OGB-5N (filled circles; 200 µM; n=3), Fluo-4 (open triangles; 100 µM; n=3) or both OGB-5N (200 µM) and fura-2 (open squares; 50 µM; n=4). Responses are given relative to the response to the first APW.
Figure 4.

Simulations of fluorescence transients.

Calcium currents shown in Figure 3 were taken as inputs for calculating the fluorescence transients in a single-compartment model of the calyx of Held. Parameters are given in Methods. Terminal contained OGB-5N (200 µM; A), Fluo-4 (100 µM; B) or a combination of OGB-5N (200 µM) and fura-2 (50 µM; C). Responses are given as percentage of total dye concentration. Insets show overlay of fluorescence transients evoked by the first (black) and the last APW (gray), enlarged to emphasize the difference in time course. D: Ratio between calcium influx and amplitude of the fluorescence transient, relative to the first APW. Black circles, OGB-5N; Open triangles, Fluo-4; Open squares, OGB-5N plus fura-2.