Post-tetanic potentiation in the rat calyx of Held synapse

Ron L. P. Habets and J. Gerard G. Borst

Department of Neuroscience, Erasmus MC, University Medical Center Rotterdam, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands

We studied synaptic plasticity in the calyx of Held synapse, an axosomatic synapse in the auditory brainstem, by making whole-cell patch clamp recordings of the principal cells innervated by the calyces in a slice preparation of 7- to 10-day-old rats. A 5 min 20 Hz stimulus train increased the amplitude of excitatory postsynaptic currents (EPSCs) on average more than twofold. The amplitude of the synaptic currents took several minutes to return to control values. The post-tetanic potentiation (PTP) was accompanied by a clear increase in the frequency, but not the amplitude, of spontaneous EPSCs, which returned to baseline more rapidly than the potentiation of evoked release. The size of the readily releasable pool of vesicles was increased by about 30%. In experiments in which presynaptic measurements of the intracellular calcium concentration were combined with postsynaptic voltage clamp recordings, PTP was accompanied by an increase in the presynaptic calcium concentration to about 210 nM. The decay of the PTP matched the decay of this increase. When the decay of the calcium transient was shortened by dialysing the terminal with EGTA, the PTP decay sped up in parallel. Our experiments suggest that PTP at the calyx of Held synapse is due to a long-lasting increase in the presynaptic calcium concentration following a tetanus, which results in an increase in the release probability of the vesicles of the readily releasable pool. Although part of the PTP can be explained by a direct activation of the calcium sensor for phasic release, other mechanisms are likely to contribute as well.

(Received 12 November 2004; accepted after revision 2 February 2005; first published online 3 February 2005)
Corresponding author J. G. G. Borst: Department of Neuroscience, Erasmus MC, University Medical Center Rotterdam, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands. Email: g.borst@erasmusmc.nl

The calyx of Held is a large glutamatergic nerve terminal that synapses onto glycinergic principal cells in the medial nucleus of the trapezoid body (MNTB). The MNTB acts as a sign-inverting relay in the process of localizing sound (Grothe, 2003). The view of a synapse that faithfully transmits the presynaptic action potentials is at odds with the multitude of different forms of synaptic plasticity that have been observed in a slice preparation of the MNTB (von Gersdorff & Borst, 2002). During brief stimulus trains, the responses generally depress. If the release probability is lowered, a short-term facilitation of responses is uncovered (Borst *et al.* 1995). It is not yet known whether this synapse displays post-tetanic potentiation (PTP) following longer stimulus trains.

PTP is a form of synaptic enhancement with a duration in the order of minutes (Fisher *et al.* 1997; Zucker & Regehr, 2002). In several preparations PTP is accompanied by a long-lasting increase in the presynaptic calcium concentration, which decays with a similar time course to the PTP. We will refer to this long-lasting increase in calcium concentration as 'residual calcium', a term coined for the calcium ions that linger in the terminal after an action potential, which are essential for short-term facilitation (Katz & Miledi, 1968). However, in contrast to the increase in presynaptic calcium concentration involved in facilitation, part of the sustained calcium transient following a tetanus may be due to exchange of sodium ions that accumulate in the presynaptic cytoplasm during the tetanus for calcium ions (Lev-Tov & Rahamimoff, 1980). The sustained calcium increase is thought to be insufficient to directly activate the calcium sensor for phasic release, both in the case of short-term facilitation and of PTP (Zucker & Regehr, 2002). However, a direct test of the involvement of the phasic calcium sensor in short-term facilitation in the calyx of Held synapse showed that it is responsible for up to 30% of the increase in transmitter release (Felmy *et al.* 2003). In the case of PTP, several alternative mechanisms have been proposed to contribute to the increase in transmitter release, including an increase in calcium influx during an action potential, saturation of an endogenous calcium buffer, the presence of a separate, high-affinity calcium sensor, an increase in the number of releasable vesicles, or the modification of their release probability through an effect of second messengers such as protein kinase C (PKC) or protein kinase A (PKA).

The calyx of Held synapse has distinct advantages for studying the mechanisms of short-term plasticity (von Gersdorff & Borst, 2002). Presynaptic calcium dynamics

 DOI: 10.1113/jphysiol.2004.079160

have been well characterized (Meinrenken *et al.* 2003). It is possible to discriminate between changes in release probability and changes in the readily releasable pool (Schneggenburger *et al.* 2002). In this paper, we show that PTP can be induced at the calyx of Held synapse. We explore different possible mechanisms, including changes in action potential waveform, a direct activation of the presynaptic calcium sensor, changes in the releasable pool and postsynaptic changes.

A preliminary account of the data has been published in abstract form (Habets *et al.* 2003).

Methods

Preparation of slices

Preparation of slices and electrophysiological measurements were done as previously described (de Lange *et al.* 2003). Animal procedures were in accordance with guidelines provided by the animal committee of the Erasmus MC.

In brief, 7- to 10-day-old Wistar rats were decapitated without prior anaesthesia. The brainstem was dissected and immersed in ice-cold saline containing (mM): 125 NaCl, 2.5 KCl, 3 $MgSO_4$, 0.1 $CaCl_2$, 1.25 NaH_2PO_4, 0.4 ascorbic acid, 3 *myo*-inositol, 2 pyruvic acid, 25 D-glucose, 25 $NaHCO_3$ (Merck); pH 7.4 when bubbled with carbogen (95% O_2, 5% CO_2); osmolarity 320 mosmol l^{-1}. Transverse slices of 200 μm thickness were cut with a vibratome (Vibratome, St Louis, MO, USA). Slices were transferred to a holding chamber containing normal Ringer solution, which had the same composition as the solution that was used for slicing, except that the concentrations of $CaCl_2$ and $MgSO_4$ were 2 and 1 mM, respectively. Slices were incubated for 30 min at 37°C. Thereafter, they were kept at room temperature until they were used.

Electrophysiological recordings

Slices were transferred to a recording chamber, which was continuously (2 ml min^{-1}) perfused with normal Ringer solution. In some experiments kynurenic acid (2 mM; Tocris, Bristol, UK) was added, to reduce postsynaptic receptor saturation and desensitization. Neurones 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 (0.1 ms, 0.03–0.5 mA) in the midline by a bipolar electrode (Frederic Hear & Co, Bowdoinham, ME, USA). 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, USA). Pipette solutions contained (mM): 125 potassium gluconate, 20 KCl, 10 Na_2-phosphocreatine, 4 MgATP, 0.3 Na_2GTP, 10 Hepes (Sigma) and 0.05 fura-2 (Molecular Probes, Eugene, OR, USA) or 0.5 EGTA for pre- or postsynaptic recordings, respectively. The 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 (2 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 (Wavemetrics, Lake Oswego, OR, USA).

Imaging

Terminals were prefilled with fura-2 for 10 min via the patch pipette. Only cells in which a gigaohm outside-out patch formed after retraction were selected for analysis. The tissue was illuminated through a 40× objective (NA 0.8, Olympus, Tokyo, Japan) by a monochromator (Polychrome IV; 8 nm bandwidth, TILL Photonics, Martinsried, Germany). Emission light was filtered through a 525/80 bandpass filter and detected with a cooled CCD camera (Sensicam, PCO, Kelheim, Germany). Every 30 s, a set of two images was taken at 360 nm (isosbestic) and at 380 nm (calcium-sensitive wavelength). Images were integrated for 100 ms and binned 4 × 4 on the CCD chip.

Calcium concentrations were calculated using a standard equation for ratiometric dyes (Grynkiewicz *et al.* 1985):

$$[Ca^{2+}] = K_{eff}\frac{(R - R_{min})}{(R_{max} - R)}, \quad (1)$$

where R is the background-corrected fluorescence ratio F_{360}/F_{380}; R_{max} (4.64 ± 0.98, $n = 3$) and R_{min} (0.74 ± 0.02, $n = 3$) are the fluorescence ratios in terminals filled with pipette solution plus 1 mM $CaCl_2$ or 10 mM EGTA, respectively, and $K_{eff} = K_d\,(R_{max}/R_{min})$. For the dissociation constant (K_d) of fura-2, a value of 273 nM was assumed (Helmchen *et al.* 1997).

Data analysis

The average EPSC amplitude at a stimulation frequency of 0.1 Hz was taken as baseline. The amount of PTP was calculated as the percentage increase of the average amplitude of the first three EPSCs after tetanic stimulation relative to the average amplitude of the last three EPSCs before the tetanus. The readily releasable pool (RRP) size was estimated by summing the EPSC amplitudes evoked by a 100 or 200 Hz train of action potentials, after

subtraction of the steady state component (Elmqvist & Quastel, 1965; Schneggenburger *et al.* 1999). An estimate of the release probability (P_r) was obtained by dividing the EPSC amplitude by the RRP size. The decay ($A(t)$) of the increases in release of calcium was fitted with a single exponential function with time constant τ:

$$A(t) = A_0 e^{(-t/\tau)} + B, \quad (2)$$

where A_0 is the increase in amplitude directly after the tetanus ($t = 0$) and B is the average amplitude during the baseline period.

Spontaneous release events were identified using Clampfit 9.0 (Axon Instruments) by a template made of averaged, manually selected, spontaneous EPSCs.

Data are given as mean ± standard error of the mean (S.E.M.). Statistical comparisons were done using Student's *t* test.

Theory

Relation between the time course of residual calcium and PTP. In this section we will explore theoretically whether changes in the intracellular calcium concentration, the affinity of the calcium sensor for calcium or the power relation between calcium and transmitter release may differentially affect the time course of the decay of the potentiation of spontaneous and of action potential-evoked release.

Phasic transmitter release depends strongly on the local calcium transient experienced by the releasable vesicles during an action potential. For low release probabilities, it has been observed at many synapses that release is proportional to the intracellular calcium concentration raised to a power m of about 4 (Augustine, 2001). However, for increasing calcium concentration, this release probability will eventually reach a maximum, at which point an action potential releases all vesicles of the readily releasable pool. As a simple approximation, we therefore assume that the release probability of the vesicles in the readily releasable pool during an action potential (P_r) is described by a Hill equation (Reid *et al.* 1998):

$$P_r = 1/(1 + F^m), \quad (3)$$

and $F = K_d/[Ca^{2+}]$. K_d is the $[Ca^{2+}]$ at which half-maximal activation of the calcium sensor occurs.

Although there are more realistic schemes available for the binding of Ca^{2+} to the calcium sensor, this equation does provide an excellent fit of Fig. 2*D* in Meinrenken *et al.* (2003; results not shown). From this equation it is apparent that a potentiation of evoked release can be due to a change in m, K_d and/or in $[Ca^{2+}]$.

We define β as the fractional change in F after a tetanus:

$$F = F_c - \beta F_c, \quad (4)$$

where F_c is the value of F before the tetanus.

The release probability will then be:

$$P_r = 1/\left(1 + (F_c(1 - \beta))^m\right). \quad (5)$$

As long as $\beta \ll 1$, the first Taylor polynomial, $f(1 - \beta) = f(1) - \beta f'(1)$:

$$P_r \approx 1/\left(1 + (F_c)^m\right) + \beta m (F_c)^m/\left(1 + (F_c)^m\right)^2 \quad (6)$$

represents a good approximation of this function. Therefore, for constant m, as long as changes in F are small, they can be well approximated by a linear function of the relative changes in F. If these changes are linearly dependent on residual calcium, the change in release probability of action-potential-driven release after the tetanus can also be approximated by a linear function of residual calcium. For most terminals, m is around 4 and P_r ranges between 0.05 and 0.5 (Augustine, 2001; Zucker & Regehr, 2002). This means that F ranges between 1 and 2. Therefore, a 5% increase or decrease in F (due to a change in K_d or [Ca]) will lead to a 10–20% change in P_r. The linear approach is accurate within 10% for F between 1 and 2, as long as the changes in F are less than 10%.

Analogously, one can define γ as the fractional change in m after the tetanus. In that case:

$$P_r = 1/\left(1 + F^{m_c(1-\gamma)}\right), \quad (7)$$

where m_c is the value of m before the tetanus. For $\gamma \ll 1$, the first Taylor polynomial

$$P_r \approx 1/(1 + F^{m_c}) + \gamma m F^{m_c} \ln(F)/(1 + F^{m_c})^2 \quad (8)$$

again proves to be a good approximation, with small changes in m leading to $\ln(F)$ times larger changes in P_r than the same fractional changes in F. This means that for spontaneous release, the relative increase will be clearly larger than for evoked release, since F is much smaller in the latter case. The linear approach is accurate to within 10% for values of F between 1 and 2, as long as changes in m are less than 15%.

Consequences for decay time course. From eqns (6) and (8) we conclude that as long as the fractional changes in F or in m after the tetanus are small, the potentiation is well approximated by a linear function of β or γ, respectively. What does this mean for the time course of PTP? As an example, we assume that the change in F (or in m) returns to its original value with the same time course as the residual calcium. Then, if the decay of residual calcium is well approximated by a single exponential function with time constant τ_{Ca}, the decay of PTP will also show an exponential decay, with time constant τ_{PTP} equalling τ_{Ca}.

A comparison of the effect of changes in K_d and [Ca] after the tetanus on spontaneous and evoked release yields some interesting differences. Assuming that the same sensor is responsible for evoked and for spontaneous

release, it can be seen that small fractional changes in F will lead to approximately m times larger changes in release. Therefore, an (isolated) change in K_d that leads to a 10% change in evoked release will lead to a change in the spontaneous frequency that is also only about 10%. The same is obviously not true for changes in $[Ca^{2+}]$. An increase in the calcium transient that is experienced by a vesicle of the readily releasable pool during an action potential can be due to residual calcium, depletion of calcium buffers that compete with the phasic calcium sensor, or an increased calcium influx. The latter two will not affect spontaneous release. In contrast, changes in evoked release due to a direct effect of residual calcium will lead to a much larger effect on the spontaneous release, since the relative change of the calcium concentration will be much larger for the spontaneous release. What does this mean for the decay of the potentiation of spontaneous release? As long as the residual calcium concentration is much smaller than the K_d of the calcium sensor, F^m in eqn (3) is much larger than 1 and eqn (3) can be approximated by:

$$P_{r,spont} \approx ([Ca^{2+}]/K_d)^m. \quad (9)$$

As long as residual calcium is much larger than basal calcium, the contribution of basal calcium to release and the contribution of calcium-independent spontaneous release can both be neglected. In the absence of changes in K_d, if spontaneous release after the tetanus is proportional to residual calcium raised to the power m, it follows that the decay of the spontaneous release rate to basal values after the tetanus will be proportional to the decay of residual calcium raised to a power m:

$$P_{r,spont}(t) \propto (e^{-t/\tau_{Ca}})^m = e^{-mt/\tau_{Ca}}. \quad (10)$$

Therefore, as long as $m > 1$, spontaneous release is expected to decay more rapidly than evoked release under these conditions.

In conclusion, synaptic potentiation that is caused by a direct effect of residual calcium on the phasic calcium sensor is predicted to result in differential decay of the potentiation of spontaneous and evoked release. Additional, indirect effects may also differentially affect spontaneous and evoked release. A decrease of competing endogenous calcium buffer due to saturation (Neher, 1998), or an increase in calcium influx due to calcium current facilitation will specifically promote evoked release. A change in K_d of the phasic calcium sensor will also affect spontaneous release; however, the effects are predicted to be relatively small.

Results

Post-tetanic potentiation at the calyx of Held

To study plasticity at the calyx of Held synapse, the axons leading to the calyces were stimulated with a 20 Hz tetanus for 5 min. During the tetanus, the EPSCs showed prominent synaptic depression (Fig. 1). However, after the train, the amplitude of the EPSCs was increased by $123 \pm 22\%$ (mean ± S.E.M.; range 25–452%; $n = 23$). In the example shown in Fig. 1*B*, the EPSC amplitude at a holding potential of −80 mV increased from −1.6 nA before the 20 Hz stimulation to −4.7 nA after the tetanus. The increase in EPSC size decayed back to baseline over a time course of minutes, as shown in detail below, and was therefore classified as post-tetanic potentiation (Fisher *et al.* 1997; Zucker & Regehr, 2002).

The presynaptic action potential

A change in the shape of the presynaptic action potential will result in a change in calcium influx. During high frequency trains, an increase of the action potential width will broaden calcium influx at this synapse (Borst & Sakmann, 1999). Since EPSC size critically depends on calcium influx, we measured the action potential during and after tetanic stimulation. However, during presynaptic whole-cell current clamp recordings, the EPSCs suffered from use-dependent rundown (data not shown). The presynaptic action potential was therefore monitored either in cell-attached recordings (Fig. 1*A* and *C*) or as a prespike (Forsythe, 1994) in postsynaptic recordings (Fig. 1*B* and *D*). During the tetanus the prespike amplitude (Fig. 1*B* and *D*, middle) was reduced, often disappearing into the noise. Only half of the cells fired action potentials throughout the 5 min 20 Hz tetanus. In the other half, presynaptic action potential failures were apparent. Action potential failures were more pronounced in 7- and 8-day-old rats than in 9- or 10-day-old rats. However, there was no clear correlation between the ability of the terminal to follow the stimulus and the amount of PTP. One minute after the tetanus, the difference between the negative and the positive peak of the prespike was significantly decreased to $76 \pm 5\%$ of control ($P < 0.01$; $n = 19$). At the same time, the time between the negative and the positive peak of the prespike increased by $25 \pm 11\ \mu s$ ($P < 0.05$). In whole-cell recordings, changes in action potential shape were small (results not shown).

We conclude that the PTP was accompanied by a change in the presynaptic action potential shape, which is expected to result in a change in calcium influx. The relative change in prespike amplitude was not correlated with differences in the amount of PTP between experiments. After the tetanus, the interval between the stimulation artifact and the prespike increased by at least 0.1 ms in 15 of 19 experiments (Fig. 1*B*). Following the tetanus, the average increase was 0.44 ± 0.09 ms ($P < 0.01$; $n = 19$). The interval between the peak of the presynaptic cell-attached recording and the peak of the EPSC also increased significantly.

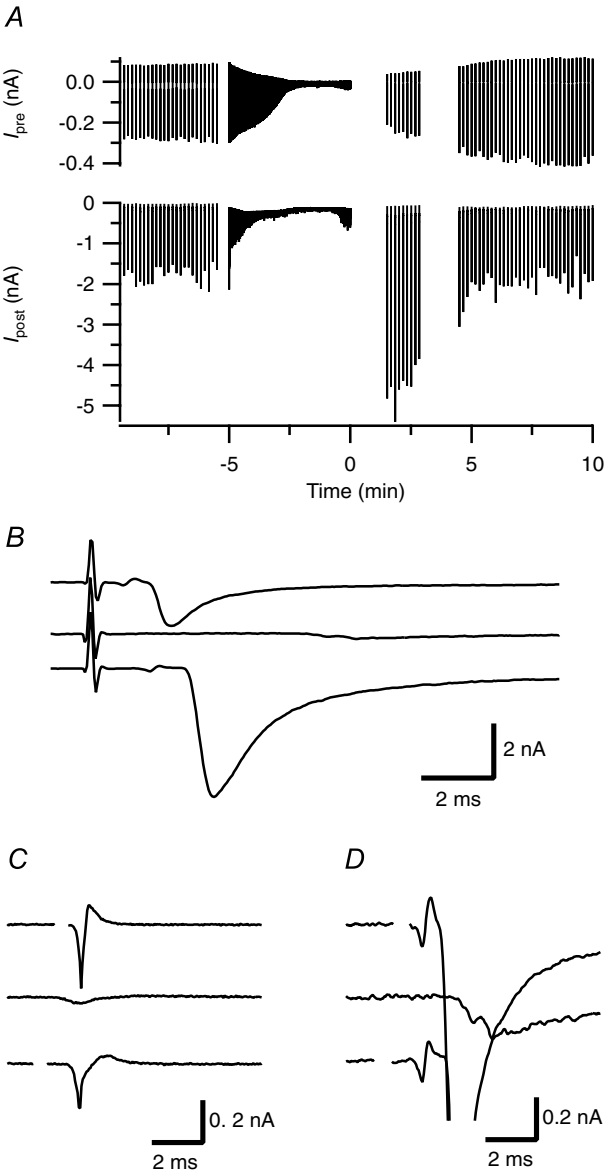

Figure 1. Post-tetanic potentiation at the calyx of Held synapse
Cells were stimulated by an electrode placed in the ventral midline of the slice. A baseline period of 0.1 Hz stimulation preceded a 20 Hz tetanus of 5 min. One to two minutes after the tetanus, EPSCs were again evoked at 0.1 Hz. Before and after the tetanus, stimulation was interrupted to measure spontaneous release. *A*: top traces, presynaptic recording in cell-attached configuration; bottom traces, EPSCs simultaneously recorded in postsynaptic whole-cell configuration. *B*, postsynaptic recording. Top trace, last EPSC of the baseline period. Middle trace, response to last stimulus of the 5 min 20 Hz tetanus. Bottom trace, first EPSC, evoked 1 min after the tetanus. *C*, presynaptic traces at the same time points as the signals shown in *B*, shown at high magnification to illustrate the changes in the cell-attached presynaptic action potential. *D*, enlargement of the prespikes preceding the EPSCs shown in *B*. Signals in *C* and *D* were aligned on the negative peak of the recorded presynaptic action potential. In *A, C* and *D*, stimulation artifacts were removed. In the postsynaptic recordings shown in *A*, prespikes were removed as well.

Spontaneous release

To discriminate between pre- and postsynaptic mechanisms for the generation of PTP, we measured the amplitude and frequency of spontaneous release in principal cells before and in the first minute after the tetanus (Fig. 2*A* and *B*). Although changes in amplitude of the spontaneous EPSCs comparable to the results shown in Fig. 2*C* could be found in four out of eight experiments, on average the amplitude of the spontaneous EPSCs was 35.5 ± 1.6 pA before and 37.2 ± 2.5 pA after the tetanus, which was not significantly different (Fig. 2*D*; $P = 0.25$).

In contrast to the lack of changes in the average amplitude of the spontaneous EPSCs, their frequency

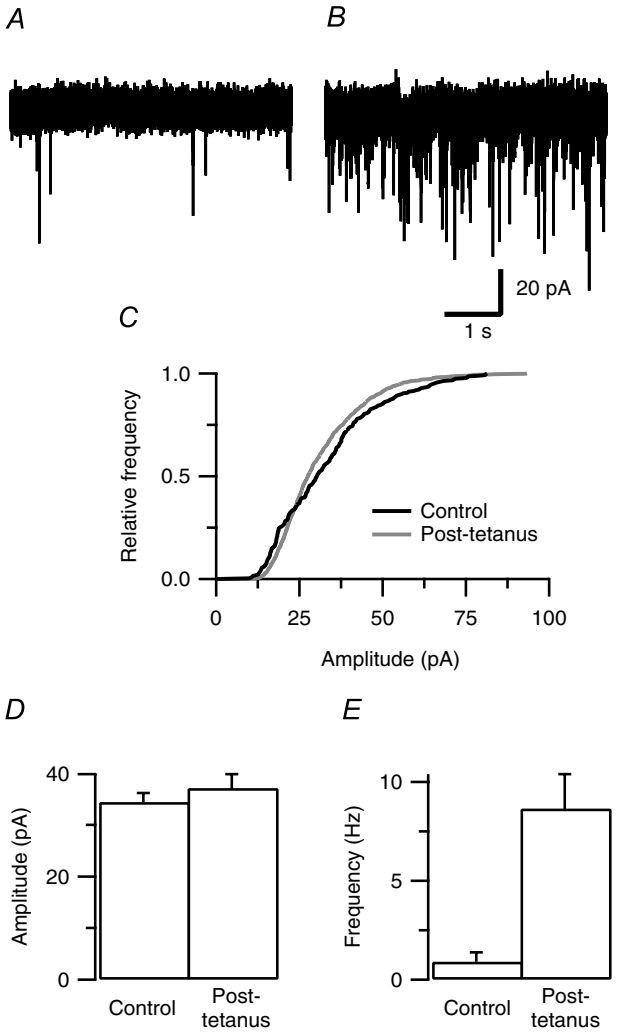

Figure 2. Increase of spontaneous release after a 5 min 20 Hz tetanus
A, postsynaptic voltage clamp recording before the tetanus. *B*, increase in frequency of spontaneous EPSCs directly following the tetanus. *C*, cumulative amplitude distribution of the spontaneous EPSCs of the experiment illustrated in *A* and *B*. *D*, average amplitude of the spontaneous EPSCs (8 cells). *E*, mean of the average frequency of spontaneous EPSCs ($n = 8$).

clearly increased (Fig. 2*B*). On average the frequency increased from 0.91 ± 0.45 Hz before the tetanus to 8.65 ± 1.6 Hz after the tetanus (Fig. 2*E*; $n = 8$).

The increase in frequency of spontaneous events following a tetanus, without a significant effect on their size, indicates that PTP at the calyx of Held synapse – similar to the situation at most other synapses that have been studied (Zucker & Regehr, 2002) – is a presynaptic form of synaptic plasticity.

Pool size and release probability

After establishing that the mechanism underlying PTP had a presynaptic origin we considered two mechanisms: an increase in the number of vesicles immediately available for release (the 'readily releasable pool', RRP) or an increase in the probability of release of the available vesicles (P_r). To distinguish between these two mechanisms, we estimated the RRP and P_r from the amplitudes of EPSCs elicited by a high frequency train before and after tetanization (Schneggenburger *et al.* 1999; Wu & Borst, 1999; Fig. 3*A*. We waited 1 min after the tetanus to ensure that replenishment of the RRP was complete (Wu & Borst, 1999). After the tetanus, a small ($22 \pm 6\%$, $n = 9$) but significant ($P < 0.01$) increase in the RRP estimate was seen (Fig. 3*B*). To minimize possible confounding effects of postsynaptic receptor saturation and desensitization, we repeated these experiments in the presence of the competitive glutamate receptor antagonist kynurenic acid (Wu & Borst, 1999; Neher & Sakaba, 2001; Wong *et al.* 2003). Kynurenic acid (2 mM) reduced the amplitude of the EPSCs to $5.1 \pm 0.8\%$ ($n = 6$) of control. In agreement with earlier results (Wong *et al.* 2003), the pre-tetanus estimate of the RRP size increased and the estimate of P_r decreased in the presence of the drug. Under these conditions, tetanization increased the RRP estimate by $31 \pm 22\%$, which was similar to the increase in the absence of kynurenic acid. However, the largest contribution to PTP was an increase in release probability (Fig. 3*B* and *C*). The relative increase in release probability was even larger in kynurenic acid ($153 \pm 31\%$, $n = 6$) than in Ringer solution ($51 \pm 12\%$, $n = 9$, $P < 0.01$). Probably, the release probabilities in normal Ringer solution were overestimated (Neher & Sakaba, 2001; Wong *et al.* 2003). The larger increase in release probability in kynurenic acid therefore can probably be attributed to a better estimation of the pool size, due to reduction of desensitization of post-synaptic glutamate receptors, rather than a direct effect of kynurenic acid on PTP induction.

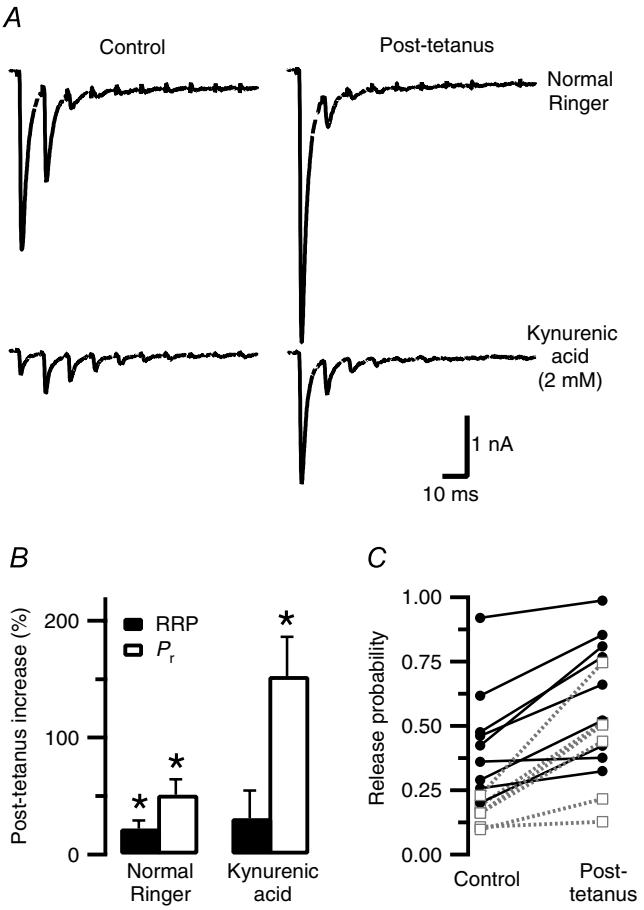

Figure 3. Readily releasable pool
The size of the readily releasable pool (RRP) of vesicles was estimated from a high frequency train. EPSC peak amplitudes were summed and corrected for replenishment. Replenishment was estimated from the responses to the last stimuli and was assumed to be constant. Release probability (P_r) was calculated by dividing the first EPSC amplitude by the RRP estimate. *A*: top, EPSCs in response to 100 Hz stimulus trains before (Control) and after (Post-tetanus) a 20 Hz tetanus of 5 min; bottom: same stimulation in the presence of 2 mM kynurenic acid. *B*, average increase in the RRP and P_r in normal Ringer solution ($n = 9$) and in kynurenic acid ($n = 6$). *C*, release probability of the individual experiments. Black lines are the data points measured in normal Ringer solution. Grey dotted lines were measured in the presence of kynurenic acid.

Decay of the PTP

In a minority (5 of 19) of the experiments in which decay of the potentiation of evoked EPSC amplitudes after the tetanus was monitored, a fast and a slow component could be discerned. The fast component had a time constant of less than 1 min and may be an augmentation phase. These experiments showed particularly large increases in EPSC amplitude after the tetanus ($> 116\%$). In the other experiments, the fast component was less apparent and the decay could be approximated by a single exponential function with a time constant of 9 ± 2 min ($n = 14$).

After the tetanus, the frequency of spontaneous release decayed much faster than the amplitude of the evoked release. In four cells, evoked and spontaneous release were measured in consecutive trains within the same cell. In each case the decay time constant of spontaneous release was smaller than for evoked release. To compare

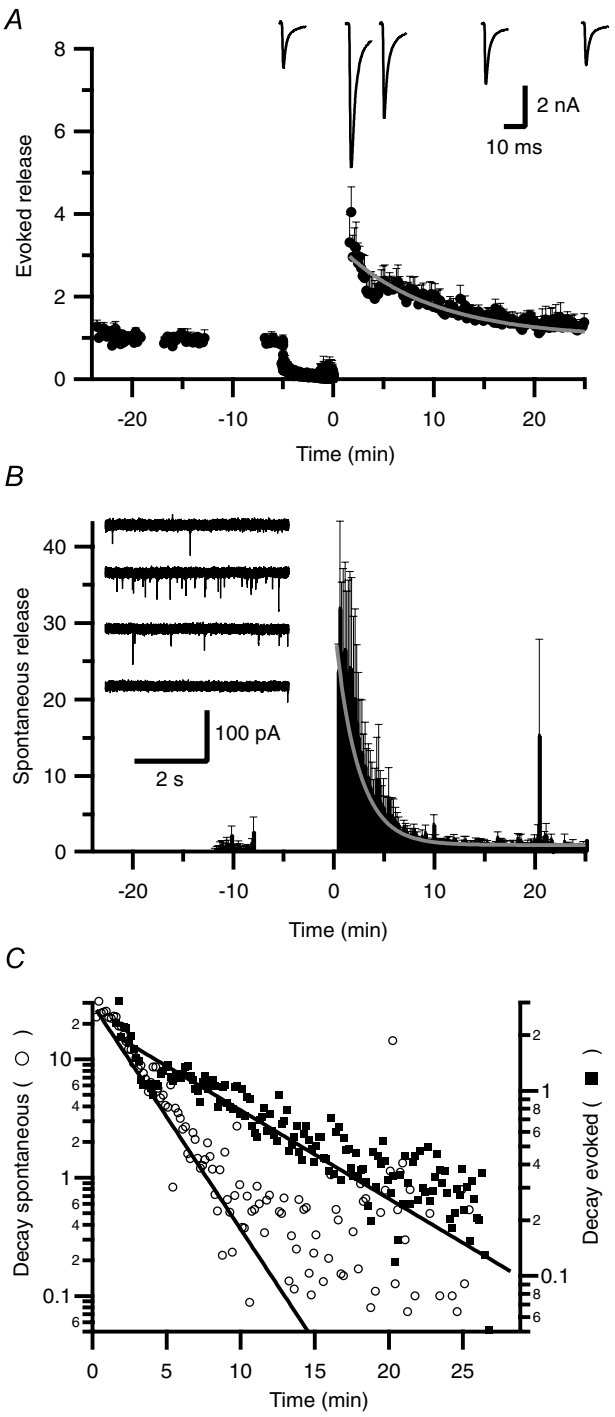

Figure 4. Decay of post-tetanic potentiation
A, the evoked EPSC amplitudes of four cells were normalized to baseline and the average was plotted *versus* time after the tetanus ($n = 4$ + S.E.M.). A single exponential function back to baseline was fitted through the data (grey line). Time constant was 9.1 min. Example traces can be seen in the inset, from left to right: last EPSC before the tetanus and 1.4, 5, 15 and 25 min after the tetanus. *B*, in the same cells as shown in *A*, a second tetanus was given, but now only the spontaneous release was monitored. Spontaneous release was binned (bin size 10 s), normalized to baseline, and fitted with a single exponential function. Time constant was 2.3 min (grey line). Example traces, before the tetanus and 1.4, 5 and 15 min after the tetanus, can be seen in the inset. *C*, semilogarithmic plot of the decay of spontaneous (○) and evoked (■) release following the tetanus. The single exponential fits in *A* and *B* are shown as continuous lines.

their time courses, in these four experiments both the normalized, averaged amplitude of evoked release and the normalized, average frequency of spontaneous release were fitted with a single exponential function. The time constants were 9.1 min for evoked release and 2.3 min for spontaneous release (Fig. 4*A* and *B*). In Fig. 4*C* the decay phase of the traces shown in Fig. 4*A* and *B* is displayed on semi-logarithmic scales to illustrate the difference in their decay.

Induction characteristics of the tetanus

Since the decay time constant of potentiation in the neuromuscular junction depends on the frequency and duration of the tetanus (Van der Kloot & Molgó, 1994), we investigated whether the induction characteristics were the same for both types of release. We varied the number of stimuli in a 20 Hz tetanus between 100 and 6000 and measured the amount of PTP after the stimulus. Figure 5*A* shows an exceptionally long experiment, in which five different tetani could be presented. The amount of potentiation clearly depended on the number of stimuli in the tetanus. On average, evoked release was already elevated after 500 stimuli and it was close to maximal at 2000 stimuli (Fig. 5*B*). Spontaneous release was probably still far from maximal following the longest stimulus train (Fig. 5*C*). In contrast to PTP in the endplate (Lev-Tov & Rahamimoff, 1980), the decay time constant of evoked release did not depend on the number of stimuli.

Presynaptic [Ca^{2+}] dynamics

In several preparations, it has been shown that PTP depends on residual calcium (Zucker & Regehr, 2002). We therefore compared changes in the presynaptic calcium concentration, measured with fura-2 in preloaded terminals, with the EPSC amplitudes after induction of PTP (Fig. 6). During the tetanus, the high-affinity calcium indicator fura-2 approached saturation rapidly. Therefore, the concentrations during the tetanus reached the micromolar range and could not be accurately measured. After the tetanus, the calcium concentration decayed back to baseline biphasically. The rapid phase was not accurately measured. One to two minutes after the tetanus, the average calcium concentration was increased to 210 ± 60 nM from a resting concentration of 41 ± 5 nM. It subsequently decreased to resting levels with a time constant of 8.5 ± 2.1 min (Fig. 6*B*; $n = 4$). The corresponding EPSC amplitudes showed normal potentiation (Fig. 6*C*). In the same experiments, the time

constant of PTP decay was 5.2 ± 1.0 min. If the EPSC amplitude was plotted against $[Ca^{2+}]$, the average slope of the best line fit was 35 ± 13 pA nM^{-1} ($n = 4$; Fig. 6*D*). A fit with a power law function yielded, on average, an exponent of the power function that was close to 1 (0.72 ± 0.16; $n = 4$; Fig. 6*E*), confirming that the relation between the residual $[Ca^{2+}]$ and PTP was close to linear. Since both the amount of PTP and its decay time constant did not differ significantly from undialysed terminals, we conclude that the calcium measurements did not interfere with PTP induction.

The decay of the presynaptic calcium concentration and of the PTP had a similar time course (Fig. 6*D* and *F*), suggesting that residual calcium plays an important role in the increase of transmitter release. To test this hypothesis, we interfered with the normal presynaptic calcium

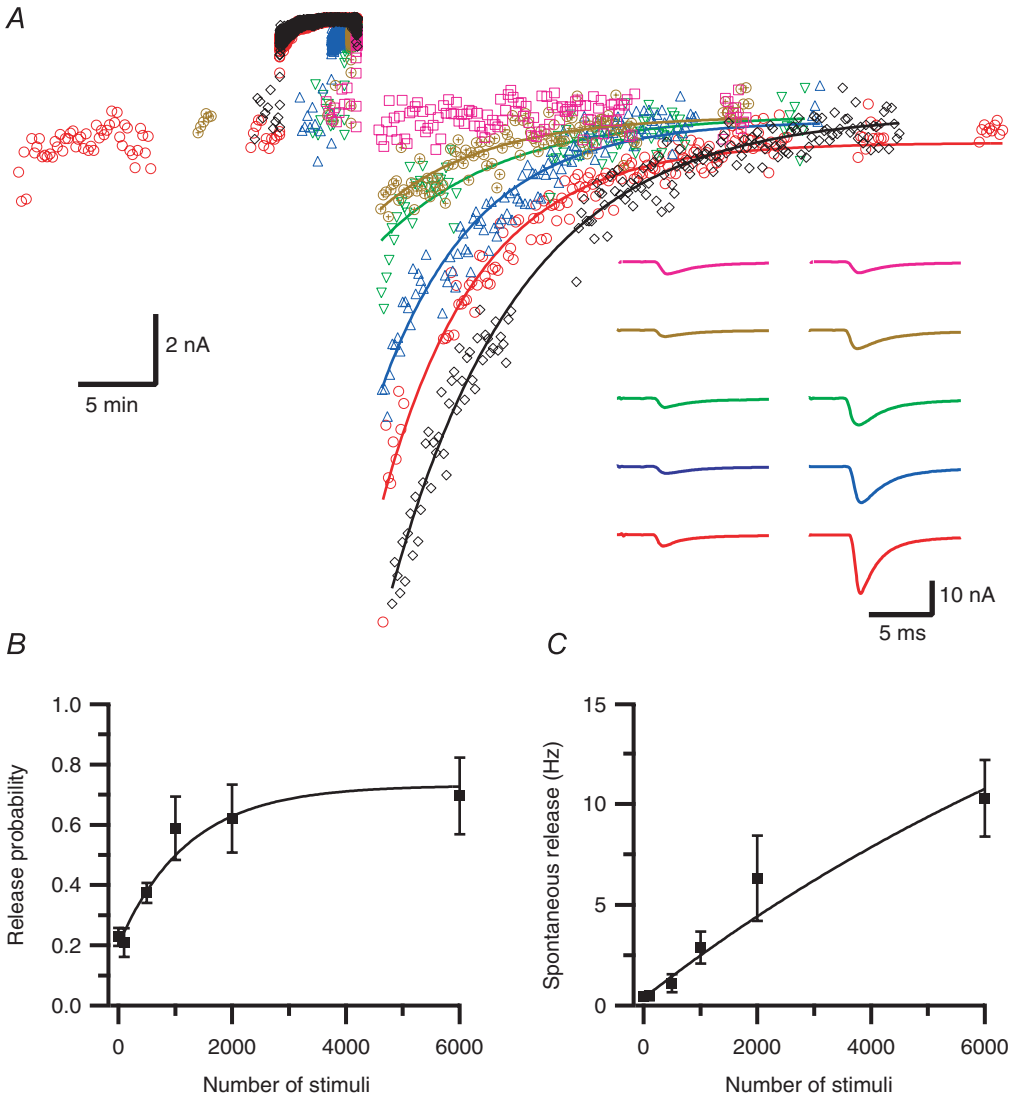

Figure 5. Induction characteristics
The number of stimuli in a 20 Hz tetanus was varied between 100 and 6000. After each tetanus, the cell was stimulated at a frequency of 0.1 Hz until the EPSCs had returned to baseline. *A*, EPSC amplitudes before, during and after the tetanus are plotted. In *A*, five tetani of different duration were applied: 100 (□, pink), 500 (⊕, brown), 1000 (▽, green) and 2000 stimuli (Δ, blue). The experiment was started (○, red) and ended (◇, black) with a train of 6000 stimuli. The data points were fitted to baseline with a single exponential function (continuous lines), with time constants of 500 and 370 s (6000), 370 s (2000), 480 s (1000) and 410 s (500 stimuli). Inset: examples of the EPSCs. Left traces were measured before, right traces after, the tetanus, which contained from top to bottom, 100, 500, 1000, 2000 and 6000 stimuli, respectively. *B*, plot of the average release probability *versus* the number of stimuli in the tetanus. ($n = 6$ for 0 and 6000 stimuli, $n = 3$ for the other stimuli). Release probability was calculated by dividing the EPSC amplitude by the pool size estimate. *C*, plot of the average spontaneous release frequency in the first minute after the tetanus. In *B* and *C*, the data were fitted with an exponential function (continuous lines).

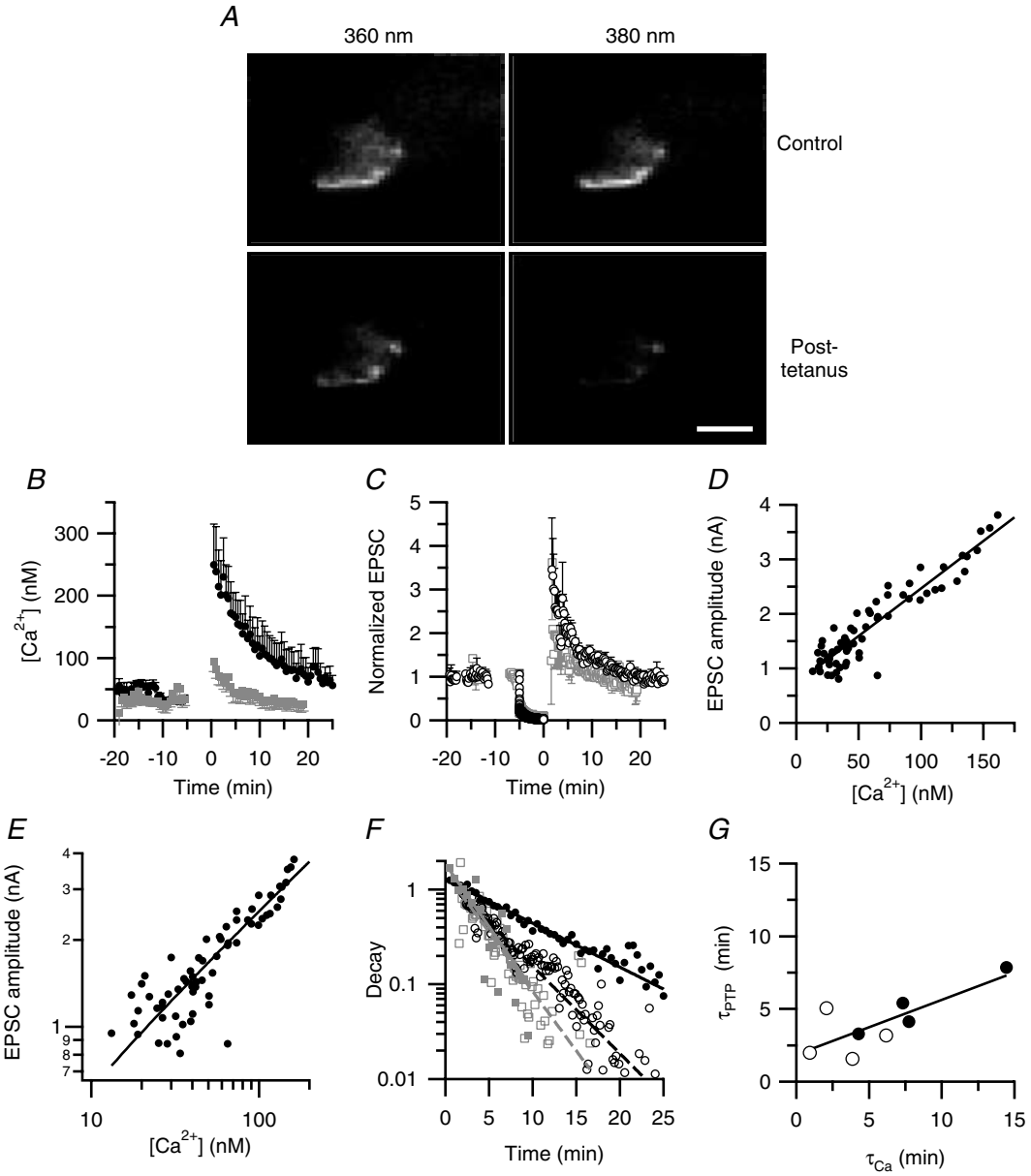

Figure 6. Relation between residual calcium and PTP time course
A, a terminal was filled with 50 μM fura-2. Images were obtained at 360 nm (isosbestic wavelength; left) and 380 nm (calcium-sensitive wavelength; right). At the excitation wavelength of 380 nm, fura-2 fluorescence decreased upon calcium binding. Calibration bar, 10 μm. The last pair of images before (top) and the first after the tetanus (bottom) are shown. *B*, mean calcium concentration for cells filled with fura-2 (black symbols, $n = 4$), or with fura-2 plus EGTA (grey symbols, $n = 5$). During the tetanus ($t = -5$ to 0 min) the high-affinity calcium dye approached saturation in both conditions (data points not shown). *C*, mean normalized EPSC amplitudes for cells filled with fura-2 (black) or fura-2 plus EGTA (grey). *D*, relation between EPSC size and $[Ca^{2+}]$ during the decay phase of PTP for an individual experiment. Continuous line has a slope of 17 pA nM^{-1} ($r = 0.93$). *E*, same data as in *D*, now shown on a double-logarithmic plot. Continuous line is a fit of the relation between EPSC amplitude and residual calcium with a power function: EPSC amplitude $= K_1[Ca^{2+}]^m + K_2$, where K_1 and K_2 are scaling constants. Best fit was obtained for $m = 0.55$. *F*, semi-logarithmic plot of the decay of calcium and EPSC size. Symbols correspond to the symbols used in panels *B* and *C*. Both the $[Ca^{2+}]$ and the EPSC sizes are normalized to their respective average values during the time period when the first three EPSCs after the tetanus were measured (1.5–2 min after the tetanus). Continuous black line is the fit of $[Ca^{2+}]$ in 50 μM fura-2 with a single exponential function with time constant 9.4 min. Continuous grey line is the fit of $[Ca^{2+}]$ in 50 μM fura-2 plus 1 mM EGTA, with time constant 3.3 min. Dashed black line is the fit of EPSC decay in the absence of EGTA, with time constant 4.7 min. Dashed grey line is the fit of EPSC decay in the presence of EGTA, with time constant 3.5 min. *G*, relation between PTP decay and residual calcium. A linear correlation ($r = 0.78$) was found (continuous line; $P < 0.05$).

dynamics by preloading the terminal with the slow calcium buffer EGTA. At a concentration of 1 mM, EGTA decreased the EPSC amplitudes by $27 \pm 13\%$ ($n = 5$) compared to the cell-attached configuration, in agreement with earlier results (Borst & Sakmann, 1996). The resting calcium levels were similar in the presence of EGTA (40 ± 12 nM; $n = 5$). After the tetanus the calcium concentration was significantly lower (70 ± 10 nM) than in the absence of EGTA. The average calcium concentration decayed to baseline much faster in the presence of EGTA compared to cells filled with only fura-2 (Fig. 6*B*; $n = 5$). In two experiments, no PTP was observed. In the other four experiments, potentiation of both evoked (Fig. 6*C*) and spontaneous release (not shown) were still largely intact. Both the decay of PTP and of residual calcium were clearly faster in the presence of EGTA (Fig. 6*F* and *G*). The observation that both sped up in parallel suggests that residual calcium and PTP are causally related. A comparison of all experiments in which calcium concentration and PTP time course were monitored indeed showed that the slow phase of PTP decay depended on residual calcium (Fig. 6*G*).

Since the potentiation of the evoked responses decayed not much more rapidly than the decay of residual calcium, the decay of the potentiation of the spontaneous events must have been more than threefold faster than residual calcium. A possible explanation for the difference in the decay of evoked and spontaneous release potentiation is that the relative increase in calcium concentration is much larger for the spontaneous events, which are normally triggered at the resting calcium concentration, than for the evoked events, which are normally triggered by a calcium concentration in the micromolar range (Bollmann *et al.* 2000; Schneggenburger & Neher, 2000). From the Theory section of Methods we concluded that as long as the fractional increases in the calcium concentration triggering release are small, as expected for the summation of residual calcium with action potential-evoked transients, the decay of potentiation becomes a linear function of residual calcium (eqn (6)), whereas for large fractional increases, as in the case of the difference between basal calcium and residual calcium, the decay is faster, due to the non-linear dependence of transmitter release on calcium (eqn (10)). However, a simple calculation shows that a direct activation of the phasic calcium sensor provides an insufficient explanation for the observed potentiation. The average increase in calcium after the tetanus was only about 170 nM. If phasic release is driven by brief calcium transients with an amplitude of about 8.9 μM (Bollmann *et al.* 2000), an increase of at most 9% ($((8.9 + 0.17)/8.9)^{4.4} \times 100\%$) is predicted. We therefore also considered whether changes in the apparent affinity of the calcium sensor for release would be compatible with the observed differences in the decay of spontaneous and evoked release after the tetanus (see Theory section for details). This simulation reproduced some key features of our experimental findings. A 20% change in the K_d was needed to reproduce the observed amount of PTP. The resulting change in F was too large for the linear approximation (eqn (6)) to be valid, therefore the apparent PTP decay time constant will be somewhat smaller than the decay time constant of residual calcium (Fig. 7*A*), as was also experimentally observed (Fig. 6). The residual calcium leads to a relatively large change in $[Ca^{2+}]$ for spontaneous release. As a result, the observed decay time constant will be clearly smaller than that of the evoked release (Fig. 7*A*), as was also experimentally observed (Fig. 4). We therefore find that an increase of the affinity of the calcium sensor for calcium of about 2 μM per 170 nM residual calcium would be compatible with both the amount of PTP we observed and the differences in decay of spontaneous and evoked release (Fig. 7). However, our experiments do not allow us to discriminate between a change in K_d or m of the calcium sensor and an increase in calcium influx or calcium buffer depletion, which would selectively potentiate evoked release, thereby protracting its decay phase.

Discussion

Cells in the MNTB show post-tetanic potentiation when stimulated with a tetanus of 500 or more action potentials at 20 Hz. The EPSCs returned to baseline in minutes. This enhancement of the evoked responses was accompanied by an increase in the frequency of spontaneous EPSCs. We show that the major mechanism was an increase in the release probability and that the time courses of the decay of PTP and of the residual calcium were similar, but that the spontaneous release decayed about threefold faster.

Physiological relevance of the stimulation protocol

A 25 s tetanus at 20 Hz was sufficient to induce PTP. During *in vivo* recordings, spontaneously recorded activities are almost as high (Kopp-Scheinpflug *et al.* 2003). A comparison is not straightforward. Firstly, we recorded at room temperature, since the rapid, large synaptic currents are difficult to measure at physiological temperatures (Borst *et al.* 1995). In the rabbit superior cervical ganglion, both the time course and the amplitude of PTP were shown not to be highly temperature dependent (Zengel *et al.* 1980). Secondly, we recorded in brain slices from animals a few days before the onset of hearing and it is not known what the electrical activity of this synapse is at this age. *In vivo* recordings before the onset of hearing are therefore needed to assess the physiological relevance of the stimuli used in this paper.

PTP is due to an increase in release probability

PTP was accompanied by an increase in the frequency but not the amplitude of spontaneous EPSCs. Both clearance of glutamate from the synaptic cleft (Otis *et al.* 1996) and recovery from desensitization of glutamate receptors (Joshi *et al.* 2004) most likely are complete within seconds after the tetanus, so they probably did not affect the measurements of the amplitudes of the spontaneous EPSCs during the first minute after the tetanus. The lack of a change in spontaneous EPSC size indicates that PTP was not caused by increases in vesicle filling (Ishikawa *et al.* 2002) or increases in the postsynaptic sensitivity to glutamate. PTP was largely unaltered when synaptic transmission was reduced by about 95% by a glutamate antagonist, arguing against the involvement of a retrograde messenger (Bao *et al.* 1997; Kushmerick *et al.* 2004). We tested two possible mechanisms for the increase in the number of vesicles that were released by an action potential, an increase in the readily releasable pool (RRP) and an increase in the release probability of the vesicles in the readily releasable pool (P_r). Measurements of the size of RRP suggested a modest increase following the tetanus. From these experiments we conclude that PTP in the MNTB is mostly due to an increase in the release probability of the vesicles in the RRP. This increase was quite large: following a 5 min 20 Hz tetanus, P_r was approximately doubled. This increase may even have been underestimated, due to saturation of the postsynaptic receptors.

Role of changes in action potential waveform following a tetanus

The prespike, the capacitatively coupled presynaptic action potential measured in postsynaptic voltage clamp recordings (Forsythe, 1994), was decreased following the tetanus. The amplitude of the prespike, which is a measure of the rate of rise and fall of the presynaptic action

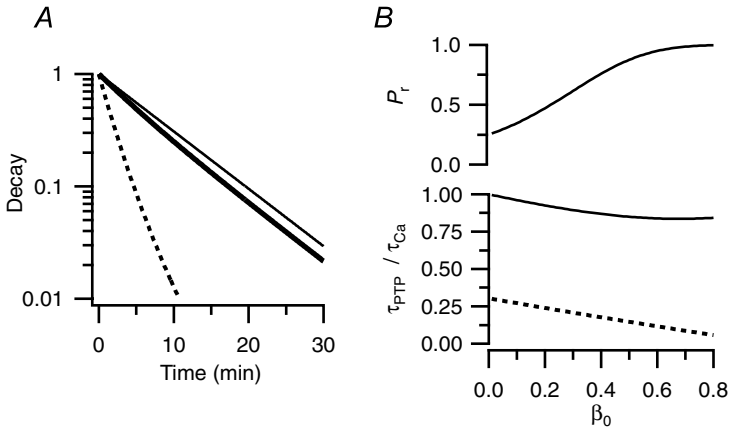

Figure 7. Simulation of PTP decay
A, simulation of the decay of evoked and spontaneous release according to a release model based on the Hill equation (eqn (3); see Theory section in Methods for details). A good fit of the curve in Fig. 2*D* of Meinrenken *et al.* (2003), which describes the probability that a vesicle of the readily releasable pool is released as a function of the peak calcium concentration reached during an action potential, can be obtained with $K_d = 11.4\ \mu$M and $m = 4.4$ (results not shown). For a release probability of 0.25, a concentration of 8.9 μM is then needed (Bollmann *et al.* 2000). Basal calcium concentration was 40 nM. In addition, for the simulation it was assumed that *F*, which is the ratio of K_d and [Ca], depended linearly on the residual calcium. As a result, a decrease of *F* after the tetanus recovered with the same time course as residual calcium. Continuous thin line gives the decay of residual calcium (time constant 8.5 min). Continuous thick line is the decay of evoked release (apparent time constant 7.3 min). Dotted line is the decay of spontaneous release (apparent time constant 2.0 min). Decays were normalized to the respective amplitudes at $t = 0$. To increase P_r twofold, as was experimentally observed during PTP, the change in *F* after the tetanus (β, eqn (4)) would have to be about 0.2 (i.e. *F* would have to be about 20% smaller than control) directly after the tetanus, which translates as an increase in the apparent affinity of the calcium sensor of about 2 μM. *B*: top, release probability of releasable vesicles (P_r) as a function of the fractional change in *F* directly after a tetanus (β_0); bottom, simulation of the relation between the ratio of the apparent time constant for decay of PTP and the time constant for decay of residual calcium (τ_{PTP}/τ_{Ca}) and β_0. Continuous line is computed for evoked release, dotted line has been computed for spontaneous release. Simulations were performed as described in *A*, with residual calcium immediately after the tetanus in each case 170 nM, while K_d was varied to get the appropriate changes in *F*. *F* decayed with the same time course as residual calcium. For large values of β, evoked release saturated and – in contrast to what was experimentally observed – plateaued after the tetanus. To be able to describe the decay of PTP satisfactorily by a single exponential function, the fit was therefore restricted to the period starting 5 min after the tetanus.

potential, decreased by about 25%. This change will affect both amplitude and time course of the calcium influx during an action potential. In an earlier study, we tested the effect of changes in action potential shape, as occur during high-frequency trains. Halving the rate of rise and fall, which leads to a halving of the prespike amplitude, results in a small increase of the calcium influx of about 8% and an increase in release of about 20% (Borst & Sakmann, 1999). We therefore conclude that the changes in presynaptic action potential probably were smaller than in the earlier study and therefore resulted in an even smaller change in the calcium influx.

Effect of residual calcium

The decay of the presynaptic calcium concentration following a tetanus was much slower than after a single action potential, after which clearance takes only a few hundred milliseconds (Helmchen *et al.* 1997). The slower decay following a tetanus could be due to a reverse action of the Na^+–Ca^{2+} exchanger, which may allow calcium ions to enter the cell when pumping out the sodium ions that accumulate in the presynaptic terminal during a tetanus (Lev-Tov & Rahamimoff, 1980; Zhong *et al.* 2001). It could also originate from an intracellular source, such as mitochondria (Billups & Forsythe, 2002), which may release calcium following a tetanus (Tang & Zucker, 1997; Yang *et al.* 2003).

The observation that the time course of the decay of the residual calcium after the tetanus largely matched the decay of the PTP suggests that the two are causally related. Recently, it was observed that the time course of the decay of calcium after a brief stimulus and short-term facilitation also matched at the calyx of Held (Felmy *et al.* 2003). Although residual calcium has generally been implicated in PTP, the precise relation between the two is still largely unclear. Similar to what we observed, at the crayfish neuromuscular junction (Delaney *et al.* 1989) and the chick ciliary ganglion (Brain & Bennett, 1995) the time courses of PTP decay and residual calcium match. Our conclusion that residual calcium and PTP are causally related at the calyx of Held is strengthened by the experiment in which we added the calcium buffer EGTA to the terminal. Under these conditions, the residual calcium decay and the PTP sped up in parallel and the linear correlation between PTP decay and residual calcium decay still held true. Similar results have been obtained in *Aplysia* (Kretz *et al.* 1982). We conclude that it is therefore likely that residual calcium caused the increase in release probability.

Different decay of potentiation of spontaneous and evoked release

The potentiation of spontaneous release decayed about three times more rapidly than the evoked release. Since the latter followed the decay of residual calcium, the spontaneous release must have decayed much more rapidly than residual calcium. This is also observed at the crayfish neuromuscular junction (Zucker & Lara-Estrella, 1983; Delaney *et al.* 1989). In contrast, at the frog neuromuscular junction (Zengel & Magleby, 1981) and the *Aplysia* sensory-motor neurone synapse (Eliot *et al.* 1994), decay of spontaneous and evoked release have a similar time course.

In the Theory section of Methods, we have explored possible causes for the difference in the decay of spontaneous and evoked release. In general, we observed that as long as changes in the calcium concentration, the affinity (K_d) and the power exponent (m) of the calcium sensor are relatively small, the time course of potentiation will largely follow the time course of the changes in these parameters. If any of these parameters shows a clear change ($> 10\%$), this linear approximation is no longer valid. From our data it is clear that residual calcium after the tetanus is much larger than the resting calcium concentration. Firstly, a potentiation of calcium influx or a depletion of calcium buffers would selectively affect evoked release. In addition, the results of simulations (Fig. 7) indicate that changes in the residual calcium differentially affect the time course of evoked and of spontaneous release. The relative change in the calcium signal that triggers spontaneous release will be much larger after the tetanus than the relative change in the calcium signal that triggers evoked release. For small changes, the potentiation is predicted to follow the time course of residual calcium (eqn (6)), for large changes potentiation is predicted to decay more rapidly (eqn (10)), as experimentally observed.

The potentiation of spontaneous release will only decay clearly faster than the potentiation of evoked release if the power law of the calcium dependence has an exponent that is larger than one. This condition provides a possible explanation for the observed difference in the dependence of the decay of spontaneous release in the crayfish and in the frog neuromuscular junction. Asynchronous release at the crayfish neuromuscular junction depends on at least the third power of presynaptic calcium concentration increases (Ravin *et al.* 1997), whereas the calcium dependence of asynchronous release in the frog neuromuscular junction appears to be much more shallow (Angleson & Betz, 2001).

From our simulations we conclude that residual calcium can result in the observed differences in the decay of the potentiation of spontaneous and evoked release at the calyx of Held, both as a direct effect and as an indirect effect. The possible indirect effects include calcium buffer depletion (Felmy *et al.* 2003), a facilitation of calyceal calcium channels (Borst & Sakmann, 1998; Cuttle *et al.* 1998) or an activation of second messengers, which may change the K_d of the calcium sensor (Hori *et al.* 1999; Sakaba & Neher,

2001; Wu & Wu, 2001; Kaneko & Takahashi, 2004). We will next discuss these different possibilities in more detail.

Direct activation of the calcium sensor

Our results confirm earlier results showing that a moderate, sustained increase in presynaptic calcium concentration is both a necessary and sufficient condition to induce PTP (Zucker & Regehr, 2002). Measurements of the calcium sensitivity of transmitter release at the calyx of Held (Bollmann *et al.* 2000; Schneggenburger & Neher, 2000) suggest that the increases that we observed during the decay phase of the PTP are sufficient to affect evoked or spontaneous release. In the experiments of Bollmann *et al.* (2000), a uniform rise of the calcium concentration to 0.5 μM resulted in an increase in the frequency of small EPSCs that was clearly larger than the increases we observed in the first minute after the tetanus. Although smaller increases than to 0.5 μM were not studied, this suggests that the increases in spontaneous EPSCs that we observed could be due to a direct activation of the calcium sensor that is responsible for phasic release. We emphasize that a direct activation of the calcium sensor due to residual calcium cannot be the major cause of PTP. With a 'typical' calcium concentration of 8.9 μM seen by the vesicles that are released during an action potential (Bollmann *et al.* 2000), a linear summation with the residual calcium of 170 nM will lead to a potentiation of only about 9%, even if it is assumed that the relation between calcium and release is described by a power law with a power m of 4.4. Nevertheless, Felmy *et al.* (2003) showed that submicromolar elevations of the calcium concentration can lead to larger increases in evoked release than the amount of PTP observed in the present study. They suggested that buffer depletion leads to supra-linear addition of residual calcium with the calcium transients during an action potential. Candidates for this calcium buffer are still being investigated (Felmy & Schneggenburger, 2004).

Role of changes in presynaptic calcium currents

An earlier study reported post-tetanic depression (PTD) rather than PTP at the calyx of Held (Forsythe *et al.* 1998). This PTD was due to inactivation of calcium currents. In dual whole-cell recordings, we also observed depression instead of potentiation following a tetanus. The use-dependent rundown of release that we observed in presynaptic whole-cell recordings could be related to washout of a cytoplasmic factor. This rundown precluded a direct measurement of the action potential-driven calcium influx during PTP.

The calyceal calcium currents facilitate calcium-dependently (Borst & Sakmann, 1998; Cuttle *et al.* 1998). They activate more rapidly in the presence of residual calcium. A change in calcium influx during an action potential following the tetanus cannot be solely responsible for PTP at the calyx of Held, since spontaneous release showed a prolonged increase as well. Direct measurements of the calcium influx during an action potential after establishment of PTP are necessary to quantify the contribution of facilitation and inactivation of calcium currents conclusively.

Involvement of second messengers

Calcium could indirectly affect release by activating a second messenger. For example, protein kinase C (PKC) is a good candidate because it is involved in PTP in the hippocampus (Alle *et al.* 2001; Brager *et al.* 2003), because its activity depends on Ca^{2+} and because PKC activation has been shown to increase release probability at this synapse without a large effect on RRP (Hori *et al.* 1999; Wu & Wu, 2001). The presynaptic protein Munc13 could also be involved, largely for the same reasons as PKC (Hori *et al.* 1999). An increase in cAMP will also increase the release probability in the calyx of Held (Sakaba & Neher, 2001; Kaneko & Takahashi, 2004). Since cAMP results at the same time in a substantial increase of the RRP, whereas we observed only a modest increase, this second messenger cannot be exclusively involved in PTP.

If any of these second messengers acts at a late 'maturation' step, thereby decreasing the fraction of 'reluctant' vesicles (Wu & Borst, 1999; Sakaba & Neher, 2003), the apparent calcium sensitivity of these vesicles is expected to increase. Our simulations showed that, apart from a change in calcium influx or a depletion of calcium buffers, a calcium-dependent change in K_d or m of the calcium sensor may also lead to a differential decay of the potentiation of spontaneous and of evoked release, similar to what we observed experimentally. Therefore, apart from pharmacological experiments, experiments in which the calcium sensitivity of release during PTP is measured would aid in the further delineation of the mechanisms that govern PTP at the calyx of Held synapse.

References

Alle H, Jonas P & Geiger JR (2001). PTP and LTP at a hippocampal mossy fiber-interneuron synapse. *Proc Natl Acad Sci U S A* **98**, 14708–14713.

Angleson JK & Betz WJ (2001). Intraterminal Ca^{2+} and spontaneous transmitter release at the frog neuromuscular junction. *J Neurophysiol* **85**, 287–294.

Augustine GJ (2001). How does calcium trigger neurotransmitter release? *Curr Opin Neurobiol* **11**, 320–326.

Bao JX, Kandel ER & Hawkins RD (1997). Involvement of pre- and postsynaptic mechanisms in posttetanic potentiation at *Aplysia* synapses. *Science* **275**, 969–973.

Billups B & Forsythe ID (2002). Presynaptic mitochondrial calcium sequestration influences transmission at mammalian central synapses. *J Neurosci* **22**, 5840–5847.

Bollmann JH, Sakmann B & Borst JGG (2000). Calcium sensitivity of glutamate release in a calyx-type terminal. *Science* **289**, 953–957.

Borst JGG, Helmchen F & Sakmann B (1995). Pre- and postsynaptic whole-cell recordings in the medial nucleus of the trapezoid body of the rat. *J Physiol* **489**, 825–840.

Borst JGG & Sakmann B (1996). Calcium influx and transmitter release in a fast CNS synapse. *Nature* **383**, 431–434.

Borst JGG & Sakmann B (1998). Facilitation of presynaptic calcium currents in the rat brainstem. *J Physiol* **513**, 149–155.

Borst JGG & Sakmann B (1999). Effect of changes in action potential shape on calcium currents and transmitter release in a calyx-type synapse of the rat auditory brainstem. *Philos Trans R Soc Lond B Biol Sci* **354**, 347–355.

Brager DH, Cai X & Thompson SM (2003). Activity-dependent activation of presynaptic protein kinase C mediates post-tetanic potentiation. *Nat Neurosci* **6**, 551–552.

Brain KL & Bennett MR (1995). Calcium in the nerve terminals of chick ciliary ganglia during facilitation, augmentation and potentiation. *J Physiol* **489**, 637–648.

Cuttle MF, Tsujimoto T, Forsythe ID & Takahashi T (1998). Facilitation of the presynaptic calcium current at an auditory synapse in rat brainstem. *J Physiol* **512**, 723–729.

Delaney KR, Zucker RS & Tank DW (1989). Calcium in motor nerve terminals associated with posttetanic potentiation. *J Neuroscience* **9**, 3558–3567.

de Lange RP, de Roos AD & Borst JGG (2003). Two modes of vesicle recycling in the rat calyx of Held. *J Neurosci* **23**, 10164–10173.

Eliot LS, Kandel ER & Hawkins RD (1994). Modulation of spontaneous transmitter release during depression and posttetanic potentiation of *Aplysia* sensory-motor neuron synapses isolated in culture. *J Neurosci* **14**, 3280–3292.

Elmqvist D & Quastel DMJ (1965). A quantitative study of end-plate potentials in isolated human muscle. *J Physiol* **178**, 505–529.

Felmy F, Neher E & Schneggenburger R (2003). Probing the intracellular calcium sensitivity of transmitter release during synaptic facilitation. *Neuron* **37**, 801–811.

Felmy F & Schneggenburger R (2004). Developmental expression of the Ca^{2+}-binding proteins calretinin and parvalbumin at the calyx of Held of rats and mice. *Eur J Neurosci* **20**, 1473–1482.

Fisher SA, Fischer TM & Carew TJ (1997). Multiple overlapping processes underlying short-term synaptic enhancement. *Trends Neurosci* **20**, 170–177.

Forsythe ID (1994). Direct patch recording from identified presynaptic terminals mediating glutamatergic EPSCs in the rat CNS, *in vitro*. *J Physiol* **479**, 381–387.

Forsythe ID, Tsujimoto T, Barnes-Davies M, Cuttle MF & Takahashi T (1998). Inactivation of presynaptic calcium current contributes to synaptic depression at a fast central synapse. *Neuron* **20**, 797–807.

Grothe B (2003). New roles for synaptic inhibition in sound localization. *Nat Rev Neurosci* **4**, 540–550.

Grynkiewicz G, Poenie M & Tsien RY (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260**, 3440–3450.

Habets RLP, Elgersma Y & Borst JGG (2003). Post-tetanic potentiation at the calyx of Held synapse. *Abstr Soc Neurosci* 902.6.

Helmchen F, Borst JGG & Sakmann B (1997). Calcium dynamics associated with a single action potential in a CNS presynaptic terminal. *Biophys J* **72**, 1458–1471.

Hori T, Takai Y & Takahashi T (1999). Presynaptic mechanism for phorbol ester-induced synaptic potentiation. *J Neurosci* **19**, 7262–7267.

Ishikawa T, Sahara Y & Takahashi T (2002). A single packet of transmitter does not saturate postsynaptic glutamate receptors. *Neuron* **34**, 613–621.

Joshi I, Shokralla S, Titis P & Wang LY (2004). The role of AMPA receptor gating in the development of high-fidelity neurotransmission at the calyx of Held synapse. *J Neurosci* **24**, 183–196.

Kaneko M & Takahashi T (2004). Presynaptic mechanism underlying cAMP-dependent synaptic potentiation. *J Neurosci* **24**, 5202–5208.

Katz B & Miledi R (1968). The role of calcium in neuromuscular facilitation. *J Physiol* **195**, 481–492.

Kopp-Scheinpflug C, Lippe WR, Dorrscheidt GJ & Rübsamen R (2003). The medial nucleus of the trapezoid body in the gerbil is more than a relay: comparison of pre- and postsynaptic activity. *J Assoc Res Otolaryngol* **4**, 1–23.

Kretz R, Shapiro E & Kandel ER (1982). Post-tetanic potentiation at an identified synapse in Aplysia is correlated with a Ca^{2+}-activated K^+ current in the presynaptic neuron: evidence for Ca^{2+} accumulation. *Proc Natl Acad Sci U S A* **79**, 5430–5434.

Kushmerick C, Price GD, Taschenberger H, Puente N, Renden R, Wadiche JI, Duvoisin RM, Grandes P & von Gersdorff H (2004). Retroinhibition of presynaptic Ca^{2+} currents by endocannabinoids released via postsynaptic mGluR activation at a calyx synapse. *J Neurosci* **24**, 5955–5965.

Lev-Tov A & Rahamimoff R (1980). A study of tetanic and post-tetanic potentiation of miniature end-plate potentials at the frog neuromuscular junction. *J Physiol* **309**, 247–273.

Meinrenken CJ, Borst JGG & Sakmann B (2003). Local routes revisited: the space and time dependence of the Ca^{2+} signal for phasic transmitter release at the rat calyx of Held. *J Physiol* **547**, 665–689.

Neher E (1998). Usefulness and limitations of linear approximations to the understanding of Ca^{++} signals. *Cell Calcium* **24**, 345–357.

Neher E & Sakaba T (2001). Combining deconvolution and noise analysis for the estimation of transmitter release rates at the calyx of Held. *J Neurosci* **21**, 444–461.

Otis T, Zhang S & Trussell LO (1996). Direct measurement of AMPA receptor desensitization induced by glutamatergic synaptic transmission. *J Neurosci* **16**, 7496–7504.

Ravin R, Spira ME, Parnas H & Parnas I (1997). Simultaneous measurement of intracellular Ca^{2+} and asynchronous transmitter release from the same crayfish bouton. *J Physiol* **501**, 251–262.

Reid CA, Bekkers JM & Clements JD (1998). N- and P/Q-type Ca^{2+} channels mediate transmitter release with a similar cooperativity at rat hippocampal autapses. *J Neurosci* **18**, 2849–2855.

Sakaba T & Neher E (2001). Preferential potentiation of fast-releasing synaptic vesicles by cAMP at the calyx of Held. *Proc Natl Acad Sci U S A* **98**, 331–336.

Sakaba T & Neher E (2003). Involvement of actin polymerization in vesicle recruitment at the calyx of Held synapse. *J Neurosci* **23**, 837–846.

Schneggenburger R, Meyer AC & Neher E (1999). Released fraction and total size of a pool of immediately available transmitter quanta at a calyx synapse. *Neuron* **23**, 399–409.

Schneggenburger R & Neher E (2000). Intracellular calcium dependence of transmitter release rates at a fast central synapse. *Nature* **406**, 889–893.

Schneggenburger R, Sakaba T & Neher E (2002). Vesicle pools and short-term synaptic depression: lessons from a large synapse. *Trends Neurosci* **25**, 206–212.

Tang Y & Zucker RS (1997). Mitochondrial involvement in post-tetanic potentiation of synaptic transmission. *Neuron* **18**, 483–491.

Van der Kloot W & Molgó J (1994). Quantal acetylcholine release at the vertebrate neuromuscular junction. *Physiol Rev* **74**, 899–991.

von Gersdorff H & Borst JGG (2002). Short-term plasticity at the calyx of Held. *Nat Rev Neurosci* **3**, 53–64.

Wong AY, Graham BP, Billups B & Forsythe ID (2003). Distinguishing between presynaptic and postsynaptic mechanisms of short-term depression during action potential trains. *J Neurosci* **23**, 4868–4877.

Wu LG & Borst JGG (1999). The reduced release probability of releasable vesicles during recovery from short-term synaptic depression. *Neuron* **23**, 821–832.

Wu X-S & Wu L-G (2001). Protein kinase C increases the apparent affinity of the release machinery to Ca^{2+} by enhancing the release machinery downstream of the Ca^{2+} sensor. *J Neurosci* **21**, 7928–7936.

Yang F, He XP, Russell J & Lu B (2003). Ca^{2+} influx-independent synaptic potentiation mediated by mitochondrial Na^{+}-Ca^{2+} exchanger and protein kinase C. *J Cell Biol* **163**, 511–523.

Zengel JE & Magleby KL (1981). Changes in miniature endplate potential frequency during repetitive nerve stimulation in the presence of Ca^{2+}, Ba^{2+}, and Sr^{2+} at the frog neuromuscular junction. *J Gen Physiol* **77**, 503–529.

Zengel JE, Magleby KL, Horn JP, McAfee DA & Yarowsky PJ (1980). Facilitation, augmentation, and potentiation of synaptic transmission at the superior cervical ganglion of the rabbit. *J Gen Physiol* **76**, 213–231.

Zhong N, Beaumont V & Zucker RS (2001). Roles for mitochondrial and reverse mode Na^{+}/Ca^{2+} exchange and the plasmalemma Ca^{2+} ATPase in post-tetanic potentiation at crayfish neuromuscular junctions. *J Neurosci* **21**, 9598–9607.

Zucker RS & Lara-Estrella LO (1983). Post-tetanic decay of evoked and spontaneous transmitter release and a residual-calcium model of synaptic facilitation at crayfish neuromuscular junctions. *J Gen Physiol* **81**, 355–372.

Zucker RS & Regehr WG (2002). Short-term synaptic plasticity. *Annu Rev Physiol* **64**, 355–405.

Acknowledgements

We thank Dr A. D. G. de Roos (Amsterdam) for help with pilot experiments on PTP, Dr A. Rodriguez-Contreras (Rotterdam) and Dr M. Postema (Rotterdam) for their helpful comments on an earlier version of this manuscript and the latter also for deriving eqn (6). This study was supported by a Pionier grant of the Netherlands Organization for Scientific Research (NWO) and by a Human Frontier Science Program collaborative grant.