Kinetics of Synaptic Depression and Vesicle Recycling after Tetanic Stimulation of Frog Motor Nerve Terminals

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ABSTRACT We measured the time courses of two key components of the synaptic vesicle cycle during recovery from synaptic depression under different conditions, and used this and other information to create a kinetic model of the vesicle cycle. End plate potential (EPP) amplitudes were used to follow recovery from synaptic depression after different amounts of tetanic stimulation. This provided an estimate of the time course of vesicle mobilization from the reserve pool to the docked (readily releasable) pool. In addition, FM1–43 was used to measure the rate of membrane retrieval after tetanic stimulation, and the amount of membrane transferred to the surface membrane. This provided a measure of the rate of refilling of the reserve pool with recycled vesicles. The time courses of both synaptic depression and endocytosis were slowed by prolonged tetanic stimulation. This behavior could be fitted by a simple model, assuming a first-order kinetics for both vesicle endocytosis and mobilization. The results show that a nearly 20-fold decrease in the rate constant of endocytosis greatly delays refilling of the depleted reserve pool. However, to fully account for the slower recovery of depression, a decrease in the rate constant of vesicle mobilization from the reserve pool of about sixfold is also required.

INTRODUCTION

Repetitive nerve stimulation often leads to a decrease in postsynaptic potentials called synaptic depression. In principle, depression could be due to a decrease in presynaptic transmitter release or a decrease in the sensitivity of postsynaptic receptors to transmitters (or both). At the frog neuromuscular junction, a decrease in transmitter release underlies synaptic depression (Del Castillo and Katz, 1954; Betz, 1970; for reviews see Zucker, 1989; Van der Kloot and Molgó, 1994), and the sensitivity of postsynaptic receptors to transmitters, measured as the size of the miniature end plate potentials (MEPPs), does not change during depression (Del Castillo and Katz, 1954; Zengel and Sosa, 1994). A decrease in transmitter release can be caused by a depletion of the readily releasable pool (for simplicity here we call it docked vesicle pool), or a decrease in the release probability of each docked vesicle (or both). Depression can often be relieved by reducing the level of transmitter release, suggesting a depletion of the docked vesicle pool during depression (Zucker, 1989). A decrease in the release probability of docked vesicles may be caused by a decrease in presynaptic calcium influx evoked by an action potential or a direct modulation of the release machinery downstream of calcium influx. Endogenous ATP and its derivatives, such as adenosine, may mediate these effects (for reviews see Van der Kloot and Molgo, 1994; Wu and Saggau, 1997) and have been shown to mediate depression during low, but not high, frequency stimulation under conditions in which the bath adenosine level was kept low (Zengel and Sosa, 1994). At the frog neuromuscular junction, the depletion hypothesis remains the most widely accepted model of depression induced by high-frequency stimulation.

Neuromuscular synaptic depression induced by a short stimulus train recovers within several seconds, following first-order kinetics (Takeuchi, 1958; Betz, 1970), whereas longer stimulus trains often produce slower recovery rates from depression (Birks and MacIntosh, 1961; Elmqvist and Quastel, 1965; Rosenthal, 1969; Lass et al., 1973). Similar results have been obtained at nerve-nerve synapses, including cultured hippocampal neurons (Liu and Tsien, 1995; Rosenmund and Stevens, 1996) and isolated retinal bipolar cell terminals monitored with the patch-clamp capacitance technique (von Gersdorff and Matthews, 1997). This might suggest a gradual depletion of the reserve vesicle pool from which the docked vesicle pool is replenished. Consistent with this idea, the total amount of transmitter release is decreased during repetitive stimulation when the reserve vesicle pool is reduced at the *Drosophila* neuromuscular junction of the temperature-sensitive endocytosis mutant *Shibire* (Koenig et al. 1989), and at hippocampal synapses of synapsin I knock-out mice (Ryan et al., 1996).

Synaptic vesicles can be divided functionally into at least three pools, two entirely intracellular (the readily releasable or "docked" pool and the much larger "reserve" pool) and one exposed to the extracellular fluid (i.e., during the time after exocytosis but before endocytosis, the "fused" pool). These are illustrated in Fig. 1. However, it is not clear, quantitatively, how recovery from synaptic depression is related to depletion of the reserve vesicle pool, the kinetics of endocytosis from the fused to the reserve vesicle pool, and the kinetics of mobilization from the reserve pool to the docked pool (k_1 and k_2, respectively, in Fig. 1). We addressed these questions by measuring the time course of synaptic depression with intracellular recording techniques and measuring the rate of endocytosis and the degree of

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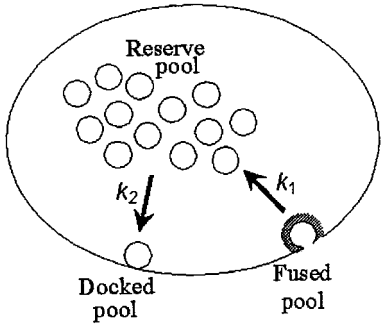

FIGURE 1 Synaptic vesicles are modeled as residing in three pools during recycling after a tetanus: the reserve pool, the docked pool, and the fused pool. Vesicles are mobilized from the reserve to the docked pool with a rate constant k_2, and are endocytosed from the fused pool and recycle into the reserve pool with a rate constant k_1.

depletion of the reserve vesicle pool by FM1–43 imaging techniques. We found that a slower recovery of synaptic depression was accompanied by a slower endocytosis and more significant depletion of the reserve vesicle pool. A model was constructed with first-order kinetics between three pools of vesicles as shown in Fig. 1. In this model, the time course of synaptic depression can be analytically calculated with all of the initial parameters experimentally measured. Such a calculated time course fit well with the observed time course of synaptic depression, suggesting that the dynamics of vesicle recycling determines the speed of replenishment of the docked vesicle pool and thus the time course of synaptic depression. Parts of this work have previously been published (Wu and Betz, 1995, 1996).

MATERIALS AND METHODS

Most methods were generally the same as those described previously (Betz and Bewick, 1993; Wu and Betz, 1996). Frog (*Rana pipiens*) cutaneus pectoris nerve-muscle preparations were dissected and mounted in a Sylgard-lined chamber containing normal frog Ringer's solution (mM: 115 NaCl; 2 KCl; 1.8 $CaCl_2$; 2.4 $NaHCO_3$). The muscle nerve was drawn into a suction electrode for electrical stimulation. For recording of endplate potentials (EPPs), curare (final concentration 1.5 mg/ml) was added to the bath solution to prevent contraction of the muscle. The pipette (20–30 MΩ) for intracellular recording of EPPs was filled with 3 M potassium acetate. EPPs were displayed on a Tektronix oscilloscope and stored and analyzed on a PC running Axobasic. EPPs were sampled every 0.5–50 s.

Fluorescence images of the selected surface terminals were obtained with a Nikon upright epifluorescence microscope equipped with a 40× water immersion (0.75 NA) objective lens, a 100-W Hg lamp, 5–100% neutral density transmission filters, excitation filters (435/10 nm), dichroic mirrors (455 nm), and emission filters (540/100 nm). Images were captured with a Photometrics PXL 200 camera (1–2.5 s exposure, gain 4) and processed with Inovision Isee software and Hannaway software in a Silicon Graphics Indigo 2 computer. All images were background subtracted.

A curve-fitting program using the principle of the least-squares fitting was used to fit single exponential curves and to select best parameters such as t and k_2. Measurements are expressed as mean ± 1 SEM.

FM1–43 imaging

Imaging data are taken from earlier published work (Wu and Betz, 1996). A variation of a protocol developed by Ryan and colleagues (Ryan et al., 1993; Ryan and Smith, 1995) was used to measure the time course of endocytosis (Wu and Betz, 1996). The muscle nerve was first stimulated at 30 Hz for a certain period of time (10, 60, or 300 s). Then, after a variable delay time, FM1–43 (4 μM) was added to the bath solution and left until virtually all endocytosis had stopped (1, 3, or 20 min for 10, 60, and 300 s stimulation, respectively). For each delay time, several nerve-muscle preparations were subjected to the same treatment. In each muscle, four or five of the brightest surface terminals were selected for fluorescence imaging. The outline of each selected terminal was marked, and the summed fluorescence intensity of all pixels inside the outline was calculated. After correction of background fluorescence, the average value of the summed fluorescence intensity from those four or five selected terminals from each preparation was obtained. The dye also passively and faintly stained the nerve membrane without stimulation (Wu and Betz, 1996). Thus the fluorescence intensity from dye loading after stimulation was corrected by passive loading without stimulation (Wu and Betz, 1996).

RESULTS

Prolonged stimulation caused slower recovery from synaptic depression. Tetanic trains of stimuli at 30 Hz and different durations were given to induce synaptic depression. Typical results from one experiment are shown in Fig. 2, *A–C*; graphs of EPP amplitudes before, during, and after 30-Hz stimulation are shown for trains lasting 10, 60, and 300 s (Fig. 2, *A*, *B*, and *C*, respectively). Average recovery rates from depression from all experiments (n = 5–6 for each train duration) are shown in Fig. 2, *D–F*. EPP amplitudes were normalized to those before tetanic stimulation. EPP amplitudes recovered to different levels, depending on the duration of stimulation. After relatively short trains (Fig. 2, *A* and *D*), recovery was incomplete. After long trains (Fig. 2, *C* and *F*), recovery from depression was followed by a phase of potentiation. With intermediate trains (Fig. 2, *B* and *E*), some terminals showed incomplete recovery from depression, and others showed potentiation.

The second phase (potentiation) is likely to arise during or immediately after the tetanic stimulation. For example, with low calcium solution to avoid synaptic depression, posttetanic potentiation develops immediately after stimulation (Zucker, 1989; Wu and Betz, unpublished results). Thus we assume that the recovery of EPPs mainly reflects the time course of synaptic depression, and that the potentiation mechanism does not change.

EPPs in Fig. 2, *D* and *E*, were best fit with a single exponential curve with a time constant of 10.7 and 27.9 s, respectively. For better comparison, the mean EPP of the recovery phase in Fig. 2, *D–F*, was first normalized to the maxium level to which it recovered and then superimposed in Fig. 2 *G*. Clearly, the recovery of EPP amplitudes depends on the duration of stimulation.

Endocytic rate depends on the stimulation duration

To understand how synaptic vesicle recycling may affect the recovery time course of synaptic depression, we used FM1–43 to measure the rate of endocytosis and the amount of vesicle membrane remaining at the plasma membrane and in the reserve pool before and after the tetanic stimu-

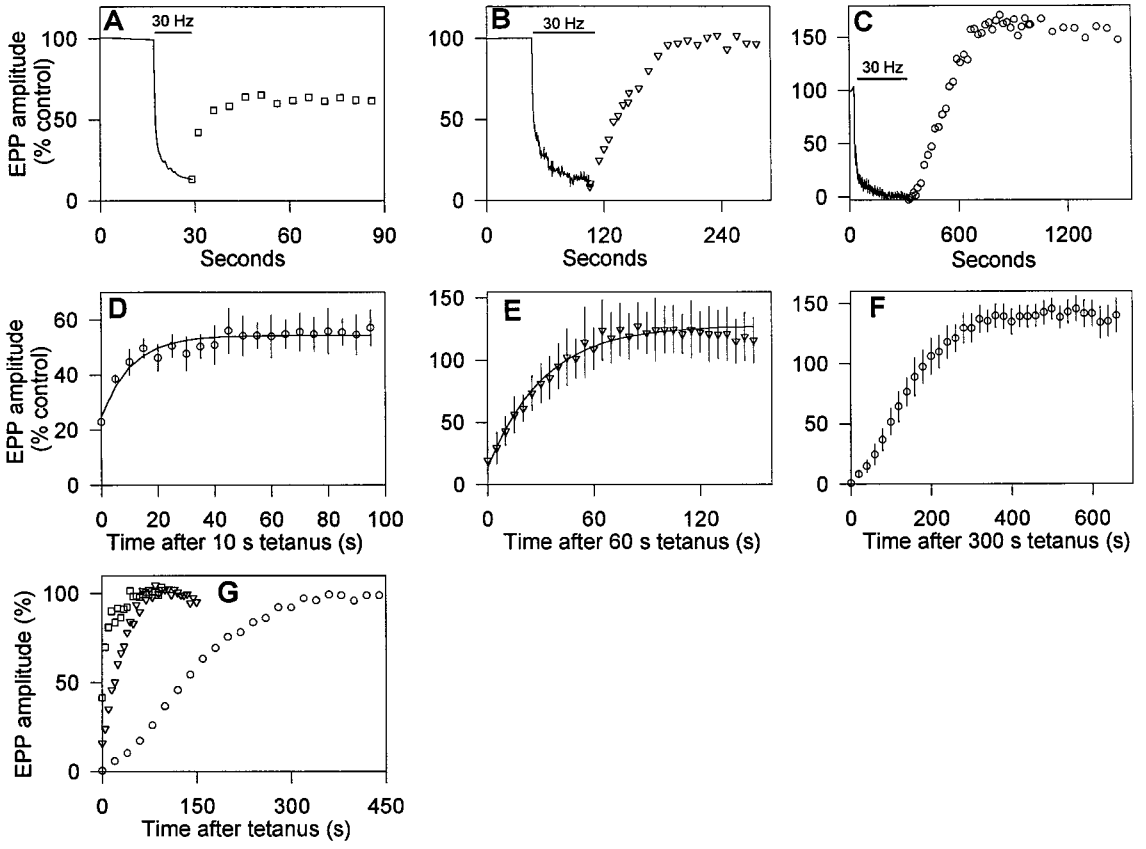

FIGURE 2 The time course of recovery from synaptic depression depends on the duration of nerve stimulation. (*A–C*) Typical examples of EPPs subjected to 10 s (*A*), 1 min (*B*), and 5 min (*C*) of stimulation. EPPs were evoked at 30 Hz during the times marked by the horizontal line, and then sampled once each 5–50 s after stimulation (*symbols*). (*D–F*) The mean amplitude of EPPs (normalized to the amplitude before the tetanus) from five or six terminals with standard errors (*error bars*) was plotted against time after stimulation. The lines in *D* and *E* are exponential fits with time constants of 10.7 and 27.9 s, respectively. (*G*) The mean values of EPPs in *D–F* were first normalized to the maximum values to which EPPs recovered, and then superimposed. □, 10-s train; ▽, 60-s train; ○, 5-min train.

lation. Most of these results have been published (Wu and Betz, 1996) and are summarized in Fig. 3 *A*. Each point represents the average brightness of four to six terminals in one muscle, normalized to the average brightness at zero time. FM1–43 was added after the tetanus ended; the interval between the end of the tetanus and the addition of dye is called the "delay time." Fig. 3 *A* shows the endocytosis time course after tetanic stimulation for 300 s. This procedure was repeated for tetanus durations of 10 s and 60 s (Wu and Betz, 1996). As the delay time increased, the fluorescence intensity of the terminals decreased; the time course could be fit reasonably well with a single exponential for each duration of stimulation (Fig. 3 *A*, *solid line*) and represents the time course of endocytosis after the tetanus. The rate of endocytosis depended on the duration of the stimulus train (τ = 23, 57, and 460 s for trains lasting 10, 60, and 300 s, respectively). These results show that the endocytosis time constant increases as the duration of stimulation is prolonged, and the increase is similar to the increase in the time course of synaptic depression, as illustrated in Fig. 3 *B*.

These data provided information only about the kinetics of endocytosis. To measure the amount of membrane shifted

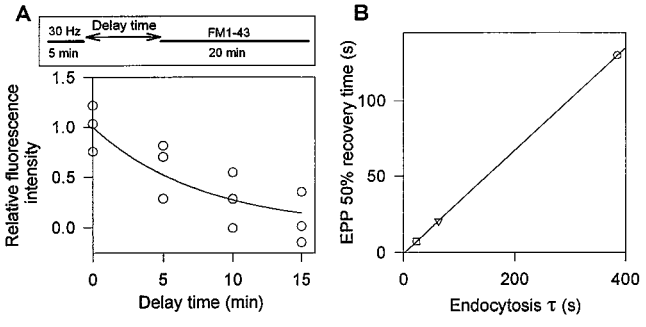

FIGURE 3 Synaptic vesicle endocytosis slows after prolonged stimulation. (*A*) The time course of endocytosis (data from Wu and Betz, 1996). Each point shows the average fluorescence intensity of terminals in one muscle, normalized to the average brightness at zero delay time. The protocol is shown at the top. The nerve was stimulated at 30 Hz for 5 min, and then FM1-43 was added after a variable delay time. The FM1-43 was left on for 20 min to ensure that all endocytic vesicles formed after the dye was added would be labeled (see also Wu and Betz, 1996). The data were fit with a single exponentially decaying curve with a time constant of 460 s. (*B*) Data from Figs. 2 and 3 are compared. □, 10-s train; ▽, 60-s train; ○, 5-min train. The endocytic time constants and the EPP 50% recovery times increased proportionately as the train duration increased. The solid line is a linear regression.

to the surface during the tetanus (the fused pool), we compared the brightnesses at zero delay times with the maximum brightness (all vesicles stained with FM1–43). Maximum labeling of vesicles was achieved by stimulating the nerve at 30 Hz for 5 min, with FM1–43 present for 20 min, starting from the beginning of stimulation ($n = 4$ preparations; data not shown). Because stimulation at higher frequencies or for longer times (or both) did not result in significantly brighter fluorescence, we take this brightness to represent the maximum possible labeling of nerve terminals (Wu and Betz, 1996). In Fig. 3 *A*, the average of the points at zero delay time was 74% as bright as the maximum brightness, suggesting that immediately after the 5-min tetanus, 74% of all synaptic vesicles were externalized. After shorter trains, the comparable numbers were 8% (after a 10-s train) and 14% (after a 60-s train) of the maximum brightness (Wu and Betz, 1996).

Construction of a kinetic model

These initial values of the fraction of vesicles in the fused pool at the end of tetanic stimulation, combined with the kinetic values of endocytosis and depression recovery rates, provided sufficient data to develop a quantitative model of synaptic vesicle recycling after tetanic stimulation. The following assumptions were made:

1. Synaptic vesicles exist in one of three states: the fused pool (f), the reserve pool (r), or the docked pool (d).

2. There are two irreversible reactions between pools of vesicles:

$$f(\text{fused}) \rightarrow r(\text{reserve}) \rightarrow d(\text{docked})$$

with rate constants k_1 (fused → reserve) and k_2 (reserve → docked).

3. The sum of vesicles in all three pools is constant at all times, i.e., $f + r + d = 1$.

4. At rest, nearly all vesicles are in the reserve pool, R_r. Previous electron microscopic data suggest that the size of the docked pool (D_r) is ~5% of the total vesicles (see Van der kloot and Molgó, 1994, for a review). Thus $R_r \cong 0.95$, $D_r \cong 0.05$, and $F_r = 0$. Immediately after the end of stimulation (at a delay time of 0), the values of f, r, and d are F_0, R_0, and D_0, respectively.

5. The docked pool d is saturable, the maximum being that observed at rest, D_r.

6. EPP amplitude is proportional to the size of the docked pool. This implies that the release probability of each docked vesicle and the quantal size of EPP remain constant during recovery from synaptic depression. The effect of spontaneous release, which would escape electrophysiological detection, can be ignored because of its relatively small contribution compared to evoked release during tetanic stimulation (Wu and Betz, 1996).

Thus we have the following differential equations:

$$df/dt = -k_1 f \tag{1}$$

$$dr/dt = k_1 f - k_2(D_r - d)r \tag{2}$$

$$dd/dt = k_2(D_r - d)r \tag{3}$$

In Eq. 2, the rate of vesicle movement from the reserve to the docked pool is $k_2(D_r - d)r$, i.e., the product of k_2, the relative number of the empty docking sites ($D_r - d$), and the size of the reserve pool (r). Based on assumptions 3–5, the relative number of empty docking sites ($D_r - d$) is maximally only 5% of the total number of vesicles, much smaller than the size of the reserve pool (r). The vesicle movement from the reserve to the docked pool would not significantly affect the size of the reserve pool. Therefore, the term $k_2(D_r - d)r$ in Eq. 2 is neglected. Equation 2 becomes

$$dr/dt = k_1 f \tag{4}$$

Solving Eqs. 1, 3, and 4, we obtain

$$f = F_0 \exp(-k_1 t) \tag{5}$$

$$r = R_r - F_0 \exp(-k_1 t) \tag{6}$$

$$d = D_r - (D_r - D_0) * \exp\left(\frac{F_0 k_2}{D_r k_1}\right) * \exp\left[-\frac{k_2 R_r}{D_r} t - \frac{F_0 k_2}{D_r k_1} * \exp(-k_1 t)\right] \tag{7}$$

As described in Eq. 7, the time course of EPP recovery after stimulation can be calculated provided that the parameters R_r, D_r, F_0, D_0, k_1, and k_2 can be experimentally estimated. As described above (assumption 4), $R_r \cong 0.95$ and $D_r \cong 0.05$ (the exact values of D_r and R_r do not affect the calculation of the time course of the size of d pool as long as $R_r \gg D_r$). The fraction of fused vesicles at the end of tetanic stimulation, F_0, was estimated by comparing the brightness of terminals stained with zero delay time with maximally stained terminals, as described above. Values of F_0 were 0.08 (10-s train), 0.14 (60-s train), and 0.74 (5-min train). EPP amplitudes at the end of a stimulus train provided a measure of D_0 (Fig. 2 *G*). Values were expressed as a fraction of the entire vesicle pool, based on the assumption that the docked pool at rest, D_r, contained 5% of all vesicles. Values of D_0 were 0.0208 (10-s train), 0.00805 (60-s train), and 0.000275 (5-min train). k_1 is the reciprocal of the endocytosis time constant, which was measured as shown in Fig. 3 *A*.

The last parameter, k_2, was estimated from Eq. 3 when r could be treated as a constant (i.e., after a short stimulus train). In such a condition, Eq. 3 can be solved as

$$d = D_r - (D_r - D_0) * \exp(-rk_2 t) \tag{8}$$

Thus the EPP (d) will recover with a time constant of $1/(rk_2)$. This time constant can be experimentally estimated as shown in Fig. 2 *D*. With a 10-s stimulation, the amount of membrane in the fused pool (F_0 (10 s) = 0.08 ± 0.01) was only a small fraction of the total vesicles. Thus the reserve pool r could be approximated as a constant ($R_0 \cong$

$1 - D_0 - F_0 = 1 - 0.02 - 0.08 = 0.9$). According to Eq. 8, the docked pool, d, recovers with a time constant of $1/(0.9k_2)$. The docked pool was estimated from EPPs as shown in Fig. 2 *D*, where the recovery time course could be fit with a single exponential curve with a time constant of 10.7 s. Thus k_2 (10 s) = $1/(0.9 \times 10.7\ \text{s}) = 0.104\ \text{s}^{-1}$. A similar case holds for the 60-s train. After stimulation for 60 s, F_0 (60 s) = 0.14 ± 0.01. Thus r could also be approximated as a constant ($R_0 = 1 - 0.008 - 0.14) \cong 0.85$). With similar methods, k_2 (60 s) was estimated to be $1/(0.85 \times 27.9\ \text{s}) = 0.0422\ \text{s}^{-1}$, which is significantly smaller than k_2 (10 s). After a 5-min train, k_2 (300 s) could not be similarly estimated, because 74% of the vesicle membrane had been shifted to the fused pool, greatly depleting the reserve pool ($R_0 = 1 - 0.0003 - 0.74 \cong 0.26$).

Using a constant value of k_2 obtained from the 10 s train data, we attempted to fit the observed rates of recovery from depression by using Eq. 7. The results from fitting clearly showed a slower recovery from depression after prolonged stimulation (Fig. 4 *A*). However, they greatly underestimate the observed recovery rates (Fig. 4 *A*). This suggests that k_2 is not independent of the duration of stimulation. Thus k_2 was estimated from Eq. 7 for each of the three train durations individually (Fig. 4 *B*). The equation cannot be solved explicitly for k_2 (it is transcendental for k_2). Instead, a curve fitting program using least squares fitting was used. The values of k_2 ($k_{2,10\ \text{s}} = 1/10.4$ s, $k_{2,60\ \text{s}} = 1/26.9$ s, $k_{2,300\ \text{s}} = 1/64.9$ s) were similar to those estimated with Eq. 8, and the depression recovery curves were fit with reasonable accuracy (Fig. 4 *B*). These values of k_2 are approximately proportional to the duration of stimulation (Fig. 3 *B*). These results suggest that k_2, like k_1, also varies with stimulation duration, and that a decrease in k_2 may contribute to the slower recovery of synaptic depression after longer stimulation.

In summary, prolonged stimulation slows the rates of both vesicle mobilization and, in particular, endocytosis (Fig. 4 *C*). The mobilization rate is about six times slower after a 5-min train (30 Hz), compared to after a 10-s train. The slowing of endocytosis, however, is more than three times greater; endocytosis is ~20 times slower after a 5-min train, compared to after a 10-s train. Thus, with increasing stimulation, as the reserve pool of vesicles becomes depleted, the slowing in the rate of recovery from synaptic depression can be ascribed mainly to a profound slowing in the rate of endocytosis and consequent slowing in the replenishment of the reserve pool of vesicles.

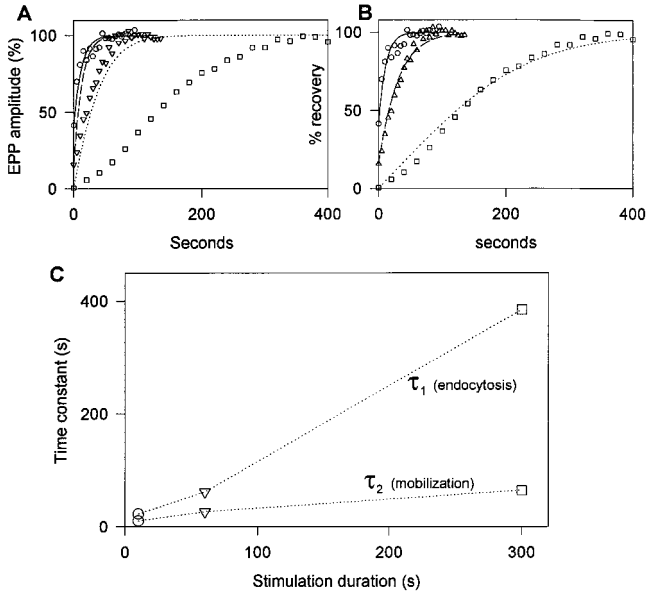

FIGURE 4 The time constants for vesicle mobilization and, especially, endocytosis increase as the train duration is prolonged. (*A*) EPP recovery is plotted as in Fig. 2 *G* (○, 10-s train; ▽, 60-s train; □, 5-min train). The lines are fits predicted by assuming that the time constant of vesicle mobilization is constant, that is, the value (9.7 s) as determined for the 10-s train data (——). The predicted fits for the 60-s train (– – –) and especially for the 5-min train (·····) are severe underestimates. (*B*) Fits to the same data are improved if the mobilization time constant is determined for each curve (10.4 s, 26.9 s, and 64.9 s for trains of 10 s, 60 s, and 5 min, respectively). (*C*) Values of the endocytic time constant (τ_1) and the mobilization time constant (τ_2) are plotted as a function of duration of stimulus train. Note that τ_1 increases much more than does τ_2.

DISCUSSION

We found that longer tetanic nerve stimulation is followed by a slower recovery of EPP amplitude (Fig. 2), a slower rate of endocytosis (Fig. 3), and a greater depletion of the reserve vesicle pool. A simple explanation for these results is that depletion of the reserve pool at the end of stimulation and the slower rate of endocytosis slow down the replenishment of the reserve pool from the fused vesicle pool, and thus decrease the replenishment of the docked pool from the reserve pool (Fig. 4). A simple model can account quantitatively for these experimental observations. The model comprises three vesicle pools: the fused, the reserve, and the docked pool, with rate constants of endocytosis (k_1) and mobilization (k_2) (Fig. 1). With some basic and reasonable assumptions as discussed in the Results and below, the time course of recovery of synaptic depression can be calculated analytically as described in Eq. 7. All of the parameters in this equation can be experimentally estimated by recording EPPs and by imaging nerve terminals stained with FM1–43. The results reveal that a decrease in the rate constant of endocytosis and depletion of the reserve pool significantly slow the time course of depression after tetanic stimulation (Fig. 4). In addition, a decrease in the rate constant of mobilization is required to account fully for the slower time course of depression. In the following, we justify the assumptions in the model. When the assumption does not hold, a decrease in the rate constant of mobilization may mean something else.

Assumptions in the model

This model divides vesicles into three pools (assumption 1), which is almost certainly an oversimplification. Previous electron microscopic studies (for a review see Heuser, 1989)

suggested that endocytosed vesicles may first cycle into cisternae from which nascent vesicles bud, although recent evidence for a simpler route involving only a single budding process (from the plasma membrane) has been presented (Takei et al., 1996). In addition, in adrenal chromaffin and other cells, morphologically docked granules and vesicles may need to pass through several stages of biochemical maturation or "priming" that involve, for example, ATP hydrolysis and sensitivity to pH before becoming fully release-competent (Parsons et al., 1995). We could not have distinguished any of these processes in our experiments. The observed decrease in the rate constant of mobilization and docking (k_2) could reflect changes in any of these processes.

We ignored the effect of the posttetanic increase in MEPP frequency, because it is much less than the amount of evoked release, and so would have a negligible effect on estimates of the size of the fused pool. The increased spontaneous release after repetitive stimulation is a curious phenomenon. At a time when evoked release is profoundly depressed, spontaneous release is elevated (Zengel and Sosa, 1994). If those spontaneously released quanta were used instead to replenish evidently empty docking sites, the rate of recovery from synaptic depression would be increased. However, because there are no quantitative data regarding this process, the effect of spontaneous release is neglected.

The assumption that the size of the docked pool is saturable is different from the corresponding assumption in a two-step model developed for neuroendocrine cells, in which the size of the docked pool was limited by introducing a reverse movement from the docked to the reserve pool (Heinemann et al., 1993). Such undocking events have been visualized directly in adrenal chromaffin cells (Steyer et al., 1997). Whether such undocking events occur at the frog neuromuscular junction, where vesicle docking and release sites are more regularly arranged, is unknown. Exocytosis occurs from active zones that are aligned transversely in the terminal and are separated by $\sim 1\ \mu$m (Couteaux and Pécot-Dechavassine, 1970; Heuser et al., 1974, 1979; Wernig, 1976; Propst and Ko, 1987; Zefirov et al., 1995; see also Heuser, 1989, for a review). Vesicles dock like two strands of beads in a double row at each active zone; signs of vacant docking sites are uncommon. Based on these experimental data, we limited the size of the docked pool by assuming a finite number of docking sites at terminals. This assumption also permits an experimental estimate of the mobilization rate constant (k_2; Fig. 2, *D* and *E*).

We also assumed that the release probability of each docked vesicle remains constant during recovery from synaptic depression; this assumption requires careful scrutiny, because several other events occur during synaptic depression. These include changes in $[Ca^{2+}]_i$, effects of accumulation of extracellular adenosine, and changes in presynaptic calcium currents. For example, an increase in the residual calcium concentration after a tetanic stimulation (Wu and Betz, 1996) could, in principle, increase either the release probability or the number of docked vesicles (or both). (A prolonged increase in the calcium concentration during tetanic stimulation might also cause adaptation of the calcium sensor that triggers release (Hsu et al., 1996), resulting in a decrease in the release probability of a docked vesicle. Currently, we do not know whether there is a change in the release probability of the docked vesicle after tetanic stimulation. An increase in the release probability would produce an overestimate in the rate constant of mobilization (k_2), while a decrease in the release probability leads to a decrease in k_2 in our model. Thus our result that the slower recovery of depression is partly due to a decrease in k_2 could actually reflect a decrease in k_2 and/or a decrease in the release probability.

Endogenous adenosine may mediate synaptic depression during low-frequency (e.g., 1 Hz) stimulation when the basal adenosine level in the bath is minimized by several precedures, including rapid dual superfusion (Redman and Silinsky, 1993, 1994). However, when the basal adenosine level is not minimized, synaptic depression under low- and high-frequency stimulation is not related to endogenous adenosine (Meriney and Grinnell, 1991; see also Ribeiro and Sebastiao, 1987; Bennett et al., 1991). We did not use rapid perfusion to minimize the basal adenosine, and we induced synaptic depression by high- (30 Hz) but not low-frequency nerve stimulation. Thus the synaptic depression we observed is not mediated by endogenous adenosine.

Whether or not a decrease in the presynaptic calcium current contributes to synaptic depression after a tetanic stimulation at the frog neuromuscular junction is not clear. However, at the squid giant synapse (Charlton et al., 1982) and the goldfish bipolar synapse (von Gersdorff and Matthews, 1996), where presynaptic calcium currents can be directly recorded, inactivation of calcium current was found not to play a significant role in synaptic depression. These results are consistent with the hypothesis that depression is mainly due to depletion of the docked vesicle pool.

Assumption 6 implies that the quantal size of EPP remains constant during and after tetanic stimulation. However, accumulated evidence suggests that quantal size might decrease by $\sim$20–30% (on average) during tetanic stimulation (Van der Kloot and Molgó, 1994; Glavinovic, 1995). If this is the case in our experiments, we would underestimate the size of the docked pool at the end of stimulation (D_0) by using amplitudes of EPP. However, the EPP amplitude was close to 0 at the end of 60 s and 300 s of stimulation. Thus the estimate of D_0 in these two conditions was practically not affected. Only at the end of 10 s of stimulation would D_0 be underestimated by $\sim$20–30%. which would slightly affect the estimate of mobilization rate constant k_2. Furthermore, whether and for how long there is a decrease in quantal size after tetanic stimulation is not known. In short, if there is a decrease in quantal size after tetanic stimulation, it would result in an overestimate of depletion in the docked pool, and thus may partly explain why the prediction of the recovery of depression with a fixed rate constant of mobilization is not sufficient (Fig. 4 *A*).

Vesicles are actively mobilized during repetitive stimulation (for a review, see Zucker, 1989). We did not measure those rates because our imaging and electrophysiological data were acquired after the end of the stimulation period. Surprisingly, the mobilization (docking) rate constants that we measured decreased, rather than increased, after prolonged stimulation. The biological significance of such a phenomenon may be to protect the synapse from excessive transmitter release and depletion of the vesicle pool during strong nerve muscle activities. In summary, our results show that the rates of synaptic vesicle endocytosis and mobilization vary inversely as a function of presynaptic nerve activity. The rate constants of endocytosis and mobilization, and the degree of depletion of the reserve vesicle, determine the time course of synaptic depression. Thus regulation of the rates of endocytosis and docking may also offer neurons various forms of synaptic plasticities.

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REFERENCES

Bennett, M. R., S. Karunanithi, and N. A. Lavidis. 1991. Probabilistic secretion of quanta from nerve terminals in toad (*Bufo marinus*) muscle modulated by adenosine. *J. Physiol. (Lond.).* 433:421–434.

Betz, W. J. 1970. Depression of transmitter release at the neuromuscular junction of the frog. *J. Physiol. (Lond.).* 206:629–644.

Betz, W. J., and G. S. Bewick. 1993. Optical monitoring of transmitter release and synaptic vesicle recycling at the frog neuromuscular junction. *J. Physiol. (Lond.).* 460:287–309.

Birks, R. I., and F. C. MacIntosh. 1961. Acetylcholine metabolism of a sympathetic ganglion. *Can. J. Biochem. Physiol.* 39:787–827.

Charlton, M. P., S. J. Smith, and R. S. Zucker. 1982. Role of presynaptic calcium ions and channels in synaptic facilitation and depression at the squid giant synapse. *J. Physiol. (Lond.).* 323:173–93.

Couteaux, R., and M. Pécot-Dechavassine. 1970. Vesicules synaptiques et poches au niveau des "zones actives" de la jonction neuromusculaire. *C. R. Acad. Sci. D.* 271:2346–2349.

Del Castillo, J., and B. Katz. 1954. Statistical factors involved in neuromuscular facilitation and depression. *J. Physiol. (Lond.).* 124:574–585.

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

Glavinovic, M. I. 1995. Decrease of quantal size and quantal content during tetanic stimulation detected by focal recording. *Neuroscience.* 69:271–281.

Heinemann, C., L. von Rüden, R. H. Chow, and E. Neher. 1993. A two-step model of secretion control in neuroendocrine cells. *Pflügers Arch.* 424:105–112.

Heuser, J. E. 1989. Review of electron microscopic evidence favouring vesicle exocytosis as the structural basis for quantal release during synaptic transmission. *Q. J. Exp. Physiol.* 74:1051–1069.

Heuser, J. E., T. S. Reese, M. J. Dennis, Y. Jan, L. Jan, and L. Evans. 1979. Synaptic vesicle exocytosis captured by quick freezing and correlated with quantal transmitter release. *J. Cell Biol.* 81:275–300.

Heuser, J. E., T. S. Reese, and D. M. D. Landis. 1974. Functional changes in frog neuromuscular junctions studied with freeze-fracture. *J. Neurocytol.* 3:109–131.

Hsu, S.-F., G. J. Augustine, and M. B. Jackson. 1996. Adaptation of Ca^{2+}-triggered exocytosis in presynaptic terminals. *Neuron.* 17:501–512.

Koenig, J. H., T. Kosaka, and K. Ikeda. 1989. The relationship between the number of synaptic vesicles and the amount of transmitter released. *J. Neurosci.* 9:1937–1942.

Lass, Y., Y. Halevi, E. M. Landau, and S. Gitter. 1973. Post-tetanic potentiation and facilitation do not share a common calcium dependent mechanism. *Nature New Biol.* 244:155–157.

Liu, G., and R. W. Tsien. 1995. Properties of synaptic transmission at single hippocampal synaptic boutons. *Nature.* 375:404–408.

Meriney, S. D., and A. D. Grinnell. 1991. Endogenous adenosine modulates stimulation-induced depression at the frog neuromuscular junction. *J. Physiol. (Lond.).* 443:441–455.

Parsons, T. D., J. R. Coorssen, H. Horstmann, and W. Almers. 1995. Docked granules, the exocytic burst, and the need for ATP hydrolysis in endocrine cells. *Neuron.* 15:1085–1096.

Propst, J. W., and C.-P. Ko. 1987. Correlations between active zone ultrastructure and synaptic function studied with freeze-fracture of physiologically identified neuromuscular junctions. *J. Neurosci.* 7:3654–3664.

Redman, R. S., and E. M. Silinsky. 1993. A selective adenosine antagonist (8-cyclopentyl-1,3-dipropylxanthine) eliminates both neuromuscular depression and the action of exogenous adenosine by an effect on A1 receptors. *Mol. Pharmacol.* 44:835–840.

Redman, R. S., and E. M. Silinsky. 1994. ATP released together with acetylcholine as the mediator of neuromuscular depression at frog motor nerve endings. *J. Physiol. (Lond.).* 477:117–127.

Ribeiro, J. A., and A. M. Sebastiao. 1987. On the role, inactivation and origin of endogenous adenosine at the frog neuromuscular junction. *J. Physiol. (Lond.).* 384:571–585.

Rosenmund, C., and C. F. Stevens. 1996. Definition of the readily releasable pool of vesicles at hippocampal synapses. *Neuron.* 16:1197–1207.

Rosenthal, J. 1969. Post-tetanic potentiation at the neuromuscular junction of the frog. *J. Physiol. (Lond.).* 203:209–231.

Ryan, T. A., L. Li, L.-S. Chin, P. Greengard, and S. J. Smith. 1996. Synaptic vesicle recycling in synapsin I knock-out mice. *J. Cell Biol.* 134:1219–1227.

Ryan, T. A., H. Reuter, B. Wendland, F. E. Schweizer, R. W. Tsien, and S. J. Smith. 1993. The kinetics of synaptic vesicle recycling measured at single presynaptic boutons. *Neuron.* 11:713–724.

Ryan, T. A., and S. J. Smith. 1995. Vesicle pool mobilization during action potential firing at hippocampal synapses. *Neuron.* 14:983–989.

Steyer, J. A., H. Horstmann, and W. Almers. 1997. Transport, docking and exocytosis of single secretory granules observed in live chromaffin cells. *Nature.* 388:474–478.

Takei, K., O. Mundigl, L. Daniell, and P. De Camilli. 1996. The synaptic vesicle cycle: a single vesicle budding step involving clathrin and dynamin. *J. Cell Biol.* 133:1237–1250.

Takeuchi, A. 1958. The long-lasting depression in neuromuscular transmission of frog. *Jpn. J. Physiol.* 8:102–113.

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

von Gersdorff, H., and G. Matthews. 1996. Calcium-dependent inactivation of calcium current in synaptic terminals of retinal bipolar neurons. *J. Neurosci.* 16:115–122.

von Gersdorff, H., and G. Matthews. 1997. Depletion and replenishment of vesicle pools at a ribbon-type synaptic terminal. *J. Neurosci.* 17: 1919–1927.

Wernig, A. 1976. Localization of active sites in the neuromuscular junction of the frog. *Brain Res.* 118:63–72.

Wu, L.-G., and W. J. Betz. 1995. Nerve activity determines the time course of endocytosis and synaptic depression at grog neuromuscular junctions. *Soc. Neurosci. Abstr.* 22:776.

Wu, L.-G., and W. J. Betz. 1996. Nerve activity but not intracellular calcium determines the time course of endocytosis at the frog neuromuscular junction. *Neuron.* 17:769–779.

Wu, L.-G., and P. Saggau. 1997. Presynaptic inhibition of evoked neurotransmitter release. *Trends Neurosci.* 20:204–212.

Zefirov, A., T. Benish, N. Fatkullin, S. Cheranov, and R. Khazipov. 1995. Location of active zones. *Nature.* 376:393–394.

Zengel, J. E., and M. A. Sosa. 1994. Changes in MEPP frequency during depression of evoked release at the frog neuromuscular junction. *J. Physiol. (Lond.).* 477:267–277.

Zucker, R. S. 1989. Short-term synaptic plasticity. *Annu. Rev. Neurosci.* 12:13–31.