Differential Regulation of Transmitter Release by Presynaptic and Glial Ca^{2+} Internal Stores at the Neuromuscular Synapse

Annie Castonguay and Richard Robitaille

Centre de Recherche en Sciences Neurologiques and Département de Physiologie, Université de Montréal, Montréal, Canada H3C 3J7

The differential regulation of synaptic transmission by internal Ca^{2+} stores of presynaptic terminals and perisynaptic Schwann cells (PSCs) was studied at the frog neuromuscular junction. Thapsigargin (tg), an inhibitor of Ca^{2+} -ATPase pumps of internal stores, caused a transient Ca^{2+} elevation in PSCs, whereas it had no effect on Ca^{2+} stores of presynaptic terminals at rest. Tg prolonged presynaptic Ca^{2+} responses evoked by single action potentials with no detectable increase in the resting Ca^{2+} level in nerve terminals. However, Ca^{2+} accumulation was observed during high frequency stimulation. Tg induced a rapid rise in endplate potential (EPP) amplitude, accompanied by a delayed and transient increase. The effects appeared presynaptic, as suggested by the lack of effects of tg on the amplitude and time course of miniature EPPs (MEPPs). However, MEPP frequency was increased when preparations were stimulated tonically (0.2 Hz). The delayed and transient

increase in EPP amplitude was occluded by injections of the Ca^{2+} chelator BAPTA into PSCs before tg application, whereas a rise in intracellular Ca^{2+} in PSCs induced by inositol 1,4,5-triphosphate (IP_3) injections potentiated transmitter release. Furthermore, increased Ca^{2+} buffering capacity after BAPTA injection in PSCs resulted in a more pronounced synaptic depression induced by high frequency stimulation of the motor nerve (10 Hz/80 sec). It is concluded that presynaptic Ca^{2+} stores act as a Ca^{2+} clearance mechanism to limit the duration of transmitter release, whereas Ca^{2+} release from glial stores initiates Ca^{2+} -dependent potentiation of synaptic transmission.

Key words: perisynaptic Schwann cells; ATPase pump; calcium; frog neuromuscular junction; transmitter release; synaptic transmission; synapse–glia interactions; IP_3

Ca^{2+} entry through voltage-gated Ca^{2+} channels clustered at active zones (Robitaille et al., 1990; Cohen et al., 1991) is a necessary step leading to transmitter release (Zucker, 1993a; Kamiya and Zucker, 1994) in which the concentration of Ca^{2+} determines the amount of transmitter that is released (Augustine et al., 1987; Zucker, 1993b).

The release of Ca^{2+} from presynaptic internal stores also regulates the amount of transmitter that is released at various synapses (Guo et al., 1996; Peng, 1996; Smith and Cunnane, 1996; Tse et al., 1997; Li et al., 1998; Lin et al., 1998; Tse and Tse, 1998; Cao and Peng, 1999; Krizaj et al., 1999; He et al., 2000). Moreover, the ATPase pump that reloads the stores by pumping Ca^{2+} ions from the cytoplasm modulates transmitter release by participating in the clearance of Ca^{2+} , limiting its spread and duration away from the mouth of Ca^{2+} channels (Fossier et al., 1998). At the amphibian neuromuscular junction (NMJ) the release of Ca^{2+} from presynaptic stores increases asynchronous release of transmitter via a Ca^{2+} -induced Ca^{2+} release (CICR) mechanism

(Narita et al., 1998), whereas it results in a depression of transmitter release at the rat NMJ (Schwartz et al., 1999).

In addition to the presynaptic Ca^{2+} components in the regulation of transmitter release, recent evidence has revealed that perisynaptic glial cells (glial cells associated with synapses) also modulate synaptic activity (Carmignoto et al., 1998; Kang et al., 1998; Newman and Zahs, 1998; Robitaille, 1998; Araque et al., 1999). This modulation occurs via the release of Ca^{2+} from internal stores [often inositol 1,4,5-triphosphate-regulated (IP_3)], and the Ca^{2+} elevation is both necessary and sufficient for the glial modulation to occur (Araque et al., 1998, 1999; Castonguay et al., 2001). Moreover, this modulation is observed during normal synaptic activity in a frequency-dependent manner (Robitaille, 1998). This suggests that synaptic efficacy is regulated by a synapse–glia–synapse loop in which Ca^{2+} release from presynaptic and glial internal stores plays a crucial role.

Although there is now compelling evidence that transmitter release is regulated not only by internal stores of the presynaptic terminal but also by the stores of perisynaptic glial cells, there is no direct analysis of their differential contribution in the control of transmitter release. Therefore, the main goal of this work was to understand the respective roles of the presynaptic and glial Ca^{2+} stores in the regulation of transmitter release. We used thapsigargin (tg), an inhibitor of the Ca^{2+} -ATPase pump (Rasmussen et al., 1978), to block Ca^{2+} uptake into the stores of perisynaptic Schwann cells (PSCs) and nerve terminals at the frog NMJ.

We show that tg application slowed the Ca^{2+} clearance in presynaptic nerve terminals, causing an irreversible increase of miniature endplate potential (MEPP) frequency and evoked

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Correspondence should be addressed to Dr. Richard Robitaille, Département de Physiologie, Université de Montréal, P.O. Box 6128, Station "Centre-Ville," Montréal, Canada H3C 3J7. E-mail: richard.robitaille@umontreal.ca.

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transmitter release. Moreover, tg transiently elevated Ca^{2+} in PSCs by emptying their internal stores. Using specific Ca^{2+} chelator and IP_3 injections into PSCs, we show that the Ca^{2+} release from PSC internal stores potentiates transmitter release and that high frequency depression is more pronounced when the Ca^{2+} buffering capacity of PSCs is elevated.

MATERIALS AND METHODS

Labeling with fluorescent thapsigargin. Frogs (*Rana pipiens*) were anesthetized with 3-aminobenzoic acid ethyl ester (0.3 mg/gm frog; prepared in frog Ringer's solution) and then double-pithed. Then the cutaneous pectoris muscle was dissected out of the frog, along with the pectoralis proprius nerve.

To visualize binding sites of tg, we incubated nerve–muscle preparations for 5 min in normal frog Ringer's [containing (in mM): 120 NaCl, 5 MgCl_2 , 2 KCl, 1 NaHCO_3 , 15 HEPES, and 1.8 CaCl_2 pH-adjusted to 7.20 with 5N NaOH] containing fluorescent thapsigargin (f-tg; 2 μM) with 1% final dimethylsulfoxide (DMSO). Muscles then were rinsed six times with normal frog Ringer's (1% final DMSO concentration) to eliminate background fluorescence. To test the specificity of the labeling with f-tg, we applied unlabeled thapsigargin (2 μM) on the preparation for 5 min and rinsed as described above before incubation with f-tg (2 μM ; 1% final DMSO concentration). Double staining of the preparation was performed by using peanut agglutinin lectin-TRITC (PNA-T; 15 $\mu\text{g}/\text{ml}$ for 15 min in normal Ringer's) to reveal the presence of NMJs (Ko, 1987) and to determine whether f-tg labeling was located at the NMJ.

Images of f-tg and PNA-T were acquired simultaneously with the dual channel configuration of the Bio-Rad MRC-600 confocal microscope (Hercules, CA). The excitation wavelength (514 nm) was attenuated to 1% with neutral density filters. Green fluorescence emitted by f-tg was detected by one photomultiplier tube (PMT) through a bandpass filter (505–535 nm); the red signal emitted by PNA-T was detected by another PMT through a long-pass filter (cutoff at 590 nm).

Ca^{2+} imaging of nerve terminals. For specific imaging of presynaptic nerve terminals, the pectoralis proprius nerve of cutaneous pectoris muscle was dissected through a small opening in the skin of the frog and laid on the thorax of the animal. After washing the cut end of the nerve with Mg^{2+} Ringer's solution (containing 5 mM MgCl_2 , no Ca^{2+} added) to minimize the closing of the extremity of the axons, we put crystals of Ca^{2+} –green-1 dextran (MW 3000) on the cut end of the nerve and left the preparation to incubate overnight (~14 hr) at room temperature (21°C). After the incubation period the muscles were dissected from the frog, pinned down in a recording chamber, and bathed in normal Ringer's solution. Muscle contractions were prevented by blocking cholinergic receptors with α -bungarotoxin (1 μM). It has been demonstrated that α -bungarotoxin has no effect on PSC cholinergic-evoked Ca^{2+} signals (Jahromi et al., 1992; Robitaille et al., 1997).

Nerve stimulation was delivered through a suction electrode with a S88 Grass stimulator. The line scan mode of the Bio-Rad 600 confocal microscope was used to study Ca^{2+} responses elicited by brief motor nerve stimulation (100 Hz, train duration of 100 msec) or by single pulses at 0.2 Hz. Each line scan occurred at intervals of 2 msec, and 512 lines in total were acquired every scan. Twenty line scans were averaged twice during control, and 20 line scans were collected 15 min after tg application. The amplitude and duration of the average Ca^{2+} responses evoked by single stimulus were stable during the control period. When Ca^{2+} changes were monitored over longer periods of time, confocal images (192 × 128 pixels) were taken every 645 msec at the same focal plane. Presynaptic nerve terminals were monitored at rest and during prolonged motor nerve stimulation (50 or 100 Hz for 30 sec).

Changes in fluorescence were monitored with a Bio-Rad MRC 600 confocal microscope equipped with an argon ion laser producing an excitation wavelength at 488 nm. The intensity of the laser was attenuated to 1% with neutral density filters, and the emitted light was detected through a long-pass filter at 515 nm.

Ca^{2+} imaging of perisynaptic Schwann cells. For Ca^{2+} imaging of PSCs and muscle fibers, cutaneous pectoris muscles were dissected from the frogs and incubated for 90 min in a 10 μM fluo-3 AM solution prepared in normal Ringer's solution containing 1% DMSO and 0.02% pluronic acid at room temperature (21°C). After incubation the muscles were bathed in normal Ringer's solution containing TPEN [tetrakis-(2-pyridylmethyl) ethylenediamine, 20 μM] with 0.01% EtOH. The time course of fluorescence changes in PSCs and muscle fibers was monitored by using the 488 nm line of the argon ion laser (attenuated to 1%) of a

Bio-Rad MRC 600 confocal microscope, and the emitted light was detected through a long-pass filter at 515 nm. Images (192 × 128 pixels) were acquired every 645 msec before, during, and after tg application.

Electrophysiological recordings of synaptic transmission. Intracellular recordings of EPPs were performed by using glass microelectrodes (10–15 M Ω) filled with KCl (2 M). The motor nerve was stimulated by using supra-threshold pulses applied via a suction electrode, and muscle contractions were blocked with *d*-tubocurarine chloride (6 μM). The stimulation paradigm consisted of a paired pulse stimulation (10 msec) delivered at 0.2 Hz. EPPs were acquired as an average of four, using Tomahawk software (by T. A. Goldthorpe, University of Toronto, Canada). For measurements of spontaneous activity the MEPPs were recorded in normal Ringer's solution in the absence of *d*-tubocurarine chloride and were recorded in consecutive frames of 250 msec with Tomahawk software.

Procedure for BAPTA and IP_3 injection in PSCs. The procedure used to perform the injection into PSCs and record the subsequent synaptic activity has been described in detail elsewhere (Robitaille, 1998). Briefly, a microelectrode (10–15 M Ω ; filled with 1 M KCl) was inserted in the postsynaptic muscle fiber near an identified NMJ to record the synaptic activity of the whole NMJ. Then a focal electrode (2–3 M Ω , filled with Ringer's) was placed near a branch of the NMJ to record synaptic activity of only that portion of the NMJ. Finally, a third microelectrode (35–55 M Ω , filled with 500 mM K-acetate) was used to penetrate the PSC covering the nerve terminal branch recorded by the focal electrode and to inject ionophoretically (–2 to –5 nA 200 msec pulses every 500 msec for 120 sec) a solution of BAPTA (10 mM BAPTA tetrapotassium salt in 500 mM K⁺-acetate) or IP_3 (10 mM in 500 mM K⁺-acetate) into PSCs, along with a Ca^{2+} indicator (Ca^{2+} –green-1 dextran; MW 3000). This method of injection has been shown not to perturb neurotransmitter release and the activity of the PSCs (Robitaille, 1998). In cases in which synaptic depression was induced, the motor nerve was stimulated at 10 Hz for 80 sec, and the preparation was allowed to rest for 15 min before the second depression period was attempted. These procedures are known to produce stable and reproducible depression (Robitaille and Charlton, 1992; Robitaille, 1998).

Statistical analysis. All values are presented as mean $\pm$ SEM. Student's paired *t* test was used when data obtained from the same cell or NMJ were compared; a one-way ANOVA was performed when several treatments were compared. *N* indicates the number of preparations used; *n* indicates the number of cells or NMJs.

RESULTS

Thapsigargin binding sites in PSCs and presynaptic nerve terminals

Distribution of tg binding sites at the frog NMJ was examined first with f-tg (BODIPY FL-thapsigargin, Molecular Probes, Eugene, OR). Preparations also were labeled with fluorescent PNA-T to identify NMJs (Ko, 1987), and the two labels were observed simultaneously with the dual channel mode of a Bio-Rad 600 confocal microscope. As shown in Figure 1, *A* and *B*, f-tg was distributed within the PNA-T-labeled NMJs. Moreover, the staining pattern revealed that PSCs were labeled because their cell body region was heavily stained (Fig. 1*A*). No fluorescence was ever associated with the muscle fibers, indicating that these cells do not possess tg receptors or that their density is too low to be detected by this approach. However, muscle fibers were heavily labeled by fluorescent ryanodine (BODIPY FL-X ryanodine; Molecular Probes; data not shown). The labeling of f-tg appeared specific because preincubating the preparations with unlabeled tg prevented the staining normally observed with the f-tg, as indicated by the lack of green labeling at the NMJs that were identified by PNA-T staining (Fig. 1*C*).

These results indicate that tg binds to receptors located at PSCs. However, this technique cannot resolve whether there are also tg receptors in nerve terminals because the thickness of a confocal section (~4 μm) is wider than the size of the nerve terminal diameter covered by PSC processes (~2 μm). Hence, the

Figure 1. Distribution of tg labeling at the frog NMJ. *A*, False color confocal images of an amphibian NMJ labeled with f-tg ($2 \mu\text{M}$). Arrows point at PSC somata. *B*, False color confocal images of an NMJ double-labeled with f-tg (green) and PNA-T (red). The two images were acquired simultaneously by using the dual channel configuration of an MRC 600 confocal microscope and were superimposed (*Merge*) to determine the distribution of the f-tg in relation to the NMJ, as indicated by PNA-T staining. Note the presence of a PSC soma and also note that the f-tg labeling is located within the boundaries delineated by the PNA-T staining. *C*, False color confocal images of an NMJ preincubated with unlabeled tg ($2 \mu\text{M}$) for 10 min and then double-labeled with f-tg ($2 \mu\text{M}$) and PNA-T. The two images were acquired simultaneously by using the dual channel configuration of an MRC 600 confocal microscope. Note the lack of labeling when the preparations were exposed to unlabeled tg before the incubation with f-tg. Scale bars: *A*, *C*, $20 \mu\text{m}$; *B*, $10 \mu\text{m}$.

next experiments were performed to investigate the functional effects of tg on PSCs and nerve terminals.

Thapsigargin-evoked calcium responses in perisynaptic Schwann cells

The effects of tg ($2 \mu\text{M}$) on PSCs were investigated first by monitoring intracellular levels of Ca^{2+} with the membrane-permeant calcium indicator fluo-3 AM. Because a membrane-permeant indicator was used, the three compartments of the synapse (i.e., PSCs, nerve terminals, and muscle fibers) were

loaded with fluo-3 and could display intracellular Ca^{2+} changes. To minimize the interference with presynaptic terminals, we measured PSC fluorescence at the level of the cell body (Jahromi et al., 1992).

As shown in Figure 2, bath application of tg ($2 \mu\text{M}$) elicited a transient Ca^{2+} response in all of the PSCs that were tested ($N = 7$, $n = 9$). The size of the relative change in Ca^{2+} was $299 \pm 65\%$ of control. Responses occurred with a delay of 5 ± 2 min and had a duration (return to baseline) of 12 ± 2 min. This indicates that Ca^{2+} stores of PSCs were loaded with Ca^{2+} at rest and that the blockade of the ATPase pump resulted in a gradual leak of Ca^{2+} from the internal stores. The effects were irreversible because Ca^{2+} responses elicited by ATP ($50 \mu\text{M}$), an agonist that activates P2 receptors known to release Ca^{2+} from internal stores of PSCs (Jahromi et al., 1992; Robitaille, 1995), were greatly reduced or abolished (data not shown). This indicates that the internal Ca^{2+} stores could not be replenished after the effect of tg and remained empty. No changes in fluorescence were ever observed in muscle fibers after tg application. However, Ca^{2+} responses were induced in muscle fibers by using ryanodine at the same concentration ($2 \mu\text{M}$) as tg (data not shown).

Figure 2. Thapsigargin causes a transient Ca^{2+} elevation in PSCs. *A*, False color confocal images of a PSC (arrow) loaded with fluo-3 AM at rest (*Control*, before bath application of tg) and at the peak of the Ca^{2+} response elicited by the bath application of tg ($2 \mu\text{M}$). Blue indicates a low level of Ca^{2+} and red a high level. *B*, Time course of the relative changes of Ca^{2+} fluorescence in the PSC illustrated in *A* before and during tg application. Similar results were obtained in nine cells from seven preparations. Scale bar, $10 \mu\text{m}$.

Figure 3. Effects of thapsigargin on the Ca^{2+} level of presynaptic terminals. *A*, Relative changes of the basal fluorescence in a nerve terminal loaded with Ca^{2+} -green-1 dextran at rest, during the application of tg (2 μM), and in the presence of the mitochondria inhibitor CCCP (2 μM). Insets show false color images of the nerve terminal at rest (Control), during the application of tg, and in the presence of CCCP. Blue indicates low levels of Ca^{2+} and red indicates high levels. Note that tg did not cause any Ca^{2+} elevation in the nerve terminal. However, CCCP (2 μM) induced a Ca^{2+} rise, indicating that the detection method was appropriate. Scale bar, 25 μm . *B*, Relative changes of the fluorescence in a nerve terminal loaded with Ca^{2+} -green-1 dextran before, during, and after a train of stimuli (100 Hz, 30 sec; arrow) in control (black circles) and in the presence of tg (2 μM ; red circles) applied during the recovery phase of the response. Note that the presence of tg prolonged the recovery phase of the Ca^{2+} response.

Thapsigargin had no effect on nerve terminal Ca^{2+} level at rest

To test whether thapsigargin affected Ca^{2+} stores of resting nerve terminals, we selectively loaded the terminals with another fluorescent Ca^{2+} indicator, Ca^{2+} -green-1 dextran (MW 3000). To confine the Ca^{2+} indicator to nerve terminals without any possible contamination of the signal resulting from fluorescence of PSCs, we applied crystals of the indicator at the cut end of the nerve and allowed indicator molecules to diffuse overnight to the nerve terminals (16 hr). Then tg (2 μM) was applied, and the changes in nerve terminal fluorescence were monitored with a Bio-Rad 600 confocal microscope.

As shown in Figure 3*A*, there was no significant increase of fluorescence in resting nerve terminals in the presence of tg (2 μM ; 31.0 ± 6.0 pixel intensity in control and 32.9 ± 10.0 pixel intensity in tg), indicating that the level of intracellular Ca^{2+} remained unchanged ($p = 0.739$, Student's paired t test; $N = 6$,

$n = 6$). To test whether we could have detected a change in the presynaptic fluorescence level, we treated the same preparations with carbonyl cyanide *m*-cyclophenylhydrazone (CCCP), an inhibitor of mitochondrion metabolism that causes these organelles to release their internal Ca^{2+} (Tang and Zucker, 1997). Figure 3*A* also shows the rise of fluorescence over time, relative to the control level after the application of CCCP. Augmentation of the fluorescence level was obtained readily, indicating that changes induced by tg could have been detected with our method. Hence, these results suggest that tg had no effect on the basal Ca^{2+} level of the presynaptic nerve terminal at rest.

Presynaptic Ca^{2+} stores play a role in Ca^{2+} clearance during high frequency stimulation

The observation that no Ca^{2+} changes could be elicited by tg in nerve terminals indicated either that these internal stores were empty in resting conditions or that ATPase pumps sensitive to tg were lacking in the presynaptic terminals. To discriminate between the two possibilities, we challenged the presynaptic terminals with a large Ca^{2+} entry induced by repetitive high frequency stimulation to trigger the pumping of Ca^{2+} in the internal stores. In this case, tg should affect the clearance of Ca^{2+} if internal stores possess a tg-sensitive ATPase and would result in a prolonged clearance of intracellular Ca^{2+} . Preparations were stimulated at frequencies of 50 or 100 Hz for 30 sec to induce a large Ca^{2+} entry in nerve terminals. Figure 3*B* illustrates a nerve terminal Ca^{2+} response that was induced by a stimulation at 100 Hz for 30 sec (black circles). The response was characterized by a rapid rise in Ca^{2+} , followed by a fast phase of recovery that accounted for $\sim 80\%$ of the signal and a slower phase that lasted for 13 ± 3 min ($N = 5$, $n = 5$). These responses could be obtained repeatedly and showed no difference over time (data not shown). After full recovery the preparation was stimulated again, and tg was perfused immediately after the rapid phase of recovery. As shown in Figure 3*B*, the presence of 2 μM tg (red circles) significantly prolonged the second recovery phase (18 ± 3 min; $p = 0.020$, Student's paired t test). Similar results were observed with stimulation at 50 Hz. These results indicate that, after a massive Ca^{2+} entry into the nerve terminal, Ca^{2+} is pumped into the Ca^{2+} stores and that the blockade of the ATPase pump by tg leads to a reduced clearance capability resulting in a prolonged elevation of the cytoplasmic Ca^{2+} level.

Regulation of fast, phasic Ca^{2+} entry by the presynaptic ATPase pump of internal stores

Ca^{2+} transients elicited by single pulse stimulation were monitored in nerve terminals to test whether the presynaptic ATPase pumps were able to limit the duration of this phasic Ca^{2+} entry. Ca^{2+} entry in nerve terminals was monitored by using the line scan mode of a Bio-Rad MRC 600 confocal microscope that allowed us to detect changes at intervals of 2 msec. Ca^{2+} entry induced by single action potentials in nerve terminals was recorded in control and in the presence of tg in each experiment. Two series of control measurements were performed consecutively; no difference was observed between the peak and total duration of the responses ($p = 0.756$, Student's paired t test; $N = 7$, $n = 7$). Bath application of tg (2 μM) did not affect the peak amplitude of Ca^{2+} responses (24.5 ± 4.7 and $26.2 \pm 5.0\%$ in control and in the presence of tg, respectively; $p = 0.094$, Student's paired t test; $n = 7$, $n = 7$). Responses were normalized to peak amplitude for all trials before the decay time was analyzed

Figure 4. Thapsigargin prolongs Ca^{2+} clearance in nerve terminals. *A*, Relative changes in fluorescence emitted by a nerve terminal backfilled with Ca^{2+} -green-1 and obtained by using the line scan mode of the Bio-Rad 600 confocal microscope. Each record is an average of 20 individual traces normalized to peak amplitude for Ca^{2+} responses evoked by a single action potential before (*Control*) and after 15 min of

as a function of the area under the curve, to minimize the impact in variations of the size of Ca^{2+} responses on the measurement of their duration. We found that the decay time of the responses was significantly longer in the presence of tg (6011 ± 901 and $7114 \pm 882\%$ $\Delta F/F$'s in control and in the presence of tg, respectively; $p < 0.001$, Student's paired t test; $N = 7$, $n = 7$) (Fig. 4*A*). These results are consistent with the role of clearance of the presynaptic ATPase pump that efficiently regulates Ca^{2+} during a low level of activity.

The same measurements were made on Ca^{2+} entry in nerve terminals elicited by a train of 10 pulses (100 Hz/100 msec). One would predict that the Ca^{2+} entry elicited by the 10 consecutive pulses would cause a build-up of intracellular calcium, resulting in larger and prolonged Ca^{2+} responses. As shown in Figure 4*B*, the blockade of ATPase pump by tg resulted in a prolonged period during which cytoplasmic Ca^{2+} was elevated, leading to an accumulation of residual Ca^{2+} . Indeed, in these conditions not only were the resulting Ca^{2+} responses longer (from 8150 ± 188 to $10165 \pm 221\%$ $\Delta F/F$'s after normalization to peak amplitude; $N = 6$, $n = 6$; $p < 0.001$, Student's paired t test), but the peak of these responses also was increased by 76% (from 174 ± 24 to $230 \pm 12\%$ $\Delta F/F$; $p < 0.05$, Student's paired t test; $N = 6$, $n = 6$) in the presence of tg. The effects were maximal 5 ± 2 min after bath application of tg, were irreversible, and remained stable for as long as 80 min (Fig. 4*B,C*).

We next wondered whether low frequency repetitive stimulation of the motor nerve would produce a rise in the resting level of Ca^{2+} in nerve terminals. This was tested by monitoring the fluorescence of nerve terminals during low frequency stimulation of the motor nerve before and after the application of tg. Bath application of tg during low frequency stimulation of the motor nerve did not induce significant changes in resting fluorescence of presynaptic terminals where the mean resting fluorescence in pixel intensity changed from 44 ± 3 to 41 ± 4 ($p > 0.05$, Student's paired t test; $N = 3$, $n = 3$; data not shown). However, when bursts of stimulation (100 Hz/100 msec every 2 min) known to induce an accumulation of Ca^{2+} (Fig. 4*B*) were used, a significant rise in resting fluorescence was obtained (Fig. 4*D*) ($N = 5$, $n = 5$; $p < 0.001$, Student's paired t test). These results indicate that, during repetitive stimulations, the blockade of the presynaptic ATPase pump induced a detectable activity-dependent global elevation of cytoplasmic intracellular Ca^{2+} .

perfusion with tg ($2 \mu\text{M}$). Note that tg prolonged the duration of the recovery of the Ca^{2+} response. *B*, Relative changes in fluorescence emitted by a nerve terminal backfilled with Ca^{2+} -green-1 and obtained by using the line scan mode of the Bio-Rad 600 confocal. Shown are Ca^{2+} responses evoked by a brief train of stimuli (100 Hz, 100 msec) in control and 20 min after the beginning of tg ($2 \mu\text{M}$) perfusion. The control record is an average of eight individual traces. Note that both the duration and the amplitude of the Ca^{2+} responses were increased in the presence of tg. *Inset*, Average of 10 traces normalized to peak amplitude in control and after tg application. *AU*, Arbitrary units of fluorescence. *C*, From the same experiment as in *B*, the amplitude of Ca^{2+} responses collected after a 100 Hz/100 msec stimulation and plotted as a function of time before and during tg application. Note that the effect occurs rapidly and persists throughout the application of tg. *D*, Relative changes of the basal fluorescence in a nerve terminal loaded with Ca^{2+} -green-1 dextran and stimulated at 2 min intervals with trains of 100 Hz at 100 msec before and during the application of tg ($2 \mu\text{M}$). Note the elevation of the basal Ca^{2+} level in the nerve terminal a few minutes after tg application.

Figure 5. Thapsigargin potentiates transmitter release. *A*, Changes in amplitude of the first evoked EPP before, during, and after the bath application of tg ($2 \mu\text{M}$). EPPs were evoked by paired pulse stimulation (10 msec interval) at 0.2 Hz. Shown in the inset are EPPs in control and at the peak of the tg effect (Tg). Note that the potentiation of EPP amplitude by tg is transient and that a partial recovery is observed even when tg is still present in the perfusate. After the transient phase the EPP amplitude stabilized above the control level. *B*, Histograms of mean $\pm$ SEM of MEPP frequency and amplitude obtained on nonstimulated preparations

Thapsigargin potentiates transmitter release at the frog neuromuscular junction

Our results indicate that tg acted rapidly on nerve terminals to block irreversibly the Ca^{2+} pumping into the internal stores, whereas it caused a slow and delayed calcium transient that emptied Ca^{2+} stores in PSCs, also in an irreversible manner. To test whether neuromuscular synaptic transmission was affected by tg, we recorded evoked transmitter release (paired pulses, interval of 10 msec at 0.2 Hz) while tg ($2 \mu\text{M}$) was administered in the bath. Figure 5*A* shows that the amplitude of the evoked postsynaptic responses increased as a consequence of the bath application of tg ($2 \mu\text{M}$). The augmentation in the amplitude of the first EPP followed two phases: first, the amplitude of the evoked responses increased rapidly after tg administration and stabilized briefly before a second slower and transient phase occurred, which further increased EPP amplitude. Also, EPP amplitude did not return to control level and remained higher than control after the second phase. The period of maximal effect of tg will be identified as the peak effect, whereas the period after the transient phase of increase will be identified as the stable effect. The mean EPP amplitude at the peak effect in tg was 4.4 ± 0.04 mV, which represents an increase of $88 \pm 38\%$ of control value (1.89 ± 0.02 mV; $N = 6$, $n = 6$). At the stable period the mean EPP amplitude was 2.17 ± 0.01 mV ($N = 4$, $n = 4$), which represents an increase of $15 \pm 5\%$. These increases in EPP amplitude were significant at the peak and stable period (one-way ANOVA, $p < 0.05$).

Analysis of MEPPs was performed to determine whether the observed potentiation of synaptic transmission was presynaptic in origin. In the absence of nerve stimulation there was no significant difference in MEPP amplitude ($p = 0.32$; $N = 5$, $n = 5$) or frequency ($p = 0.37$; $N = 5$, $n = 5$, one-way ANOVA) between control and in the presence of tg at times corresponding to the peak or the stable period (Fig. 5*B*). MEPP amplitude and frequency were, respectively, $230 \pm 20 \mu\text{V}$ and 3.2 ± 0.7 Hz in control, $210 \pm 20 \mu\text{V}$ and 2.8 ± 0.5 Hz during the peak period, and $205 \pm 4 \mu\text{V}$ and 2.9 ± 0.7 Hz during the stable period. Also, the rise time of MEPPs in control, peak, and stable periods remained unchanged (respectively, 2.45 ± 0.3 , 2.58 ± 0.3 , and 2.43 ± 0.5 msec; $p = 0.702$, one-way ANOVA), just as the decay time (respectively, 22.9 ± 3.7 , 18.2 ± 2.7 , and 22.9 ± 1.3 msec; $p = 0.678$, one-way ANOVA) (Fig. 5*C*). These results suggest that the augmentation of EPP amplitude was attributable mainly to an increase in the number of released transmitter quanta. However, on stimulated preparations (continuous stimulation at 0.2 Hz), MEPP frequency was increased significantly in the presence of tg (3.8 ± 1.7 Hz in control and 17.7 ± 4.0 Hz in tg; $p < 0.001$, Student's paired t test; $N = 7$, $n = 7$). These results suggest that the blockade of ATPase pumps caused a local rise in Ca^{2+} , which then led to an increase in MEPP frequency.

($n = 5$) in control and at times corresponding to the peak of the tg effect and the stable recovery phase. Note that tg had no effect on MEPP amplitude and frequency. *C*, Histograms of mean $\pm$ SEM of MEPP rise time and decay time obtained on nonstimulated preparations ($n = 5$) in control and at times corresponding to the peak of the tg effect and the stable recovery phase. Note that tg had no effect on either the rise or decay time of the MEPPs. *D*, EPPs evoked by paired pulse stimuli in control and at the peak of tg ($2 \mu\text{M}$) effects. The amplitude of the first EPP recorded in the presence of tg was adjusted to fit the amplitude of the first EPP in control. Note that the amplitudes of the second EPPs of the pairs recorded in control and in the presence of tg are superimposed perfectly, suggesting that the level of facilitation was the same in both conditions.

We next tested whether paired pulse facilitation was affected by tg because it is believed to be dependent on the level of residual Ca^{2+} and tg affects the clearance of Ca^{2+} . Paired pulse facilitation was measured as the ratio $[(\text{EPP1} - \text{EPP0})/\text{EPP0}]$ between two EPPs evoked at a 10 msec interval. For each experiment 20 responses (average of four EPPs per response) were analyzed in control, at the peak, and stable periods. The measured facilitation ratio in control, at the peak, and stable periods were, respectively, 0.48 ± 0.04 , 0.44 ± 0.03 , and 0.54 ± 0.03 . These values are not significantly different from control ($p = 0.27$; $N = 4$, $n = 4$, one-way ANOVA). This is illustrated in Figure 5D, where an average of 32 pairs of EPPs for control and during the peak period are superimposed with the first EPP that was normalized for the amplitude of the control EPP. The second average EPP in the presence of tg matched the one of control, suggesting that facilitation was not affected by tg. However, unlike MEPP decay time, a slowing in the EPP decay rate was observed in the presence of tg.

PSCs contribute to potentiation of synaptic transmission

Our results indicate that the action of tg on nerve terminals alone cannot account for the effects observed on transmitter release. Indeed, we observed a slow and transient increase in transmitter release, which could not be explained solely by the rapid and steady effects of tg on the duration of presynaptic Ca^{2+} responses (Fig. 4C). Interestingly, tg elicited in PSCs a slow and transient rise in Ca^{2+} , suggesting that these cells might be responsible for the slow and transient rise in evoked EPP amplitude. We therefore hypothesized that the effects of tg on presynaptic terminals would be responsible for the rapid rise in evoked EPP amplitude, whereas the depletion of PSC internal Ca^{2+} stores by tg would cause the transient and reversible component of this response (Fig. 5A).

We tested this hypothesis by injecting a Ca^{2+} chelator directly into PSCs before tg application on the preparation. This maneuver was intended to occlude selectively the effects of tg on the injected PSCs by limiting the rise in Ca^{2+} consequent to the blockade of the ATPase pump. A multiple electrode recording technique was used to inject the chelator while monitoring transmitter release (Robitaille, 1998) (Fig. 6A). One electrode was used for intracellular recording of synaptic activity; another one focally recorded the activity in the branch of nerve terminal covered by a PSC injected with the third electrode. Hence, if indeed the Ca^{2+} elevation in PSCs was directly responsible for the slow and transient potentiation in transmitter release, the focal electrode that only records the portion of the terminal covered by the injected PSC should not present the second transient phase of potentiation of EPP amplitude, whereas an intracellular electrode that records the activity of the whole NMJ still would detect both phases because the noninjected PSCs of the NMJ (on average, four per NMJ) would present the normal Ca^{2+} response induced by tg.

We first tested whether the tg effects could be detected similarly by the focal and the intracellular electrodes. Simultaneous intracellular and focal recordings were performed after the injection of PSCs with the vehicle solution (K^+ -acetate and Ca^{2+} -green-1 dextran) before and during the bath application of tg ($2 \mu\text{M}$). As shown in Figure 6B, identical effects were monitored by the intracellular and the focal recordings ($N = 3$, $n = 3$). This indicates that the focal recordings reliably detect the effect of tg on transmitter release and that changes occurring in synaptic

efficacy caused by BAPTA injection into PSCs should be detected.

We next examined whether BAPTA injection (10 mM in the electrode) in PSCs would have any effects on transmitter release. As shown in Figure 6C, the injection of BAPTA in PSCs did not affect the amplitude of focally recorded end-plate currents (EPCs) evoked at 0.2 Hz ($650 \pm 120 \mu\text{V}$ in control and $635 \pm 112 \mu\text{V}$ after BAPTA injection; $p > 0.05$, Student's paired t test; $N = 6$, $n = 6$). This result is consistent with the observation that Ca^{2+} stores in PSCs are loaded at rest and that the release of Ca^{2+} from internal stores is triggered by higher frequencies of transmitter release (Jahromi et al., 1992; Robitaille, 1995; Bourque and Robitaille, 1998).

After bath application of tg ($2 \mu\text{M}$), the intracellular electrode that monitored the activity of the whole NMJ recorded the typical changes in EPP amplitude, that is, a rapid increase followed by a slower and transient rise indicating that tg had its full effects on synaptic transmission. However, the synaptic responses recorded by the focal electrode that originated from the portion of the nerve terminal covered by the PSC injected with BAPTA showed only a rapid and sustained potentiation of synaptic transmission (Fig. 6D). These results indicate that preventing a rise of Ca^{2+} in PSCs partially occluded tg-induced potentiation of synaptic transmission. In four experiments, the increase in EPC amplitude recorded by the focal electrode was only $22 \pm 8\%$ of control in comparison to a $75 \pm 11\%$ increase in EPP amplitude recorded by the intracellular electrode ($N = 4$, $n = 4$). The rise in EPC amplitude reflecting changes in the nerve terminal covered by the injected PSC (i.e., recorded by the focal electrode) was significantly smaller ($p = 0.01$, Student's paired t test) than the rise recorded by the intracellular electrode (activity from the whole NMJ). This shows that the period of peak amplitude was abolished completely at the portion of the nerve terminal covered by the PSC injected with BAPTA.

It is believed that internal stores in PSCs are regulated by an IP_3 receptor (Robitaille, 1995; Castonguay et al., 2000). Hence, the injection of IP_3 in PSCs should cause a Ca^{2+} transient and induce a rise in transmitter release. As shown in Figure 6E, the injection of IP_3 (10 mM in the electrode) produced an average increase in EPC amplitude of $68 \pm 12\%$ ($N = 4$, $n = 4$). Hence, specific and direct activation of glial IP_3 receptors leading to Ca^{2+} release from internal stores resulted in a potentiation of transmitter release.

PSCs modulate synaptic depression at the frog NMJ

Knowing that high frequency activity at the frog NMJ induced a Ca^{2+} elevation in PSCs, we tested whether a component of synaptic plasticity was modulated by PSCs under physiological conditions. A high frequency depression was induced by stimulating the motor nerve of the preparation while simultaneously monitoring synaptic activity by an intracellular and a focal electrode, as described above. No differences were observed between the level of control depression recorded by the intracellular and focal electrodes (respectively, $48.8 \pm 3.9\%$ and $49.1 \pm 3.5\%$; $N = 6$, $n = 6$; $p = 0.720$, Student's t test) (Fig. 7A). Moreover, depressions could be elicited repeatedly without changes in their amplitude (data not shown). After a 15 min recovery period (break in x-axis; Fig. 7A) BAPTA was injected in the PSC covering the branch of the NMJ recorded by the focal electrode, and a second depression was induced. After BAPTA injection into the PSC the depression recorded by the focal electrode was significantly greater than the depression recorded by the intracel-

Figure 6. Potentiation of synaptic transmission by PSCs. *A*, Experimental model of the three-electrode recording method. Intracellular recordings of synaptic activity generated by the whole NMJ were performed simultaneously with extracellular recordings, using a focal electrode, which monitored the portion of the presynaptic terminal covered by a PSC injected via the third electrode. *B*, EPC and EPP amplitude expressed as a percentage of change relative to control before and during the bath application of tg ($2\ \mu\text{M}$) after the injection of the PSC covering the portion of the nerve terminal recorded by the focal electrode with the vehicle solution (500 mM K^+ -acetate and $10\ \mu\text{M}$ Ca^{2+} -green-1). Changes in EPC amplitude represent the activity of the nerve terminal covered by the injected PSC, whereas changes in EPP amplitude represent changes occurring at the whole NMJ. Note that tg induced a similar increase in EPP amplitude (whole NMJ activity) and in EPC amplitude (portion of the terminal covered by the injected PSC). *C*, EPC amplitude recorded by a focal electrode located near the portion of a nerve terminal covered by a PSC before, during, and after the injection of BAPTA ($10\ \text{mM}$ in the electrode) in the PSC. EPCs were evoked by nerve stimulation at $0.2\ \text{Hz}$. Note that BAPTA injection had no effect on synaptic transmission. *D*, EPC and EPP amplitude expressed as a percentage of changes relative to control before and during bath application of tg ($2\ \mu\text{M}$) after the injection of BAPTA ($10\ \text{mM}$ in the electrode) in the PSC covering the portion of the nerve terminal recorded by the focal electrode. Note that tg induced a rapid rise, followed by a second, slower increase in EPP amplitude (whole NMJ activity) but induced only a small and rapid rise in EPC amplitude (portion of the terminal covered by the injected PSC). *E*, EPC amplitude before and after the injection of IP_3 ($10\ \text{mM}$ in the electrode) in the PSC covering the portion of the nerve terminal recorded by the focal electrode. EPCs were evoked by motor nerve stimulation at $0.2\ \text{Hz}$. Note that the injection of IP_3 in PSCs potentiated transmitter release.

lular electrode (respectively, $58.1 \pm 3.8\%$ and $48.8 \pm 3.1\%$; $p = 0.0007$, Student's t test). Furthermore, the differences between the focal and intracellular depression in control ($-0.9 \pm -0.7\%$) and after BAPTA injection ($-10.0 \pm -1.2\%$) were significantly different ($p = 0.003$, Student's paired t test) (Fig. 7*B,C*). These results indicate that PSCs can modulate synaptic transmission efficiently under physiological conditions during high frequency activity. The more pronounced depression after BAPTA injection in PSCs is consistent with the fact that the Ca^{2+} elevation in PSCs potentiated transmitter release.

DISCUSSION

In this study the differential modulation of transmitter release by presynaptic and glial internal stores has been investigated. The blockade of the ATPase pump of presynaptic internal stores

reduced the clearance of cytoplasmic Ca^{2+} , which resulted in a potentiation of transmitter release. A rise in intracellular Ca^{2+} in PSCs by either blocking the ATPase pump or inducing Ca^{2+} release by IP_3 injection also potentiated transmitter release. Moreover, synaptic depression was more pronounced after BAPTA injection in PSCs. These results demonstrate that, besides the presynaptic element, there is an important Ca^{2+} -dependent component of glial activation that intervenes in the regulation of synaptic efficacy.

Presynaptic Ca^{2+} stores participate in Ca^{2+} clearance

The blockade of the ATPase pump by tg led to a prolonged duration of Ca^{2+} responses elicited by single action potentials and to a rapid and sustained rise in EPP amplitude and MEPP frequency. The presynaptic effect of tg was stable over time, as

Figure 7. Synaptic depression is modulated by PSCs. *A*, Changes in EPC (gray trace) amplitude and EPP (black trace) amplitude expressed as a percentage of control before, during, and after the induction of high frequency depression (10 Hz/80 sec) at the frog NMJ. Note that the focal and intracellular recordings show the same amplitude of depression. After the injection of BAPTA into a PSC (break in x-axis) the focal electrode (gray trace), which records synaptic transmission under the injected PSC, shows a bigger depression than the intracellular one, thus suggesting that the presence of BAPTA in PSC blocked the potentiating effect on synaptic transmission. *B*, Mean $\pm$ SEM of synaptic depression relative to control level for intracellular and extracellular recordings before and after BAPTA injection into PSCs. Note that, after BAPTA injection, depression detected by the focal electrode was significantly greater. *C*, Mean $\pm$ SEM of the difference between intracellular and extracellular depression in control and after BAPTA injection into PSCs. Note that, after BAPTA injection, the difference between the amplitude of depression recorded by the intracellular and focal electrodes was significantly greater.

indicated by the differential occlusion of the slow component of tg effects on transmitter release after BAPTA injection in PSCs. In addition, the regulation of MEPP frequency appears activity-dependent, because no effect of tg was observed in the absence of nerve activity. This is consistent with the results of Narita et al.

(1998), who reported an activity-dependent CICR regulation of transmitter release.

Our results indicate that the presynaptic Ca^{2+} stores are empty at rest because tg had no effect on resting nerve terminals, whereas it induced a transient Ca^{2+} rise in PSCs under the same conditions. Therefore, enhancement of transmitter release cannot be accounted for by a rise in overall presynaptic resting Ca^{2+} after the depletion of nerve terminal internal stores. Rather, our data suggest that the increase in transmitter release is attributable to a local accumulation of resting Ca^{2+} after evoked synaptic activity. Indeed, the sustained rise in spontaneous release suggests that a local increase in resting Ca^{2+} occurred near the release site, although no rise in overall resting Ca^{2+} was detected during continuous, low frequency stimulation. However, a Ca^{2+} rise in the whole nerve terminal was observed as a consequence of prolonged stimulation at higher frequency. The lack of effect of tg on facilitation is consistent with the absence of rise in resting Ca^{2+} and might suggest that the local rise in resting Ca^{2+} that occurred near release sites, as suggested by the rise in MEPP frequency, was too low to affect the facilitation process.

Our results further suggest that the presynaptic ATPase Ca^{2+} pump regulates the level of cytoplasmic Ca^{2+} ions after their entry is elicited by presynaptic activity. ATPase pumps generally are associated with the smooth endoplasmic reticulum (SER; Lytton et al., 1992; Poulsen et al., 1995; Fierro et al., 1998), located away ($\sim 1 \mu\text{m}$) from release sites. This suggests that the prolongation in the duration of Ca^{2+} responses elicited by single action potentials would be caused by a reduced capacity to buffer Ca^{2+} ions escaping the active zones where Ca^{2+} channels are clustered (Blaustein et al., 1978; Robitaille et al., 1990; Cohen et al., 1991) and where Ca^{2+} concentration is high (Adler et al., 1991; Augustine et al., 1991). However, this possibility is difficult to reconcile with our observation that MEPP frequency was increased without a rise in resting fluorescence, which suggests that the elevation of resting Ca^{2+} must be spatially restricted around active zones. Moreover, the concentration of Ca^{2+} is controlled quickly and drops exponentially outside the mouth of Ca^{2+} channels, extruded by the Ca^{2+} pumps and various Ca^{2+} exchangers (Smith and Augustine, 1988; Adler et al., 1991; Llinas et al., 1992; Stanley, 1997). Hence, a necessary alternative is that tg-sensitive Ca^{2+} pumps should be localized near release sites. One possibility might be that the ATPase pumps are located in the membrane of synaptic vesicles because these organelles are located near active zones (Fig. 8) and could buffer and release Ca^{2+} within the active zone region (Grohovaz et al., 1996; Fossier et al., 1999; Lysakowski et al., 1999). This possibility is supported further by recent observations at the frog NMJ (Narita et al., 2000; Soga et al., 2000) where synaptic vesicles appear to be essential in CICR regulation of transmitter release.

Regulation of transmitter release by Ca^{2+} stores of PSCs

PSC Ca^{2+} stores are filled at rest (Jahromi et al., 1992; Robitaille, 1995) and display a Ca^{2+} elevation on activation by ACh and ATP released by the nerve terminal during synaptic activity (Jahromi et al., 1992; Robitaille, 1995; Robitaille et al., 1997). Our results further suggest that the activation of PSC receptors leads to the production of IP_3 and the activation of IP_3 receptors on internal stores. Hence, it appears that the internal stores of Ca^{2+} in PSCs serve as a signaling system in reaction to high frequency synaptic activity (Figs. 6, 7). This frequency dependence is confirmed by our observation that BAPTA injection in PSCs had no

Figure 8. Differential regulation of transmitter release by presynaptic and glial internal stores. *A*, Diagram of a cross section of a presynaptic nerve terminal covered by a perisynaptic Schwann cell (PSC). In the presynaptic terminal, a blockade of the ATPase pump with tg led to a

effect on synaptic transmission during a low level of synaptic activity (Fig. 6C).

Ca^{2+} release from PSC internal stores potentiates transmitter release because BAPTA injection in PSCs occluded the transient increase of transmitter release produced by tg, and IP_3 injection in PSCs induced a potentiation of transmitter release. It is unlikely that the potentiation of transmitter release results from the direct extrusion of Ca^{2+} from PSCs and accumulation around the nerve terminal. Indeed, the time course of tg-induced glial effects on transmitter release persists for ~45 min after the rise in Ca^{2+} in PSCs has terminated. Rather, the time course of the effects strongly suggests that the glial-mediated effects on synaptic transmission are initiated by second messenger cascades producing neuroregulatory substances (Fig. 8). Interesting candidates for such an action are prostaglandins because they are known to potentiate transmitter release of the amphibian NMJ (Madden and Van der Kloot, 1985) and their synthesizing enzymes are present in PSCs (Pappas et al., 1999). However, the presynaptic mechanisms that are targeted by this glial modulation remain unknown.

Interestingly, glial internal stores appear to be regulated by IP_3 receptors (Araque et al., 1998, 1999; Bezzi et al., 1998; this study), whereas presynaptic internal stores are associated preferentially with ryanodine receptors and are controlled by CICR mechanisms (Peng, 1996; Smith and Cunneane, 1996; Lin et al., 1998; Krizaj et al., 1999; Schwartz et al., 1999). This is consistent with the mode of activation of glial and presynaptic compartments in which PSCs, and glial cells in general, are activated by neurotransmitters via G-protein-coupled receptors, whereas presynaptic internal stores are regulated by the main event triggering release, that is, the entry of Ca^{2+} . Hence, these data indicate that synaptic efficacy is regulated differentially by the IP_3 -driven glial stores and by the presynaptic CICR mechanisms.

Glial cells as balanced feedback modulators of synaptic efficacy

The present findings further support the concept that perisynaptic glial cells play an active role in regulating synaptic transmission (Kang et al., 1998; Newman and Zahs, 1998; Robitaille, 1998;

reduction in the clearance of Ca^{2+} , whereas it resulted in a release of Ca^{2+} from the internal stores of PSCs. The release of Ca^{2+} from PSC internal stores is regulated by IP_3 receptors. *B*, Schematic representation depicting the relative contribution of the presynaptic and the glial components of tg effects on transmitter release. *C*, Proposed functional model of the ATPase pump of internal stores of presynaptic terminals, based on the results presented in this study. According to the dynamics of Ca^{2+} entry and handling around an active zone in the nerve terminal and the local changes in presynaptic resting Ca^{2+} level produced by tg, we propose that the ATPase pump is located in the membrane of synaptic vesicles located around release sites. They are located in a zone (gray zone, ~50 nm) in which the regulation of Ca^{2+} dynamics likely will affect transmitter release. Hence, this suggests that synaptic vesicles might act as an autoregulatory mechanism in transmitter release. *D*, Proposed model of the Ca^{2+} -dependent glial regulation of transmitter release. Because the duration of the PSC-dependent regulation of transmitter release by tg outlasts by several tens of minutes the duration of the resultant rise in intracellular Ca^{2+} , we propose that the PSC regulation is mediated by the Ca^{2+} -dependent production of neuromodulatory glial factors. Hence, as a consequence of synaptic activity, glial receptors would be activated, triggering IP_3 production by phospholipase C, which would result in the release of Ca^{2+} from internal stores. This Ca^{2+} then would be pumped back partially into the stores by the ATPase but also would activate Ca^{2+} -dependent second messenger cascades, leading to the production of membrane-permeant neuromodulatory substances that would reach the presynaptic nerve terminal to regulate the release machinery.

Araque et al., 1999; Castonguay et al., 2001). Indeed, during high frequency depression BAPTA injection into PSCs prevented glial Ca^{2+} -dependent potentiation, which resulted in a more pronounced synaptic depression. Moreover, it was shown at the NMJ that PSCs contribute to the production of synaptic depression and, hence, are involved in a feedback regulatory synapse–glia–synapse loop (Robitaille, 1998). According to these observations and the data presented here, it appears that PSCs at the amphibian NMJ can potentiate as well as depress transmitter release. The difference in the results obtained by Robitaille (1998) and in the present study are likely attributable to the cellular mechanisms targeted in the different experiments. Indeed, the involvement of PSCs in the regulation of synaptic efficacy was tested by interfering with G-protein activity (Robitaille, 1998), which has a large spectrum of effects because almost all PSC activities are regulated via this mechanism (Jahromi et al., 1992; Georgiou et al., 1994; Robitaille, 1995; Robitaille et al., 1997). Hence, a large number of events must have been perturbed in addition to Ca^{2+} regulation, which is the only element that has been affected in the present study.

It appears that PSCs can increase and decrease the efficacy of the synapse, using different cellular mechanisms in which the PSC-mediated potentiation of transmitter release would occur in a Ca^{2+} -dependent manner, whereas the depression would be Ca^{2+} -independent and based on other second messenger cascades. Alternatively, because an elevation of Ca^{2+} from PSC internal stores occurs during the glial-mediated potentiation and depression, a possibility is that the differential activation of PSCs may depend on the concentration and time course of the Ca^{2+} increase. Furthermore, the size and duration of Ca^{2+} responses in PSCs are graded with the level of transmitter release, as suggested by the frequency dependence of the glial Ca^{2+} responses (Jahromi et al., 1992). Hence, the balance between glial depression and potentiation may reside in the different patterns of Ca^{2+} elevation that are observed under different synaptic conditions. A similar mechanism has been reported for the selective production of long-term potentiation and depression in hippocampal neurons (Yang et al., 1999).

The evidence that PSCs can potentiate and depress transmitter release indicates that glial cells have the potential to adjust to the efficacy of the synapse according to its level of activity. This would provide perisynaptic glial cells with a unique feature to balance the efficacy of the synaptic elements in a local synapse–glia dynamic feedback loop.

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