Molecular Substrates Mediating Lanthane-Evoked Neurotransmitter Release in Central Synapses

ChiHye Chung,1 Ferenc Deák,1 and Ege T. Kavalali1,2

Departments of 1Neuroscience and 2Physiology, University of Texas Southwestern Medical Center, Dallas, Texas

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Chung C, Deák F, Kavalali ET. Molecular substrates mediating lanthanide-evoked neurotransmitter release in central synapses. J Neurophysiol 100: 2089–2100, 2008. First published August 20, 2008; doi:10.1152/jn.90404.2008. Noncanonical secretagogues such as hypertonicity or α-latrotoxin have been extremely informative in studying neurotransmission. Lanthanum and lanthanides can also trigger neurotransmitter release through an unknown mechanism. Here, we studied the effect of lanthanides on neurotransmission in hippocampal cultures. Application of 2 mM La3+ caused rapid and robust neurotransmitter release within seconds. In addition, transient application of La3+ uncovered a sustained facilitation of miniature neurotransmission. The response to La3+ was detectable at 2 μM and increased in a concentration-dependent manner <2 mM. Rapid effect of La3+ was independent of extracellular and intracellular Ca2+ and did not require La3+ entry into cells or activation of phospholipaseCβ. Synapses deficient in synaptobrevin-2, the major synaptic vesicle soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein in the brain, did not display any rapid release in response to La3+, whereas the slow facilitation of release detected after La3+ removal intact. In contrast, preincubation with intracellular Ca2+ chelators selectively attenuated the delayed release triggered by La3+. Moreover, synapses deficient in synaptotagmin-1 maintained a rapid response to La3+, suggesting that La3+-triggered neurotransmitter release does not require synaptotagmin-1 as a sensor. Therefore La3+ has two separate effects on synaptic transmission. For its rapid action, La3+ interacts with a target on the surface membrane, and unlike other forms of release, it triggers strictly synaptobrevin-2-dependent fusion, implying that in central synapses synaptobrevin-2 function is secretagogue specific. For the delayed action, La3+ may act intracellularly after its entry or through intracellular Ca2+ via a mechanism that does not require synaptobrevin-2.

INTRODUCTION

Noncanonical secretagogues such as hypertonicity or α-latrotoxin have been valuable tools in studying the mechanisms underlying neurotransmission. Hypertonicity is widely used to estimate the size of readily releasable pool (Rosenmund and Stevens 1996) and trigger neurotransmitter release independent of Ca2+ to assess selective defects in Ca2+ regulation of neurotransmission (Geppert et al. 1994). α-latrotoxin, on the other hand, helps to analyze the properties of unitary release events (Auger and Marty 1997; Bevan and Wendon 1984; Fesce et al. 1986) and has also been an extremely informative molecular bait to uncover several key components of synaptic junctions (Ushkaryov et al. 1992). Lanthanum (La3+) and other rare earth metals, collectively referred to as lanthanides, can also trigger neurotransmitter release but the mechanisms underlying their action is unknown. Previous studies showed that La3+ blocks the action potential evoked end plate potentials (Heuser and Miledi 1971; Miledi 1971) while increasing the frequency of miniature end plate potentials at the neuromuscular synapses of frog and goldfish (Bowen 1972; Curtis et al. 1986; Dekhuijzen et al. 1989; Heuser and Miledi 1971). Because these two consequences of La3+ treatment seemed inconsistent, La3+ and other lanthanides were often called to have dual action. Inhibition of evoked neurotransmission by La3+ is attributed to its potent ability to block voltage-gated Ca2+ channels (VGCCs) (Lansman 1990; Lansman et al. 1986; Reichling and MacDermott 1991). In addition, La3+ can also inhibit Ca2+ uptake by mitochondria (Mela 1969a,b), as well as by the plasma membrane Ca2+-ATPase (PMCA) (Herrington et al. 1996). However, it remains unclear how La3+ increases spontaneous neurotransmission in synapses. Moreover, the impact of La3+ on synaptic transmission in central synapses remains to be characterized. Increasing use of LaCl3 as a therapeutic agent such as in treatment for hyperphosphatemia further necessitates a better understanding of its impact on neurotransmission (Finn 2006). Therefore here we evaluated La3+’s potential as a tool to pinpoint unconventional signaling pathways regulating central synapses, which are inaccessible to canonical secretagogues.

In these experiments, La3+ application onto dissociated hippocampal cultures caused immediate neurotransmitter release (rapid effect) in a concentration-dependent manner. Interestingly, on La3+ washout, the frequency of spontaneous neurotransmission was increased, and this delayed release (delayed effect) was only slowly reversible. Other lanthanides such as praseodymium (Pr3+), gadolinium (Gd3+), erbium (Er3+), and yttrium (Y3+) could mimic La3+ effect in central synapses, implying these two actions (rapid effect vs. delayed effect) are common features of all lanthanides. Rapid effect of La3+ was independent of both extracellular and intracellular Ca2+ as well as activation of phospholipase C (PLC)β. In addition, the rapid action of La3+ was insensitive to heavy metal chelators, thus it does not require La3+ entry into the cell. In hippocampal cultures obtained from mice deficient in the synaptic vesicle soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein synaptobrevin-2, the rapid effect of La3+ was abolished, whereas the delayed effect was still detectable. In contrast, lowering internal [Ca2+] using Ca2+ chelators significantly attenuated the delayed effect while leaving the rapid La3+-triggered neurotransmission intact. Therefore La3+ has two separate effects on synaptic transmission. For its rapid action, La3+ seems to interact with a target on the surface.
membrane, triggering a SNARE-dependent fusion while La$^{3+}$ and/or [Ca$^{2+}$]$^{2-}$ is required for the delayed action. These multiple effects of La$^{3+}$ on neurotransmitter release in central synapses might mediate neurotoxic consequences of chronic exposure to La$^{3+}$ (Feng et al. 2006a,b).

**METHODS**

**Cell culture**

Dissociated hippocampal cultures were prepared from Sprague-Dawley rats or synaptotagmin-1–deficient mice pups (gift of Dr. T. C. Sudhof) as previously described (Kavalali et al. 1999). Synaptobrevin-2–deficient mice cultures were prepared at embryonic day 18, as previously described (Deak et al. 2004). Rats and mice were rapidly killed by decapitation after sedation by chilling on an ice-cold metal plate. All experiments with hippocampal cultures were performed during 14–21 days in vitro (DIV), the time period already known as the time that synapses become fully mature and represent the mature connections as in vivo (Mozhayeva et al. 2002).

**Electrophysiology**

Pyramidal neurons were voltage clamped to ~70 mV using whole cell patch-clamp technique, using an Axopatch 200B amplifier and Clampex 8.0 software (Molecular Devices, Sunnyvale, CA), filtered at 2 kHz, and sampled at 5 kHz. The pipette solution contained the following (in mM): 115 Cs-MeSO$_3$, 10 CsCl, 5 NaCl, 10 HEPES, 0.6 EGTA, 20 tetraethylammonium chloride, 4 Mg-ATP, 0.3 Na$_3$GTP, and 10 QX-314 (lidocaine N-ethyl bromide), pH 7.35, 300 mM osmol (Sigma, St. Louis, MO). A modified Tyrode solution was used as the extracellular solution with 2 mM Ca$^{2+}$ and 1 mM Mg$^{2+}$. The Tyrode solution contained the following (in mM): 150 NaCl, 4 KCl, 2 MgCl$_2$, 10 glucose, 10 HEPES, and 2 CaCl$_2$, pH 7.4, 310 mM osmol; 0 mM Ca$^{2+}$ solution had the same ionic composition except Ca$^{2+}$ concentration and contained 1 mM EGTA. To record and isolate miniature excitatory postsynaptic currents (mEPSCs), picROTOX (PTX; 50 mM; Sigma) and TTX were added to the bath solution. To chelate intracellular Ca$^{2+}$, neurons were pretreated with either 2 mM BAPTA-AM (Molecular Probes, Eugene, OR) for 30 min or 100 mM EGTA-AM (Molecular Probes) for 10 min. The exchange between extracellular solutions such as challenge with a 2 mM La$^{3+}$-containing solution was achieved by direct perfusion of solutions on the field of interest by gravity. A two-tailed unpaired t-test was used for statistical comparisons, and values are given as means ± SE.

**Lentivirus production**

A 12 amino acid–inserted synaptobrevin 2 mutant (12-ins syb 2) construct was generated as described before (Deak et al. 2006a). HEK 293 cells were transfected with the Fugene 6 transfection system (Roche Molecular Biochemicals) with the expression plasmid and two helper plasmids, which are delta 8.9 and vesicular stomatitis virus G protein (3 μg of each DNA per 75-cm$^2$ flask of HEK cells). After incubation of HEK cells at 37°C for 48 h, lentivirus-containing culture medium was harvested and filtered at a 0.45-μm pore size before use for infection. Synaptobrevin 2–deficient mice cultures were infected with 12-ins syb 2 at 4 DIV by adding 400 μl of viral suspension to each well. Patch-clamp recording was carried out at 14–21 DIV.

**Ca$^{2+}$ imaging**

Ten microliters of 4% pluronic F-127 (low UV absorbance, Molecular Probes) and 3 μM Fura-2-AM (Invitrogen, Eugene, OR) were added to growth media of 18–19 DIV hippocampal cultures, and cells were incubated for 45 min at 37°C. Cells were transferred to the chamber and washed with Tyrode’s solution, containing nominal Ca$^{2+}$ and 1 mM EGTA, for a couple of minutes. Images were obtained using DeltaRAM illuminator (Photon Technology International, Birmingham, NJ) and an IC-300 camera (Photon Technology International) at a frequency of 0.5 Hz. Baseline was recorded for 1 min before stimulation. We used 2 and 20 mM of Ca$^{2+}$ or La$^{3+}$ for stimulation. Data analysis was performed using ImageMaster Pro software (Photon Technology International).

**Statistical analysis**

ANOVA was used for statistical analysis of all multiple comparison experiments. The Student’s t-test (2-tailed) was used for pairwise comparisons.

**RESULTS**

**La$^{3+}$ application triggers rapid neurotransmitter release**

To study the effect of La$^{3+}$ on neurotransmitter release from central synapses, we used a whole cell patch-clamp technique in rat dissociated hippocampal cultures and examined the basic properties of synaptic transmission. Bath application of 2 mM La$^{3+}$ caused rapid neurotransmitter release (Fig. 1, A and B). This rapid effect of La$^{3+}$ has not been detected earlier, because in previous studies of the neuromuscular junction preparations, the La$^{3+}$ effect was monitored and analyzed not in real time but after incubation for a certain period. In these earlier experiments, the incubation times ranged between 1 and 4 min, and in some cases, synapses were incubated with La$^{3+}$ for 45 min up to hours (Curtis et al. 1986; Heuser and Miledi 1971). In contrast, here, we monitored the change of synaptic responses while bath-applying La$^{3+}$. Thus we could observe prompt neurotransmitter release, which we refer to as the rapid effect, both in excitatory and inhibitory synapses (Fig. 2). With sustained La$^{3+}$ application, the neurotransmitter release was decreased, presumably because of depletion of available vesicles akin to synaptic responses seen after hypertonic sucrose application (Moulder and Mennerick 2005; Rosenmund and Stevens 1996).

Next, we compared the kinetics of La$^{3+}$-evoked release to release evoked by hypertonic sucrose perfusion. Here, we first applied 2 mM La$^{3+}$ solution for 30 s and a +500 mM osmol hypertonic sucrose solution for 30 s after a 3-min wash period to compare La$^{3+}$ effect to the effect of hypertonicity on release (Fig. 1, A–C). The postsynaptic responses, quantified as average cumulative charge transfer induced by 2 mM La$^{3+}$ during the first 10 s of application, were almost one half of the postsynaptic charge transfer caused by hypertonic sucrose solution (n = 20; Fig. 1, D and G).

The following sets of experiments were aimed to test the origin of vesicles mobilized by La$^{3+}$. To study whether La$^{3+}$ mobilizes the same readily releasable pool (RRP) of synaptic vesicles that fuse in response to hypertonic sucrose application, we applied 2 mM La$^{3+}$ 20 s after initial application of hypertonic sucrose in the continued presence of hypertonic solution. If La$^{3+}$ induced the fusion of RRP
vesicles, we expected to observe no additional release. However, if La³⁺ mobilizes a separate set of synaptic vesicles, La³⁺ application on top of hypertonicity would be expected to trigger a comparable amount of neurotransmitter release regardless of hypertonic sucrose application. In these experiments, prior exposure to +500 mOsm hypertonic sucrose solution resulted in a 70% reduction in La³⁺-induced neurotransmitter release compared with cells that were challenged with 2 mM La³⁺ alone (compare gray bars in Fig. 1, D and F; P < 0.001). Perfusion of 2 mM La³⁺ on top of +500 mOsm hypertonic sucrose solution was able to augment release by an additional 17% (n = 6), whereas 2 mM La³⁺ typically triggered 53% of release induced by hypertonicity alone (n = 20; Fig. 1G). Taken together, these findings suggest that the pools of vesicles mobilized by La³⁺ and hypertonic sucrose are largely overlapping but not completely identical.

As briefly mentioned earlier, the rapid effect of La³⁺ was observed both in excitatory and inhibitory synapses (Fig. 2, A and B). When we varied La³⁺ concentrations from 2 μM to 2 mM, the rapid effect of La³⁺ was detectable at 2 μM and showed a concentration-dependent increase in rate and magnitude (Fig. 2). The half rise time of release (t½) at 2 μM was 11.59 ± 2.43 nC/s and reached a maximum of 1.92 ± 0.4 nC/s at 2 mM La³⁺ (Fig. 2D).

La³⁺ has been shown to activate TRPC4 and 5 channels specifically through an unknown mechanism (Jung et al. 2003), and these transient receptor potential (TRP) channels are expressed in hippocampal neurons (Chung et al. 2006). To verify that La³⁺ acts by releasing neurotransmitters presynaptically and does not have a direct postsynaptic effect, we applied La³⁺ in the presence of TTX, PTX, and CNQX to block voltage-gated Na⁺ channels, GABA receptors, and postsynaptic AMPA receptors, respectively. If La³⁺ response was caused by direct activation of postsynaptic TRP channels, we would expect a similar pattern of activity in the absence of neurotransmitter receptor activation. However, we could completely eliminate La³⁺ response with TTX, PTX, and CNQX and thus rule out the possibility that postsynaptic TRP channels were responsible for the neurotransmitter release induced by La³⁺ (Fig. 2E).

La³⁺ application does not alter the properties of unitary synaptic responses

To further evaluate the specificity of La³⁺ action on neurotransmitter release, we probed the properties of unitary transmission triggering by low concentrations of La³⁺. Here, we applied 2 μM La³⁺ because higher concentrations of La³⁺ caused several overlapping quantal events, which were hard to assess individually. Unitary release events activating a specific type of receptor were recorded with the aid of pharmacological blockers. In this way, we measured a large number of AMPA-mEPSCs, N-methyl-D-aspartate (NMDA)-mEPSCs, and mIPSCs, before and during La³⁺ application in isolation. This analysis showed no significant changes in the 10–90% rise times and amplitudes of individual events before and after La³⁺ application (Fig. 3). These results indicate that the neurotransmitter release triggered by La³⁺ is quantal in nature and not likely caused by a nonspecific disruption of presynaptic terminals or...
Europium (Eu$^{3+}$) in both excitatory and inhibitory synapses is shared by other lanthanides, we applied 2 mM of La$^{3+}$ to test whether the stimulatory action of La$^{3+}$ results in rapid neurotransmitter release both in excitatory and inhibitory synapses (Alnaes and Rahamimoff 1974; Bowen 1972; Metral et al. 1978; Molgo et al. 1991). To test whether the stimulatory action of La$^{3+}$ on neurotransmitter release from hippocampal synapses is shared by other lanthanides, we applied 2 mM of each lanthanide. Among these lanthanides, Pr$^{3+}$, Yb$^{3+}$, and Eu$^{3+}$ were able to trigger neurotransmitter release. The relative amount of average charge transfer induced by 2 mM Gd$^{3+}$, Eu$^{3+}$, Yb$^{3+}$, and Pr$^{3+}$ (n = 4, each) is plotted as a fraction of the cumulative charge transfer induced by 2 mM La$^{3+}$.

**Increased spontaneous neurotransmission after removal of La$^{3+}$**

After La$^{3+}$ washout, the frequency of mEPSCs or mIPSCs was highly increased, which we refer as the “delayed effect,” and the increased level of mPSCs was maintained for ≥10 min (Fig. 5). The ability of La$^{3+}$ to cause a sustained increase in the frequency of mPSCs has been already documented in neuromuscular junction preparations (Curtis et al. 1986; Heuser and Miledi 1971). We could also consistently observe this effect in central synapses as a delayed consequence on withdrawal of La$^{3+}$, distinct from the rapid effect we observed during La$^{3+}$ application. Delayed effect was also concentration dependent in both mEPSCs and mIPSCs (Fig. 5, A–C). Removal of 2 μM
La$^{3+}$ solution caused a 1.74 ± 0.16-fold increase in mEPSC frequency compared with rest, whereas at 2 mM, this increase was 9.91 ± 1.53-fold (n = 5–9). Interestingly, the fold increase in the frequency of mEPSCs was greater than the change in mIPSCs (Fig. 5C). The fold increase in mIPSC frequencies after 2 µM La$^{3+}$ was 1.11 ± 0.2 and reached a maximum of 4.48 ± 0.17-fold at 200 µM (n = 5–9). In contrast, we observed no significant difference in amplitudes of mEPSCs and mIPSCs (Fig. 5D).

Rapid effect of La$^{3+}$ is independent of extracellular and intracellular Ca$^{2+}$ and does not require La$^{3+}$ entry into a cell

What is the underlying mechanism of lanthanide-evoked neurotransmission at central synapses? Our data suggest that La$^{3+}$ can stimulate a releasable pool of vesicles presumably shared by the hypertonic sucrose stimulation, and La$^{3+}$ application has no detectable direct postsynaptic effect (e.g., activation of TRP channels; Fig. 1H). In the next set of experiments, we pursued how La$^{3+}$ may mobilize the RRP at central synapses. La$^{3+}$ may trigger neurotransmitter release by one of three possible scenarios. First, La$^{3+}$ might trigger Ca$^{2+}$ entry presynaptically (e.g., via presynaptic TRP channels) or cause intracellular Ca$^{2+}$ release and augment Ca$^{2+}$-dependent neurotransmission. In this case, La$^{3+}$ would require Ca$^{2+}$ as a mediator for its action. Second, La$^{3+}$ might enter into a cell and cause neurotransmitter release by substituting Ca$^{2+}$. This scenario predicts that the action of La$^{3+}$ does not require Ca$^{2+}$ but requires its entry to a cell to surrogate Ca$^{2+}$. La$^{3+}$ has a roughly similar ionic radius (3.1 Å) to Ca$^{2+}$ (2.8 Å) (Lettvin et al. 1964); thus VGCCs can be a possible entryway of La$^{3+}$ into a cell (Lansman 1990; Lansman et al. 1986). In addition, several lines of evidence including electron micrographs (Pecot-Dechavassine 1983) showed that La$^{3+}$ could also enter a cell via the Na$^{+}$/Ca$^{2+}$ exchanger (Powis et al. 1994; Reeves and Condrescu 2003; Shimmizu et al. 1997). Finally, if La$^{3+}$ neither stimulates Ca$^{2+}$ signaling nor enters into a cell for its rapid action, La$^{3+}$ might act at the surface of membrane and interact with surface receptors, which can eventually evoke neurotransmission. An earlier study suggested that La$^{3+}$ might act at superficial side of sartorius muscle (Weiss 1970). More recently, another study in synaptosomes also proposed that La$^{3+}$ may act extracellularly (Lopatina et al. 2005), possibly by modifying membrane lipid packing (Verstraeten et al. 1997).

To elucidate the underlying mechanism of prompt La$^{3+}$-evoked neurotransmission, we tested the first scenario that La$^{3+}$ may act by increasing Ca$^{2+}$-evoked neurotransmission. We carried out experiments similar to above with extracellular solution containing 1 mM EGTA and nominal Ca$^{2+}$, which is expected to remove extracellular Ca$^{2+}$ that can enter a cell on La$^{3+}$ stimulation. In addition, to test whether increase in intracellular Ca$^{2+}$ mediates rapid action of La$^{3+}$ either by release from internal stores or by attenuated uptake by mitochondria, cells were treated with 100 µM EGTA-AM for 10 min or 2 µM BAPTA-AM for 30 min before La$^{3+}$ stimulation. We examined the effect of 2 mM La$^{3+}$ in the presence of 1 mM EGTA and nominal extracellular Ca$^{2+}$. Chelating extracellular Ca$^{2+}$ as well as buffering intracellular Ca$^{2+}$ could not antagonize the rapid action of La$^{3+}$ (Fig. 6A). La$^{3+}$ could still cause a similar amount of release independent of intracellular or extracellular Ca$^{2+}$. In the presence of EGTA, the cumulative charge transfer by 2 mM La$^{3+}$ was 1.34 ± 0.39 nC, which is comparable to the control experiments shown in Fig. 1. The release induced by La$^{3+}$ after incubation with EGTA-AM or BAPTA-AM was 0.94 ± 0.14 and 1.18 ± 0.22 nC, respectively. These findings suggest that the neurotransmitter release by La$^{3+}$ is not likely caused by its potential effects on Ca$^{2+}$ signaling pathways. In agreement with these observations, depletion of Ca$^{2+}$ from internal Ca$^{2+}$ stores by for 30-min treatment of neurons with 1 µM thapsigargin did not impair release induced by subsequent application of 2 mM La$^{3+}$ (Supplementary Fig. S1).

These experiments using plasma membrane–permeable heavy metal chelators partly argue against the second possibility because EGTA-AM or BAPTA-AM is not only able to chelate Ca$^{2+}$ but also La$^{3+}$. In fact, EGTA can effectively chelate heavy metals including La$^{3+}$ even with higher affinity than it has for Ca$^{2+}$ (Siliren and Martell 1971), implying that pretreatment with EGTA-AM or BAPTA-AM could significantly eliminate intracellular La$^{3+}$ and block its action. For this reason, although we applied 2 mM La$^{3+}$ under the conditions shown in Fig. 6A, we estimate the actual concentration of La$^{3+}$ in the solution would be <2 mM. Previous studies showed that La$^{3+}$ could enter into a cell through the Na$^{+}$/Ca$^{2+}$ exchanger albeit with a slow time course around 30 s (Powis et al. 1994; Reeves and Condrescu 2003). Therefore the Na$^{+}$/Ca$^{2+}$ exchanger is not a likely mediator of La$^{3+}$ entry for its rapid action because 30 s is longer than the typical response time to La$^{3+}$ application (<5 s).

Nevertheless, to examine the second possibility more directly, we incubated cells with 200 µM Cd$^{2+}$, a potent pore

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1 The online version of this article contains supplemental data.
action of La$^{3+}$. If the rapid action of La$^{3+}$ requires its entry through Ca$^{2+}$ channels, preincubation with 200 µM Cd$^{2+}$ would be expected to impede the rapid effect of La$^{3+}$. However, 200 µM Cd$^{2+}$ did not interrupt the rapid action of La$^{3+}$ because La$^{3+}$ could trigger substantial release (1.35 ± 0.19 nC) in its presence (Fig. 6B). This finding indicates that the possible entry of La$^{3+}$ VGCCs is not required for its rapid effect.

In addition to Ca$^{2+}$ influx through VGCCs, presynaptic Ca$^{2+}$ can be regulated by several mechanisms such as the Na$^{+}$/Ca$^{2+}$ exchanger, Ca$^{2+}$ uptake and release from mitochondria, and PMCA. La$^{3+}$ is also known as a strong inhibitor of Ca$^{2+}$ uptake by mitochondria (Mela 1969a,b) and as an inhibitor of PMCA (Herrington et al. 1996), indicating that La$^{3+}$ might interfere with Ca$^{2+}$ homeostasis in presynaptic terminals in multiple ways, leading to augmentation of neurotransmitter release. Besides the activation of channels or their permeation into cells, lanthanides can also bind to Ca$^{2+}$ receptors, which are found in central nerve terminals (Smith et al. 2004), and these receptors are typically coupled to the activation of the PLC$\beta$ pathway (Breitwieser et al. 2004). To test the possibility that binding of La$^{3+}$ to Ca$^{2+}$ receptors and consequent activation of PLC$\beta$ pathway is responsible for its rapid action, we treated hippocampal neurons with U73122, a PLC$\beta$ inhibitor, for 10 min before 2 mM La$^{3+}$ stimulation. After U73122 treatment, 2 mM La$^{3+}$ still caused neurotransmitter release (1.34 ± 0.28 nC) comparable to its effect on untreated cells, indicating that U73122 treatment failed to disrupt the rapid action of La$^{3+}$ (Fig. 6B). Figure 6C summarizes the results of these experiments using 2 mM La$^{3+}$ under different conditions as a fraction of La$^{3+}$ responses in untreated cells. La$^{3+}$-evoked responses with nominal Ca$^{2+}$ and 1 mM EGTA containing extracellular solution, after EGTA-AM and BAPTA-AM treatment, were 1.19 ± 0.35 (n = 5), 0.84 ± 0.13 (n = 4), and 1.06 ± 0.20 nC (n = 6), respectively. Average postsynaptic charge transfer values after 200 µM Cd$^{2+}$ treatment or U73122 application were similar to controls [Cd$^{2+}$: 1.21 ± 0.17 nC (n = 7); U73122: 1.19 ± 0.25 nC (n = 6)].

In the next set of experiments, we evaluated the effectiveness of EGTA-AM treatment to inhibit rapid action potential−evoked release in response to 10-Hz stimulation (Fig. 6D). For this purpose, we evoked IPSCs (eIPSCs) for 1 s in 8 mM Ca$^{2+}$ before and after treatment with 100 µM EGTA-AM (Fig. 6E). These experiments showed that brief treatment with EGTA-AM is sufficient to lower the concentration of intracellular Ca$^{2+}$ and inhibit release (Fig. 6F and G). This finding further supports the premise that, if La$^{3+}$-evoked release was caused by a rise in intracellular Ca$^{2+}$, it should have been susceptible to EGTA-AM treatment.

Taken together, we ruled out two possibilities. First, the action of La$^{3+}$ does not seem to require rises in intracellular Ca$^{2+}$. Second, La$^{3+}$ entry through VGCCs or activation of PLC$\beta$ is unlikely to mediate a rapid effect of La$^{3+}$.

La$^{3+}$ entry proceeds with a slow time course and cannot account for the rapid release induced by La$^{3+}$

La$^{3+}$ might be able to enter into a cell via several poorly characterized pathways. Therefore in the next set of experiments, we aimed to monitor La$^{3+}$ entry optically using Fura-2-AM, a membrane-permeable derivative of the ratiometric
Ca\(^{2+}\)-selective fluorescent dye Fura-2. Fura-2’s affinity to La\(^{3+}\) is a 1,000-fold higher than its affinity to Ca\(^{2+}\) (Reeves and Condrescu 2003). In addition, in response to La\(^{3+}\) binding, Fura-2 shows a spectral shift (increase in fluorescence emission ratio in response to excitation at 340- vs. 380-nm wavelengths), similar to the shift seen after its Ca\(^{2+}\) binding (Reeves and Condrescu 2003).

In these experiments, Fura-2-AM was added in growth media for 45 min at 37°C (final concentration = 3 μM), and cells were transferred to the recording chamber and briefly washed with Tyrode’s solution containing nominal Ca\(^{2+}\) and 1 mM EGTA. We measured the fluorescence baseline in Tyrode’s solution in the presence of 1 mM EGTA for 1 min and applied 2 mM La\(^{3+}\) or Ca\(^{2+}\) to monitor the entry of each ion for 30 s. Because we detected a very small increase in the 340/380 ratio with 2 mM La\(^{3+}\) application, cells were challenged again with higher concentrations of La\(^{3+}\) or Ca\(^{2+}\) (20 mM) as a positive control. In this setting, we observed only a small increase in the 340/380 ratio when 2 mM La\(^{3+}\) was applied, in contrast to the rapid and robust increase in the 340/380 ratio by 2 mM Ca\(^{2+}\) (Fig. 7). This suggests that there is small, if any, entry of La\(^{3+}\) into a cell on its bath application. Second, the rise in the 340/380 ratio after La\(^{3+}\) application started slower than after Ca\(^{2+}\). The initiation of rise in the 340/380 ratio by La\(^{3+}\) is indicated by the black dotted line in Fig. 7B. Compared with the gray dotted line, which indicates the rise in the 340/380 ratio by Ca\(^{2+}\), there is a delay between the two lines, which is ≈15 s (Fig. 7B). We did not detect a significant difference between the baseline fluorescence ratios in cells treated with La\(^{3+}\) or Ca\(^{2+}\) (0.4458 for La\(^{3+}\), n = 55 from 6 coverslips and 0.434 for Ca\(^{2+}\), n = 30 from 3 coverslips). Thus it takes longer for La\(^{3+}\) to enter into a cell, and the entry occurs slower compared with the entry of Ca\(^{2+}\) (Fig. 7). This result strongly argues against the possibility that the rapid effect of La\(^{3+}\) is mediated by intracellular action of La\(^{3+}\).

After 20 mM La\(^{3+}\) application, the 340/380 ratio increased gradually, but we did not detect a decrease in the fluorescence signal on La\(^{3+}\) removal (Fig. 7A), which is consistent with the earlier observations in Chinese hamster ovary (CHO) cells (Reeves and Condrescu 2003). This finding suggests that La\(^{3+}\) is not readily extruded from cells, and it is buffered rather ineffectively once it is introduced. This observation may explain the hardly reversible nature of the delayed effect of La\(^{3+}\) on neurotransmitter release.

**Rapid effect of La\(^{3+}\) is strictly dependent on the vesicular SNARE protein synaptobrevin-2**

Our results thus far indicated that La\(^{3+}\) triggers rapid neurotransmitter release independent of its entry into cells. Thus it is likely that La\(^{3+}\) acts at an extracellular site, presumably by binding to a putative receptor or by direct modification of plasma membrane lipids to trigger neurotransmitter release (Andjus et al. 1997; Cheng et al. 1999). To test whether the form of release triggered by La\(^{3+}\) shares the same molecular machinery as the physiological action potential–driven release, we took advantage of mice deficient in the synaptic vesicle protein synaptobrevin-2. Synaptobrevin-2 (also called VAMP-2) is a SNARE protein, which interacts with the plasma membrane–bound syntaxin and SNAP-25 to trigger neurotransmitter release (Sollner et al. 1993). Together, these proteins form a four-helix bundle (Otto et al. 1997; Sutton et al. 1998). Analysis of cultured hippocampal neurons from synaptobrevin-2 (syb2) knockout mice showed less severe impairment of spontaneous and hypertonic sucrose-induced release compared with evoked neurotransmitter release (Schoch et al. 2001). In addition, loss of synaptobrevin leads to a facilitation of release during high-frequency stimulation and a defect in fast endocytosis (Deak et al. 2004).

To test whether SNARE complex–mediated fusion mechanism involving synaptobrevin-2 (syb2) mediates the rapid action of La\(^{3+}\), we applied 2 mM La\(^{3+}\) onto cortical or hippocampal cultures obtained from syb2 knockout mice. Synapses in cultures obtained from wildtype mice showed the

![Fig. 7](http://jn.physiology.org/DownloadedFrom)
same sensitivity to La$^{3+}$ as responses from wildtype rat cultures. Interestingly, the rapid effect of La$^{3+}$ was completely abolished in syb2-deficient cultures (Fig. 8B, middle), supporting the premise that rapid action of La$^{3+}$ requires functional SNARE proteins. The average amount of neurotransmitter release triggered by 2 mM La$^{3+}$ in syb2-deficient cultures was 0.05 ± 0.01 nC (n = 14), which was indistinguishable from the baseline level of activity (0.03 ± 0.01 nC; n = 4). In contrast, parallel control experiments in wildtype cultures showed normal release in response to La$^{3+}$ (0.86 ± 0.11 nC, n = 6; Fig. 8, B and C). In the same set of experiments, application of 2 mM Gd$^{3+}$ was also ineffective in triggering rapid neurotransmitter release in syb2-deficient cultures. In a separate set of experiments, we tested whether the loss of La$^{3+}$ response in syb2-deficient synapses was indeed caused by the absence of syb2 by reintroducing syb2. In syb2-deficient cultures infected with syb2, the charge transfer during 30 s of 250 μM La$^{3+}$ was 1.11 ± 0.11 nC (n = 3), nearly 60% of neurotransmitter release compared with wildtype controls. This indicates that the defect shown in syb2-deficient culture was a specific consequence of the loss of syb2.

In a recent study, the ability of several synaptobrevin mutants to rescue synaptic transmission was tested in cultures from synaptobrevin knockout mice (Deák et al. 2006a). Mutants with insertion of 12 and 24 amino acids between the SNARE motif and the transmembrane domain (TMD) of synaptobrevin-2 showed that the physical distance between the two regions of synaptobrevin is indeed critical for the rescue of evoked fusion. Surprisingly, in contrast to the mutant with insertion of 24 amino acids, the 12 amino acids insertion mutant syb2 (12-ins syb2; Fig. 8A) completely rescued spontaneous release, suggesting that constraints on SNARE function during spontaneous fusion are more flexible than for evoked fusion. Interestingly, the La$^{3+}$ effect on neurotransmission was also strictly dependent on the distance between the SNARE motif and the TMD domain of synaptobrevin-2, because La$^{3+}$ application on synaptobrevin-2–deficient cultures infected with the lentivirus expressing 12-ins syb2 could not evoke any release (Fig. 8B, bottom). This result strongly suggests that La$^{3+}$-triggered transmission shares the same strict molecular constraints as rapid action potential-evoked fusion. This is in striking contrast to spontaneous fusion or hypertonic sucrose-evoked fusion, which both persist at a reduced but readily detectable level in synaptobrevin-2–deficient synapses (Deák et al. 2004; Schoch et al. 2001).

The delayed action of La$^{3+}$ was still detectable in cultures from synaptobrevin-2–deficient mice where the baseline rate of spontaneous release was typically 10-fold lower than in wildtype cultures (Fig. 8, D–G). The delayed effect could also be elicited by Gd$^{3+}$, which also manifested a rapid synaptobrevin-2–dependent stimulation of neurotransmitter release. However, rapid application and washout of 2 mM La$^{3+}$ or Gd$^{3+}$ caused significant increase in the frequency of mPSCs in knockout and wild-type cultures, suggesting that lanthanides have two distinct effects on central synapses. The frequencies of mPSCs after 2 mM La$^{3+}$ application showed more than fivefold increase (before La$^{3+}$: 0.34 ± 0.05 Hz; after La$^{3+}$: 1.97 ± 0.32 Hz; n = 14, P < 0.01) and a twofold increase after 2 mM Gd$^{3+}$ application (before Gd$^{3+}$: 0.46 ± 0.08 Hz; after Gd$^{3+}$: 1.01 ± 0.14 Hz; n = 8, P < 0.02; Fig. 8F). The change in the amplitude of mPSCs was not statistically significant under all conditions (Fig. 8G). Thus in contrast to their rapid effects on neurotransmission, the delayed effect of La$^{3+}$ and Gd$^{3+}$ is only partially dependent on SNARE interactions.

To probe the molecular machinery underlying the rapid effect of La$^{3+}$ further, we next took advantage of synaptotagmin-1 (syt-1)-deficient mice to examine whether La$^{3+}$ may use the same pathway as rapid Ca$^{2+}$-dependent synchronous re-

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**Fig. 8.** The rapid effect of La$^{3+}$ requires synaptobrevin-2 (syb2) function whereas the delayed effect of La$^{3+}$ is only partially dependent on syb2. A–C: rapid effect of La$^{3+}$ is strictly soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) dependent. A: the construct of syb2 and modified syb2 with 12 amino acid insertion (12ins as in from Deák et al. 2006a). B: hippocampal neurons from wildtype (WT) mice showed the same response to La$^{3+}$ as rat hippocampal neurons (top). In syb2-deficient (syb2−/−) neurons, rapid effect of La$^{3+}$ is completely abolished (middle). The infection with lentivirus expressing the i12ins syb2 construct failed to rescue the rapid effect of La$^{3+}$, whereas it successfully rescued spontaneous synaptic transmission (bottom). C: the average charge transfer triggered by 2 mM La$^{3+}$ in WT (n = 6), syb-2−/− (n = 14), and syb-2−/− infected with i12ins syb2 (n = 4). The lack of fully functional syb2 reduced the rapid effect of La$^{3+}$ (***P < 0.001). D–G: delayed effect of La$^{3+}$ is only partly SNARE independent. D and E: representative traces of WT and syb-2−/− hippocampal neurons before and after 2 mM La$^{3+}$ application. F and G: the bar graphs show the changes in the frequency and amplitude of spontaneous synaptic events before and after application of 2 mM La$^{3+}$ or Gd$^{3+}$. The changes in the frequency of mPSCs by La$^{3+}$ or Gd$^{3+}$ were statistically significant (***P < 0.001, n = 14 for 2 mM La$^{3+}$; *P < 0.05, n = 8 for 2 mM Gd$^{3+}$). The change in the amplitudes of mPSCs was not significant (P > 0.9).
lease. We first stimulated cortical cultures obtained from syt-1−/− mice to confirm the genotype electrophysiologically and applied 2 mM La3+ for 30 s (Fig. 9A). Neurons obtained from Syt-1−/− were used as controls. The absence of syt-1 failed to block the rapid effect of La3+ (Fig. 9B). The cumulative charge transfer triggered during the first 10 s of La3+ application was 1.87 ± 0.49 nC in syt-1−/− (n = 4) and 1.94 ± 0.22 nC in syt-1−/− (n = 6) cultures (Fig. 9). This result suggests that, although the La3+-triggered release is strictly dependent on synaptotagmin-1 as a sensor. This finding also argues against the possibility that the rapid effect of La3+ requires La3+ entry because intracellular La3+ could readily interact with synaptotagmin-1 and trigger release.

Intracellular chelation of Ca2+ and La3+ abolishes the delayed release evoked by La3+

To further characterize the delayed effect of La3+, we quantified the mPSC frequency before and after application of 2 mM La3+ after treatment of cultures with Ca2+-chelators such as EGTA-AM or BAPTA-AM (Fig. 10, A and B). These treatments substantially attenuated the delayed release while leaving the rapid effect intact (cf. Fig. 6A). Following incubation with 100 μM EGTA-AM for 10 min, the frequency of spontaneous release was largely unchanged compared with baseline after La3+ washout (before La3+: 3.84 ± 0.73 Hz; after La3+: 4.64 ± 1.01 Hz; n = 4). Incubation with 2 μM BAPTA-AM for 30 min also significantly diminished the delayed effect of La3+ (before La3+: 1.94 ± 0.61 Hz; after La3+: 3.58 ± 0.81 Hz; n = 6). In the presence of 1 mM EGTA and nominal Ca2+ in the extracellular environment, La3+ application caused an 11-fold increase in the frequency of mPSCs (from 2.96 ± 0.49 to 34.28 ± 1.36 Hz; n = 5, P < 0.001; Fig. 10C). This finding suggests that either intracellular Ca2+ or delayed entry of La3+ (e.g., Fig. 7) but not extracellular Ca2+ is responsible for the delayed release seen after removal of La3+ (Fig. 10D). In agreement with this premise, Cd2+ was ineffective in blocking the delayed effect, which suggests that Ca2+/La3+ entry through VGCCs was not required (Fig. 10D). The delayed effect was also insensitive to a specific inhibitor of PLCβ (U73122; Fig. 10D), which suggests that Ca2+ mobilization from internal stores that could be triggered by La3+ binding to the Ca2+ receptor is an unlikely source for this effect (Smith et al. 2004). Accordingly, depletion of Ca2+ from internal Ca2+ stores by for 30-min treatment of neurons with 1 μM thapsigargin did not impair the delayed release induced by subsequent application of 2 mM La3+ (Supplementary Fig. 1).

**Discussion**

Two components of neurotransmitter release triggered by La3+

In this study, we examined the effect of La3+ and other lanthanides, in particular Gd3+, on neurotransmitter release in dissociated hippocampal cultures and found that they triggered release in a rapid phase, which occurred within seconds, followed by a slow phase of release detectable after washout, which was hardly reversible. Our data showed that the rapid effect of La3+ is not mediated by augmenting existing Ca2+-dependent release, by increasing internal Ca2+, or by La3+ entry through various putative pathways. Instead, our findings suggest that La3+ acts extracellularly and mediates rapid neurotransmitter release, which requires the SNARE-dependent fusion machinery. The most salient feature of rapid La3+-triggered release is its strict dependence on synaptobrevin-2. We could only abolish the rapid effect of La3+ in the absence of synaptobrevin-2 but not synaptotagmin-1. This is surprising because other forms of Ca2+-dependent and -independent release including release driven by hypertonic sucrose are not fully eliminated in synaptobrevin-2-deficient synapses. Synaptobrevin-2 mutant with 12 amino acid insertion was also ineffective to rescue the rapid effect of La3+, suggesting that La3+-triggered fusion events are mechanistically closely related to fast evoked release. In addition, the properties of unitary synaptic responses during La3+ application were not different from events detected during normal spontaneous neurotransmission, providing further support that La3+ triggers neurotransmitter release by activating the conventional fusion machinery.

We could also detect a delayed effect of La3+, which is characterized as an increase in the frequency of mPSCs seen after removal of La3+ as previously shown in neuromuscular junction studies (Heuser and Miledi 1971). Delayed effect of La3+ is hardly reversible and persistent even in the absence of La3+, leading us to conclude that the entry of La3+ might mediate this delayed effect. Our data showed that pretreatment with well-known Ca2+-chelators, such as EGTA-AM or BAPTA-AM, significantly attenuated the delayed effect of La3+. As mentioned earlier, EGTA and BAPTA are effective chelators of La3+ and other lanthanides, in particular Gd3+, on neurotransmitter release in dissociated hippocampal cultures and found that they triggered release in a rapid phase, which occurred within seconds, followed by a slow phase of release detectable after washout, which was hardly reversible. Our data showed that the rapid effect of La3+ is not mediated by augmenting existing Ca2+-dependent release, by increasing internal Ca2+, or by La3+ entry through various putative pathways. Instead, our findings suggest that La3+ acts extracellularly and mediates rapid neurotransmitter release, which requires the SNARE-dependent fusion machinery. The most salient feature of rapid La3+-triggered release is its strict dependence on synaptobrevin-2. We could only abolish the rapid effect of La3+ in the absence of synaptobrevin-2 but not synaptotagmin-1. This is surprising because other forms of Ca2+-dependent and -independent release including release driven by hypertonic sucrose are not fully eliminated in synaptobrevin-2-deficient synapses. Synaptobrevin-2 mutant with 12 amino acid insertion was also ineffective to rescue the rapid effect of La3+, suggesting that La3+-triggered fusion events are mechanistically closely related to fast evoked release. In addition, the properties of unitary synaptic responses during La3+ application were not different from events detected during normal spontaneous neurotransmission, providing further support that La3+ triggers neurotransmitter release by activating the conventional fusion machinery.

![Fig. 9. The rapid effect of La3+ in synaptotagmin-1 deficient (syt1−/−) mice. A: as previously characterized, the synchronized evoked release is disrupted in cultures from syt1−/− mice. B: the rapid effect of La3+ was still observed in the absence of syt1. C: the bar graph shows that the postsynaptic charge transfer during 2 mM La3+ application in syt1−/− cultures (n = 6), which was not different from the response in its heterozygote littermates (n = 4; P > 0.8).](http://jn.physiology.org/content/100/10/2097/F9)

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might mediate neurotransmitter release. Alternatively, La\(^{3+}\) might use Ca\(^{2+}\) as a mediator for its delayed action. In this case, La\(^{3+}\) contributes indirectly to delayed effect by increasing internal Ca\(^{2+}\) concentration, presumably through its ability to inhibit Ca\(^{2+}\) uptake by mitochondria (Mela 1969a,b) or the function of PMCA (Herrington et al. 1996). Therefore both internal Ca\(^{2+}\) and La\(^{3+}\) uptake can be responsible for the delayed effect. In the absence of specific tools to clearly distinguish between Ca\(^{2+}\) and La\(^{3+}\), it is difficult to conclusively pinpoint whether the delayed release triggered by La\(^{3+}\) is a direct and indirect effect of La\(^{3+}\). However, these data strongly bolster our hypothesis that the rapid effect of La\(^{3+}\) does not require intracellular Ca\(^{2+}\) or La\(^{3+}\) because it is not susceptible to the same intracellular chelators.

La\(^{3+}\) as a specific tool to study synaptobrevin-2–dependent neurotransmitter release

As indicated above, rapid neurotransmitter release triggered by La\(^{3+}\) strictly required synaptobrevin-2. This argues for a strong specificity of La\(^{3+}\)’s mechanism of action in contrast to other means to trigger neurotransmission. Most forms of release can persist in the absence of synaptobrevin-2, albeit at severely reduced levels (Deitcher et al. 1998; Schoch et al. 2001). This remaining release is thought to be triggered by an alternative vesicular SNARE(s) (Borisovska et al. 2005; Deak 2001). However, the results we present here indicate that, in the case of La\(^{3+}\)-mediated release, these noncognate SNAREs may not be able to substitute synaptobrevin-2 functionally. This finding raises two possibilities. First, different vesicular SNAREs may manifest high functional specificity for different secretagogues. Alternatively, these noncognate vesicular SNAREs are located in a distinct population of vesicles that are not mobilized in response to La\(^{3+}\). The strong specificity of La\(^{3+}\)-evoked fusion, compared with other means to trigger release, makes it a powerful tool to probe synaptobrevin-2 function in central synapses. Another advantage of La\(^{3+}\) as a tool to probe release machinery stems from the fact that most other secretagogues can cause morphological distortion of cell membranes either by inducing shrinkage (e.g., hypertonicity) or forming Ca\(^{2+}\) permeable channels (α-latrotoxin). In contrast, in our hands, the concentrations of La\(^{3+}\) used to trigger release did not have any negative impact on membrane integrity. Another key feature of La\(^{3+}\)-triggered rapid release is its persistence in the absence of synaptotagmin-1, which is consistent with the observation that rapid release triggered by La\(^{3+}\) is independent of Ca\(^{2+}\) or La\(^{3+}\) entry and may provide a useful probe to examine release independent of the fast Ca\(^{2+}\) sensing machinery.

What is the transduction mechanism that links La\(^{3+}\) to SNARE-mediated fusion machinery? As indicated above, our results suggest that La\(^{3+}\) seems to act at an extracellular site to trigger rapid SNARE-mediated neurotransmitter release, whereas the delayed release seems either to be a consequence of La\(^{3+}\)’s slow entry into the cell or an indirect effect of Ca\(^{2+}\) signaling initiated by La\(^{3+}\) binding to an extracellular receptor (or both). There are several earlier studies that suggest that neurotransmitters or neuromodulators acting on presynaptic G protein–coupled seven transmembrane domain receptors may directly regulate neurotransmission in a SNARE-dependent manner. For instance, in lamprey central synapses, interaction of Gβγ subunits with SNAP-25 during serotonergic stimulation results in kiss-and-run–type fusion events (Gerachshenko et al. 2005; Photowala et al. 2006) and restricts glutamate release, suggesting a direct interaction between a G protein–coupled receptor and the fusion machinery. In addition, recent studies propose that the time-course of acetylcholine release is regulated by the voltage-sensitive muscarinic autoreceptors (Parnas and Parnas 2007). These findings suggest that muscarinic acetylcholine receptors tonically inhibit the release machinery, and this inhibition is relieved by membrane depolarization. Thus acting through a voltage-driven conformational change in muscarinic receptors, membrane voltage can exert tight control on the timing of neurotransmitter release (Slutsky et al. 2003). Furthermore, several G protein–coupled receptors such as the Ca\(^{2+}\) receptor (Smith et al. 2004) and group I metabotropic glutamate receptors (Abe et al. 2003) are present in central nerve terminals and possess binding sites for lanthanides. Therefore La\(^{3+}\) and other lanthanides may act on these presynaptic receptors that directly impact the release machinery.

Taken together, our results suggest that lanthanides act as powerful secretagogues to induce neurotransmitter release in a Ca\(^{2+}\)-independent manner, taking advantage of the conventional SNARE-mediated release machinery. Further study of the mechanism underlying this process will not only help us better understand Ca\(^{2+}\)-independent mechanisms that mediate neurotransmission but also provide insight into how extracellular heavy metals impact synaptic transmission under physiological and pathological circumstances.
LANTHANE-DRIVEN EXOCYTOSIS

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REFERENCES


