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Pregabalin Reduces the Release of Synaptic Vesicles from Cultured Hippocampal Neurons

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ABSTRACT

Pregabalin [*S*-[+]-3-isobutylGABA or (*S*)-3-(aminomethyl)-5-methylhexanoic acid, Lyrica] is an anticonvulsant and analgesic medication that is both structurally and pharmacologically related to gabapentin (Neurontin; Pfizer Inc., New York, NY). Previous studies have shown that pregabalin reduces the release of neurotransmitters in several in vitro preparations, although the molecular details of these effects are less clear. The present study was performed using living cultured rat hippocampal neurons with the synaptic vesicle fluorescent dye probe FM4-64 to determine details of the action of pregabalin to reduce neurotransmitter release. Our results indicate that pregabalin treatment, at concentrations that are therapeutically relevant, slightly but significantly reduces the emptying of neurotransmitter vesicles from presynaptic sites in living neurons. Dye release is reduced in both glutamic acid decarboxylase (GAD)-immunoreactive and GAD-negative (presumed glutamatergic) synaptic terminals. Furthermore, both calcium-dependent release and hyperosmotic (calcium-independent) dye release are reduced by pregabalin. The effects of pregabalin on dye release are masked in the presence of L-isoleucine, consistent with the fact that both of these compounds have a high binding affinity to the calcium channel α_2-δ protein. The effect of pregabalin is not apparent in the presence of an *N*-methyl-D-aspartate (NMDA) antagonist [D(−)-2-amino-5-phosphonopentanoic acid], suggesting that pregabalin action depends on NMDA receptor activation. Finally, the action of pregabalin on dye release is most apparent before and early during a train of electrical stimuli when vesicle release preferentially involves the readily releasable pool.

Pregabalin [(*S*)-3-(aminomethyl)-5-methylhexanoic acid, synonyms *S*-[+]-3-isobutylGABA, CI-1008) is a new anticonvulsant and analgesic medication that was recently approved for adjunctive treatment of partial seizures in adults in both the United States and Europe and for the treatment of neuropathic pain from postherpetic neuralgia and diabetic neuropathy. It is both structurally and pharmacologically related to the anticonvulsant and analgesic medication gabapentin, and both compounds were originally synthesized with the hope of modulating brain GABA receptors or GABA synthetic enzymes. Subsequent studies, however, have shown that these compounds are inactive at $GABA_A$ and $GABA_B$ receptors (Piechan et al., 2004), they do not block GABA transport, and they do not alter the brain concentration of GABA in rats (Errante and Petroff, 2003). The mechanism of action of pregabalin has been characterized only partially, and in particular, the cellular and molecular details of its action to reduce neurotransmitter release are incompletely known.

The primary high-affinity binding site for both gabapentin and pregabalin in forebrain tissues is the α_2-δ type 1 auxiliary subunit of voltage-gated calcium channels (Gee et al., 1996), and this interaction seems to be required for the pharmacological actions of the medications (Taylor, 2004; Belliotti et al., 2005). The identification of the α_2-δ binding sites has lead to the speculation that pregabalin and gabapentin act pharmacologically specifically in neurons by modulating the action of synaptic calcium channels. This hypothesis is supported by several findings that pregabalin or gabapentin reduce calcium influx into synaptosomes prepared from rat or human brain (Fink et al., 2000; van Hooft et al., 2002). The exact action of gabapentin and pregabalin on calcium channel function is still a matter of controversy, with some reports suggesting that these compounds reduce currents through voltage-gated calcium channels in neurons (Alden and Garcia, 2001; McClelland et al., 2004), whereas others indicate that gabapentin has no effect on such currents (Schumacher et al., 1997; van Hooft et al., 2002; Canti et al., 2004).

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ABBREVIATIONS: GAD, glutamic acid decarboxylase; D-AP5, D(−)-2-amino-5-phosphonopentanoic acid; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline.

Despite disagreements on the effects of gabapentin on calcium currents, it is generally accepted that gabapentin and pregabalin subtly reduce calcium-dependent overflow of neurotransmitters from several different neuronal tissues and reduce synaptic responses. Neurotransmitters that are sensitive to gabapentin or pregabalin include glutamate from rat neocortex, entorhinal cortex, or hippocampus (Dooley et al., 2000; van Hooft et al., 2002; Cunningham et al., 2004; Brown and Randall, 2005), glutamate from spinal cord (Maneuf et al., 2001; Bayer et al., 2004; Kumar and Coderre, 2004), substance P and calcitonin gene-related peptide from spinal cord dorsal horn (Fehrenbacher et al., 2003), and noradrenaline from neocortex (Dooley et al., 2002). A reduction of neurotransmitter release, particularly from hyperexcited or sensitized neuronal tissues, has been proposed as a primary mechanism of gabapentin and pregabalin drug action. However, many details of these drug effects remain to be learned.

The present experiments were designed to determine whether changes in neurotransmitter release in response to pregabalin could be measured in a well-characterized system that has been used previously to determine many aspects of vesicle release and trafficking from nerve terminals in the brain, namely the release of lipophilic FM fluorescent dye from nerve terminals in primary cultures of rat hippocampus.

Materials and Methods

Cell Culture and Transfection. Primary embryonic hippocampal cultures ("Banker" style) were prepared as described previously (Goslin et al., 1998) and were used 12 to 26 days later. All procedures were approved by the Institutional Animal Care and Use Committee of Stanford University.

Confocal Microscopy of Live Neurons and Image Analysis. During imaging, neurons were kept at 37°C in Tyrode's solution (119 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 25 mM HEPES, pH 7.4) with the addition of 30 mM glucose, 1% ovalbumin, and 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (Tocris, Ellisville, MO). Neurons were transferred into the imaging solution immediately before (<3 min) the beginning of the experiment. FM 4-64 (Invitrogen, Carlsbad, CA) loading was performed by superfusing the dye (5 μM) into the imaging chamber and electrically stimulating the neurons by passing current pulses between platinum electrodes placed at opposite ends of the chamber. Temperature control was achieved by circulating air from a precision air heater in a chamber enclosing the microscope stage with the specimen and opening around the objective lens. The air escaping the chamber flows around the objective and controls its temperature as well, thus ensuring constant temperature across the specimen, which is in close thermal contact with the oil-immersion objective.

Imaging was done with a laboratory-designed laser-scanning confocal microscope using a Zeiss 40×/1.3 numerical aperture FLUAR objective (Carl Zeiss Inc., Thornwood, NY). Images were sampled at 0.286-μm pixel size and collected every 1.7 s. Images were analyzed with custom software (N. E. Ziv; Technion, Haifa, Israel). FM 4-64 fluorescence intensities were averaged over 6 × 6-pixel squares centered on presynaptic boutons.

Pharmacology. The following reagents were used: pregabalin (100 μM; Pfizer, New York, NY), isoleucine (100 μM; Sigma, St. Louis, MO), and D(−)-2-amino-5-phosphonopentanoic acid (50 μM in 50 μM NaOH; Tocris). Stock solutions (1000–2000×) were added directly to the Tyrode's solution immediately before the experiment.

Immunostaining. Cells were fixed in 4% formaldehyde and 4% sucrose in PBS at 37°C for 20 min, permeabilized in 0.3% Triton for 5 min, blocked in 5% bovine serum albumin and 5% normal goat serum in PBS at 37°C for 1 h, then incubated in primary antibody (anti-GAD, rabbit, 1:2000, in PBS with 1% normal goat serum; Chemicon, Temecula, CA) for 2 h, and finally incubated in secondary antibody (goat anti-rabbit CY-5, 1:400; Jackson Immunoresearch Laboratories, West Grove, PA) for 30 min. All of the steps, excluding fixation and blocking, were performed at room temperature.

Data Analysis. Comparisons between different conditions were performed using coverslips from the same neuronal preparation on the same day. In some cases, for the presentation of the results, data from more than 1 day were combined if there were no differences between the control experiments from these days. Two-sided Mann-Whitney test or paired t test was used for statistical analysis of data. Data are reported as mean ± S.E.M.

Results

Pregabalin Reduces Synaptic Vesicle Release as Detected with FM 4-64. The FM fluorescent membrane dyes are routinely used for the quantification of synaptic vesicle recycling. When applied extracellularly during periods of synaptic activity, FM dyes become trapped within recycled synaptic vesicles (FM loading) and can be visualized by fluorescence microscopy after washing off extracellular dye (Fig. 1). The intensity of the trapped dye is proportional to the number of recycled vesicles. Subsequent exocytosis causes release of the dye (FM unloading). The changes in dye intensity during unloading can be used to estimate the kinetics of synaptic vesicle exocytosis (Betz et al., 1992).

We loaded hippocampal synapses with FM 4-64 using electrical stimulation of 10 Hz for 30 s, and the dye was left for an additional 1 min before washing out. Thereafter, the synapses were unloaded by electrical stimulation at 50 Hz for 90 s. After 10 min of rest, a second loading/unloading protocol identical with the first one was performed, this time either in the presence or absence of pregabalin (Fig. 2A). In the "pregabalin" condition, the drug was introduced immediately after the first unloading, and it was present in the solution from approximately 20 min before the beginning of the second unloading until the end of the experiment. Normalized FM unloading curves show that

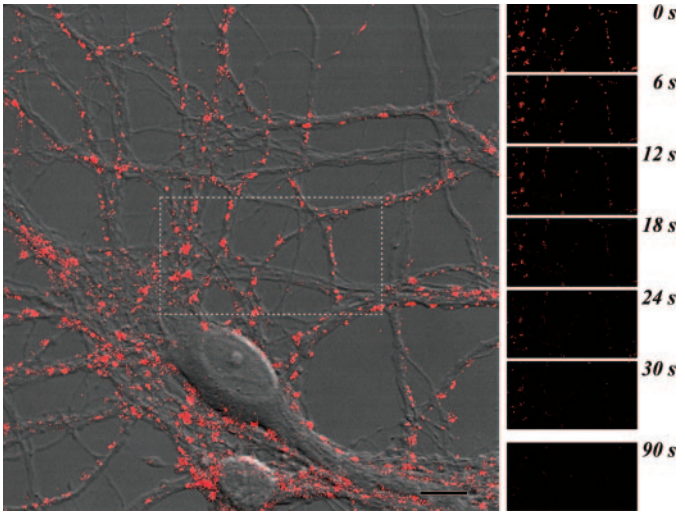

Fig. 1. Hippocampal neurons in culture, labeled with FM 4-64 (red). FM 4-64 is a marker for active synapses. The time-lapse series on the right shows the gradual release of FM 4-64 upon electrical stimulation. Scale bar, 10 μM.

whereas the first unloading is identical in hippocampal sister cultures, introduction of pregabalin in the second loading/unloading cycle reduces synaptic vesicle exocytosis (Fig. 2B). Thus, whereas in control cultures 62 ± 2% of the FM dye unloads during the second unload, this value is reduced to 56 ± 3% in the pregabalin-treated cultures. This amounts to a 9.5% reduction in FM dye unloading in the presence of pregabalin.

Both GABA and non-GABA Synapses Are Affected by Pregabalin. The hippocampal cultures used in these experiments contain approximately 90% pyramidal, glutamatergic cells, and the remaining are GABAergic interneurons (Benson et al., 1994). The synaptic population is therefore not homogenous, and the effects of pregabalin may differ depending on the synapse type. To explore this possibility, we analyzed the effects of pregabalin on presynaptic release separately in GABA-containing synapses and in non-GABA (presumably glutamatergic) synapses. A GAD antibody was used as a marker of GABA synapses. Retrospective immunolabeling of our cultures with this antibody resulted in bright punctate staining that colocalized with the live FM 4-64 labeling (Fig. 3, A–C). Comparison of normalized FM unloading curves of control and pregabalin-treated cultures showed that pregabalin reduced synaptic vesicle release in both GABA and non-GABA synapses (Fig. 3, D and E). In this series of experiments, pregabalin reduced FM unloading by 13% in GABA synapses and 12% in non-GABA synapses. Thus, the effects of pregabalin on presynaptic vesicle release do not depend on neurotransmitter type.

Pregabalin Reduces the Initial Rate of Unloading but Not Sustained Release. The results from FM unloading experiments can also be presented in a different way by calculating the rate of dye release at each time point of the experiment. The amount of dye released from one time-lapse frame to the other varies during stimulation, with a peak of release occurring shortly after the beginning of stimulation, followed by a gradual exponential decline (Fig. 4A). Pregabalin specifically reduces the initial peak rate of release without affecting subsequent release. Thus, during the initial 3.4 s of electrical stimulation, control presynaptic boutons release 15.7 ± 1.1% of the loaded FM 4-64 dye compared with pregabalin-treated boutons, which release 11.7 ± 0.5% of the dye (p = 0.015; Mann-Whitney). No statistically significant differences in the rate of release during electrical stimulation were observed at any other time point.

Pregabalin Reduces Spontaneous Release. It is noteworthy that the same Fig. 4A also suggests some differences in the spontaneous release preceding stimulation. Thus, in

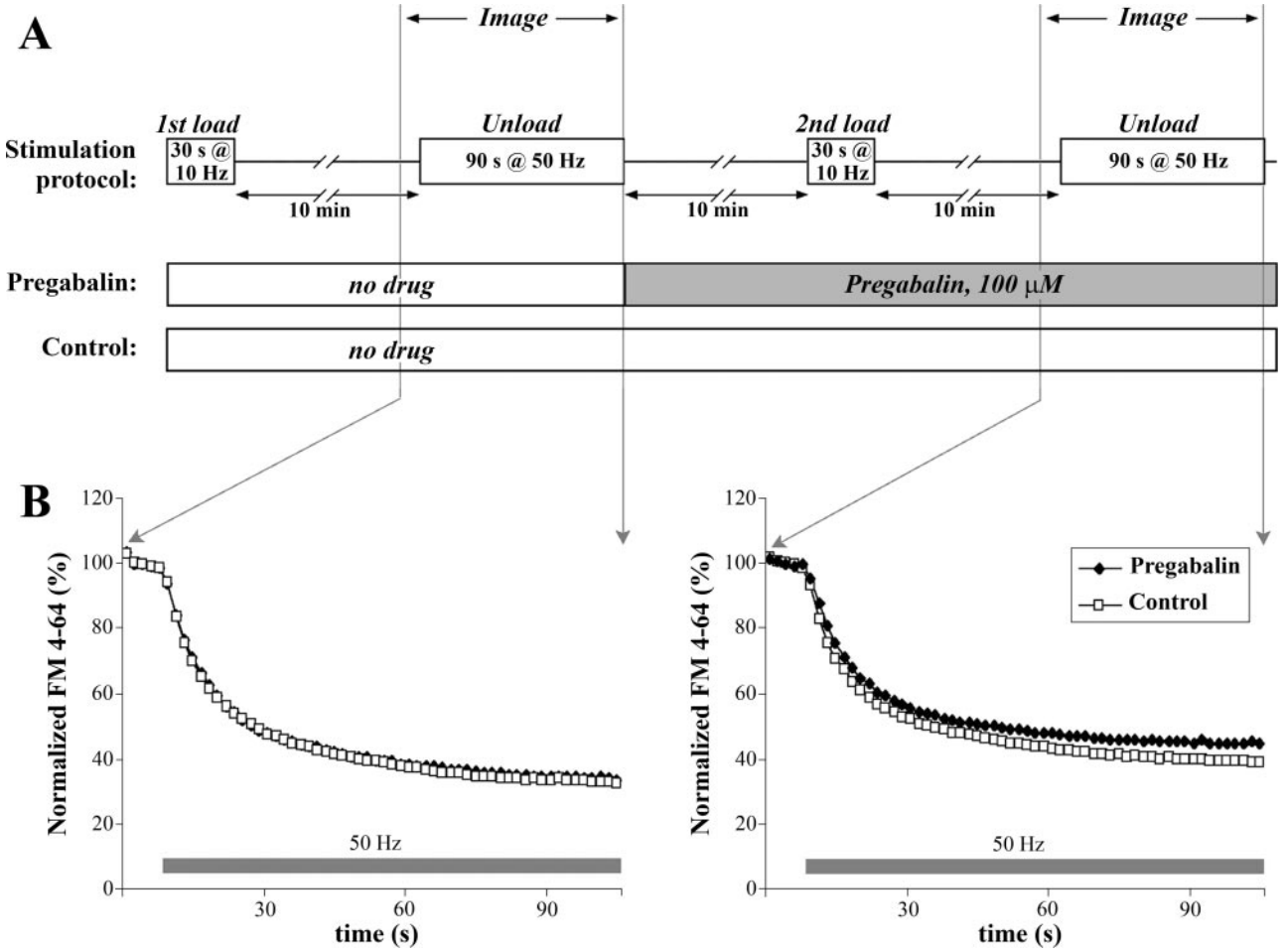

Fig. 2. Pregabalin reduces synaptic vesicle release. A, experimental protocol. Hippocampal cultures were subjected to two consecutive cycles of loading and unloading with FM 4-64 using electrical stimulation. Comparisons were made between sister cultures in the presence or absence of pregabalin (100 μM). B, unloading curves from control (seven coverslips with at least 150 boutons each) or pregabalin cultures (nine coverslips with at least 150 boutons each). FM 4-64 fluorescence intensities are normalized to the initial resting state of each presynaptic bouton and averaged for each condition. Standard errors are not presented in B because they were on the order of 1%.

the 3.4 s before the start of stimulation, control presynaptic boutons released 1.7 ± 0.3% of the loaded FM 4-64 dye compared with 0.1 ± 0.3% of the dye released from pregabalin-treated boutons ($p = 0.006$; Mann-Whitney). Because this difference is rather small, even though statistically significant, we performed further experiments to verify this observation. Using a larger sample, we analyzed the spontaneous release from presynaptic boutons during the 5 s preceding stimulation (Fig. 4B). Control presynaptic boutons released 1.9 ± 0.3% of the loaded FM 4-64 dye compared with 1.1 ±

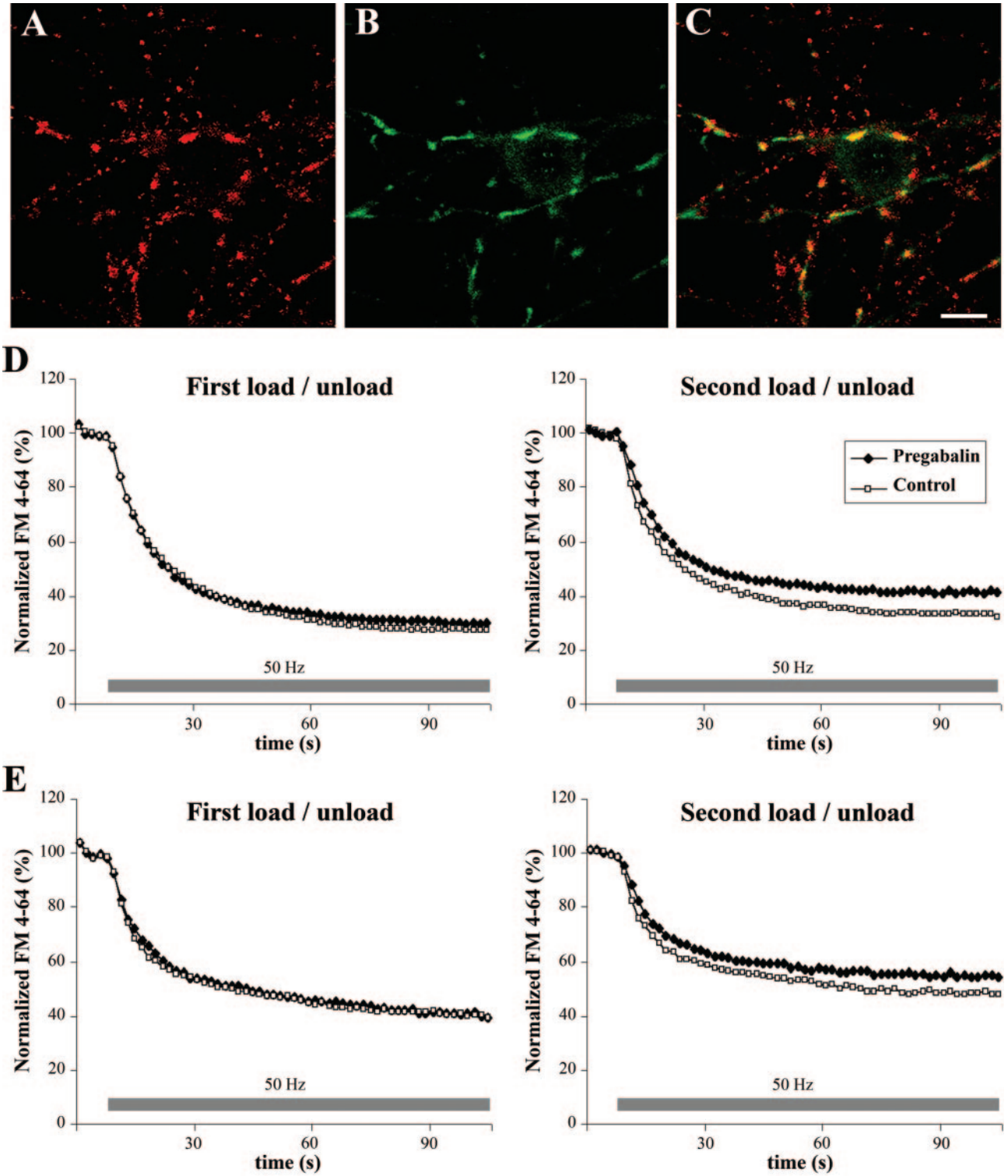

Fig. 3. Pregabalin has an effect on both GABA and non-GABA synapses. Hippocampal cultures labeled with FM 4-64 (A) (life cultures) and with GAD (B), a marker for GABA-containing structures (after fixation). C, overlay of A and B. Scale bar, 10 μM. D, unloading of GABA synapses in the presence or absence of pregabalin ($n = 192$ boutons from four coverslips for control, and $n = 273$ boutons from six coverslips for pregabalin). Same experimental protocol as presented in Fig. 2. Standard errors are not presented because they were on the order of 1%. E, unloading of non-GABA synapses ($n = 186$ boutons from four coverslips for control, and $n = 288$ boutons from six coverslips for pregabalin) from the same coverslips as D.

0.1% of the dye released from pregabalin-treated boutons ($p = 0.03$; paired t test). Another experiment (Fig. 4C) compared the spontaneous FM release from control and pregabalin-treated boutons (20-min preincubation in the drug) imaged for longer periods of time (>30 s). The pregabalin-induced reduction of spontaneous release persisted throughout the imaging period.

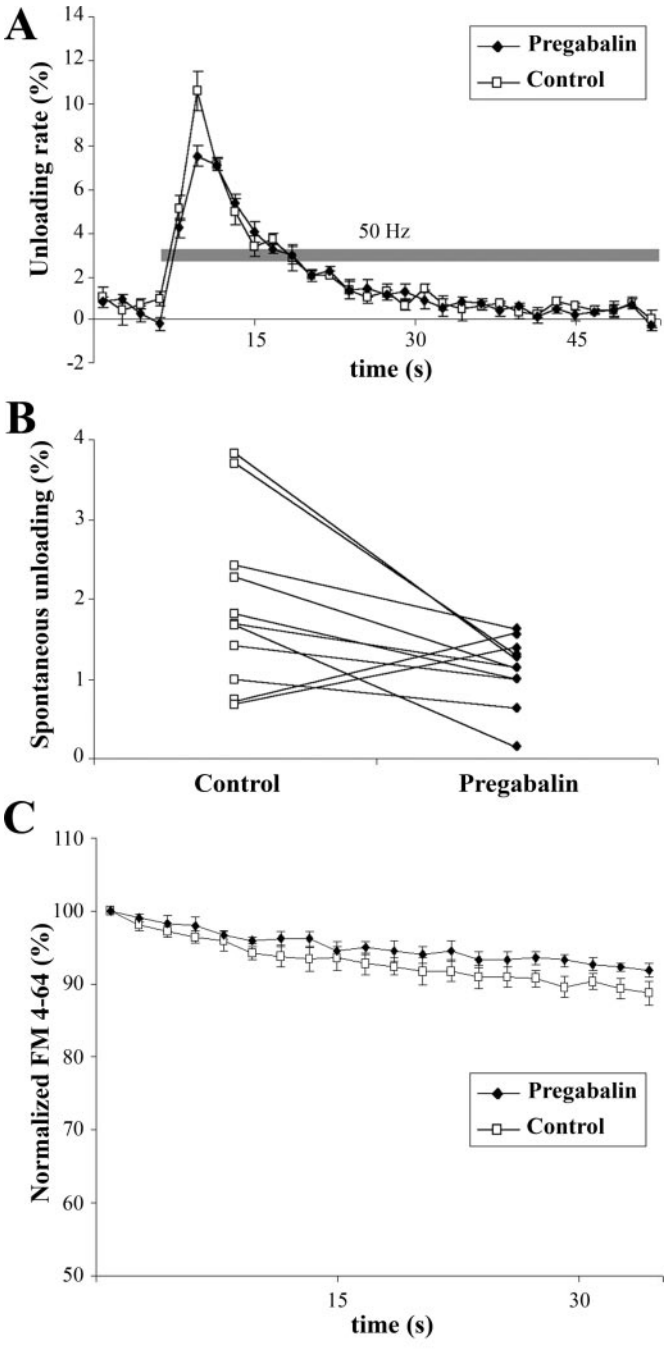

Fig. 4. Effect of pregabalin on the rate of synaptic vesicle release during stimulation and at rest. A, pregabalin (100 μM) significantly reduces the initial rate of FM 4-64 unloading during electrical stimulation. Data are from the same experiments as presented in Fig. 2. B, pregabalin also significantly reduces the spontaneous release of FM 4-64. The paired data are from experiments done on sister coverslips on the same day. Each data point represents the average unloading (as a percentage) during 5 s at rest in the presence or absence of pregabalin, as obtained from at least two coverslips with 150 synaptic boutons each. C, unloading curves from control (six coverslips with at least 50 boutons each) or pregabalin cultures (six coverslips with at least 50 boutons each) imaged in the absence of electrical stimulation. FM 4-64 fluorescence intensities are normalized to the initial resting state of each presynaptic bouton and averaged for each condition. Standard errors are presented.

Pregabalin May Preferentially Reduce Release from the Readily Releasable Vesicle Pool. The selective effect of pregabalin on spontaneous release and on the initial evoked release suggests that it may be preferentially targeting the readily releasable vesicle pool. These vesicles are immediately available for release, and they can be experimentally released by the application of hypertonic saline (Rosenmund and Stevens, 1996). To further test the effect of pregabalin on the readily releasable pool, we perfused the neuronal cultures with hypertonic sucrose solution (500 mM) in the absence of calcium (Fig. 6). Application of pregabalin significantly reduced the dye release caused by the hypertonic solution. In the first 30 s of sucrose application, control presynaptic boutons released 14 ± 1% of the loaded dye, whereas pregabalin-treated boutons released 8 ± 1% of the dye ($p = 0.01$, Mann-Whitney). After 1 min of sucrose application, the dye release was 17 ± 2% for the control boutons and 12 ± 1% for the pregabalin-treated boutons ($p = 0.04$, Mann-Whitney) (Fig. 5B). If the sucrose-stimulated release was immediately followed by electrical stimulation in the standard bath solution containing calcium, the electrically evoked release was essentially the same regardless of the presence or absence of pregabalin (Fig. 5C). These results support the idea that pregabalin affects the readily releasable pool of synaptic vesicles.

L-Isoleucine Blocks the Action of Pregabalin on Vesicle Release. L-Isoleucine and pregabalin have approximately equal affinity to the same binding site (the $\alpha_2\delta$-subunit of voltage-sensitive calcium channels) (Brown et al., 1998), and both are substrates to the system L amino acid transporter. L-Isoleucine has been shown to interfere with the action of pregabalin on the frequency of asynchronous miniature currents at neocortical glutamate synapses (Cunningham et al., 2004). We first tested the effect of L-isoleucine (100 μM) on electrically evoked presynaptic vesicle release by using the same protocol as for pregabalin (Fig. 2A). The kinetics of dye release was very similar, regardless of the presence or absence of L-isoleucine (Fig. 6A). However, when applied simultaneously with pregabalin in the same experimental protocol, L-isoleucine blocked the inhibitory effect of pregabalin on release (Fig. 6B).

Pregabalin Effects on Release Are Abolished by an NMDA Antagonist. Both positive and negative interactions between gabapentin and NMDA receptor responses have been reported in the literature (Shimoyama et al., 2000; Gu and Huang, 2002; Suarez et al., 2005), despite the fact that neither gabapentin nor pregabalin interacts with NMDA, phencyclidine, or strychnine-insensitive glycine binding sites of NMDA receptors (Piechan et al., 2004). We tested the effects of NMDA receptor blockade on the action of pregabalin in our system. The same protocol as presented in Fig. 2A was followed, but this time, the NMDA antagonist D-AP5 was present in the medium. No effect of pregabalin on the rate of synaptic vesicle release could be observed under these conditions (Fig. 7). The effect of pregabalin on spontaneous release was also abolished in the presence of D-AP5. Thus, during the 5 s preceding stimulation, control presynaptic boutons released 1.2 ± 0.4% of the loaded FM 4-64 dye

compared with 1.0 ± 0.5% of the dye released from pregabalin-treated boutons. The effects of pregabalin on both spontaneous and evoked release seem to be NMDA receptor-dependent.

Discussion

Our results confirm previous studies suggesting that pregabalin and other compounds with high-affinity binding to α_2-δ proteins reduce the release of transmitters from neuronal tissues. The reduction of neurotransmitter release observed in our study is rather small, which is consistent with previous studies and is to be expected from a drug that is usually well tolerated by patients. In addition, several novel findings emerged from our study. Thus, we show that in hippocampal cultures, both GABAergic and glutamatergic presynaptic terminals are affected by the drug. Pregabalin reduces spontaneous and evoked release, and it probably targets the readily releasable pool of synaptic vesicles. At least some of pregabalin's effects on neurotransmission seem not to require calcium influx through voltage-gated calcium channels. Our results further suggest that in this preparation, NMDA receptor activation may be required for these actions of pregabalin.

A number of studies have suggested that the primary action of pregabalin and gabapentin is reduction of neurotransmitter release, particularly from hyperexcited or sensitized neuronal tissues. However, studies that used the overflow of radioactivity from tissues prelabeled with [^{3}H]noradrenaline (Dooley et al., 2002) or [^{3}H]glutamate (Maneuf et al., 2001) from brain tissue slices could be confounded by changes in the reuptake, metabolism, or vesicle packaging of the radiolabel before or after release. These potential confounds are reduced with the FM 4-64 dye method. Other previous results showing decreased postsynaptic potentials or currents with drug treatment (Shimoyama et al., 2000; van Hooft et al., 2002; Cunningham et al., 2004; Brown and Randall, 2005) could be confounded by desensitization or other plastic changes in postsynaptic receptors. Our results confirm that pregabalin reduces synaptic vesicle release, as measured more directly at individually resolved presynaptic sites, with relatively high time resolution. This effect of pregabalin is probably mediated by an interaction with the α_2-δ type 1 auxiliary subunit of voltage-gated calcium channels because

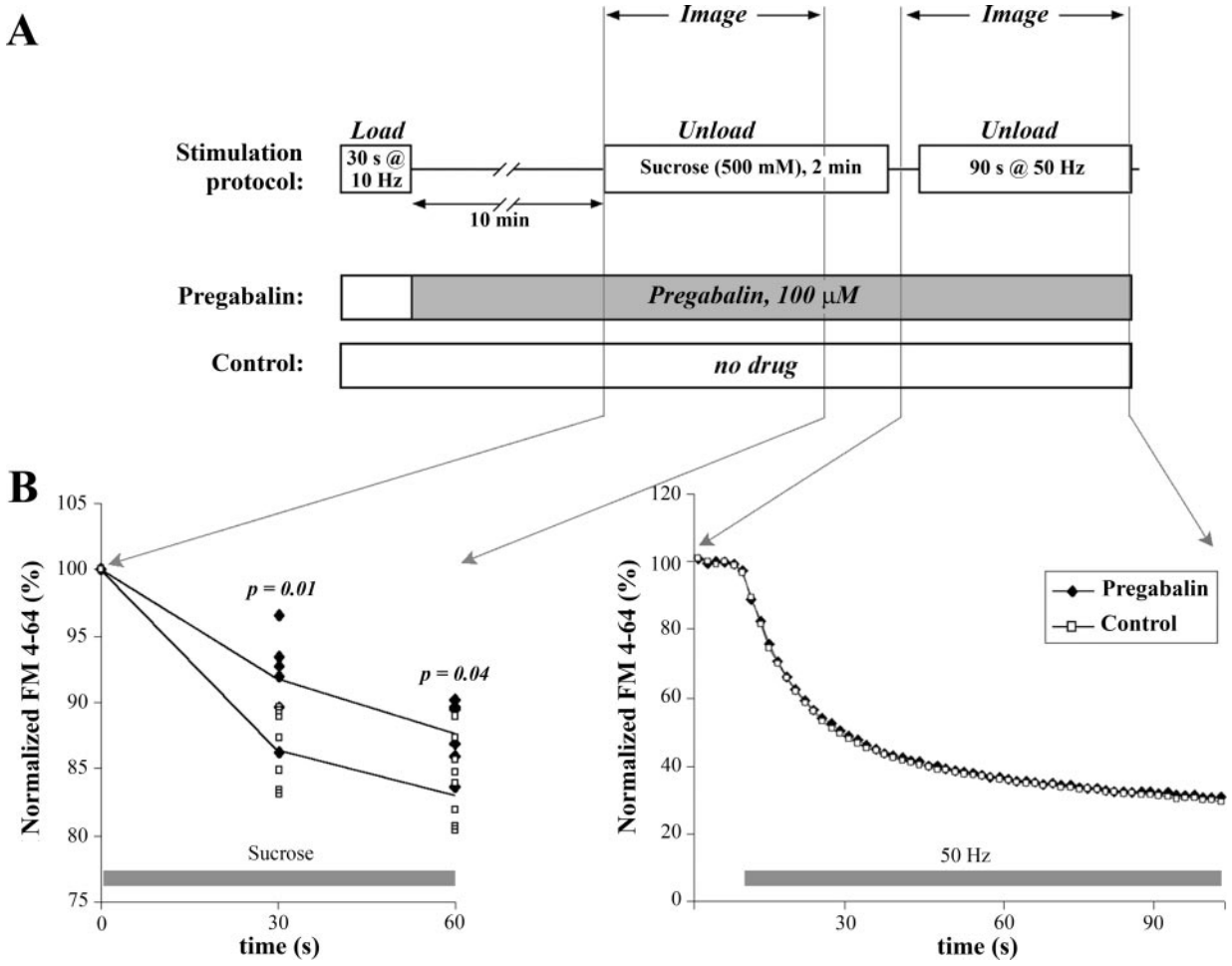

Fig. 5. Effect of pregabalin on the readily releasable pool of synaptic vesicles. A, experimental protocol. Hippocampal cultures were loaded with FM 4-64 using electrical stimulation and unloaded with application of hypertonic sucrose (500 mM) followed by electrical stimulation. Comparisons were made between sister cultures in the presence or absence of pregabalin (100 μM). B, pregabalin significantly reduces the initial synaptic vesicle release triggered by application of hypertonic sucrose in the absence of calcium. The immediately following electrically induced release is insensitive to pregabalin. Results are from seven pregabalin coverslips with at least 150 boutons each and eight control coverslips with at least 150 boutons each. Probabilities were calculated using Mann-Whitney test.

L-isoleucine, which has approximately equal affinity to the same binding site, prevented the pregabalin-induced reduction in synaptic vesicle release, and because α_2-δ type 1 is the predominant subtype present in neocortex tissues, whereas α_2-δ type 2 is more prevalent in cerebellum (Barclay et al., 2001; Bian et al., 2006).

Pregabalin Acts on Both Excitatory and Inhibitory Presynaptic Boutons. Our results are the first to suggest that pregabalin reduces the release of the inhibitory neurotransmitter GABA. This seems counterintuitive for an anticonvulsant drug, because reduced GABA transmission or GABA receptor block can cause seizure activity rather than prevent it. However, there is now evidence that GABA synapses may contribute to epileptic activity. Thus, GABAergic transmission is actually required for the spontaneous activity recorded in brain slices of human epileptic hippocampus and neocortex in vitro (Kohling et al., 1998) and in the subicular region, an examination of a subpopulation of excitatory pyramidal neurons revealed depolarizing GABA responses that were suggested to initiate epileptic discharges (Cohen et al., 2002). In light of these findings, a slight reduction of both excitatory and inhibitory neurotransmission by the action of pregabalin can be expected to significantly reduce the generation of epileptic discharges.

Therefore, results to date are consistent with a small reduction in a wide range of neurotransmitter substances by α_2-δ ligands, both excitatory and inhibitory. This agrees well with the relatively broad distribution of α_2-δ proteins in brain tissues and the localization of [^{3}H]gabapentin- (Hill et al., 1993) and [^{3}H]pregabalin-specific binding (Bian et al., 2006) in synaptic neuropil from many different brain and spinal regions, including anatomical areas in which both glutamate and GABA synaptic connections are common.

Pregabalin Action May Not Require Calcium Influx via Voltage-Gated Calcium Channels. After the identification of the α_2-δ type 1 auxiliary subunit of voltage-gated calcium channel as the major binding site for pregabalin and gabapentin in the brain (Gee et al., 1996), it was hypothesized that these drugs may affect synaptic transmission by modifying voltage-gated calcium influx. It

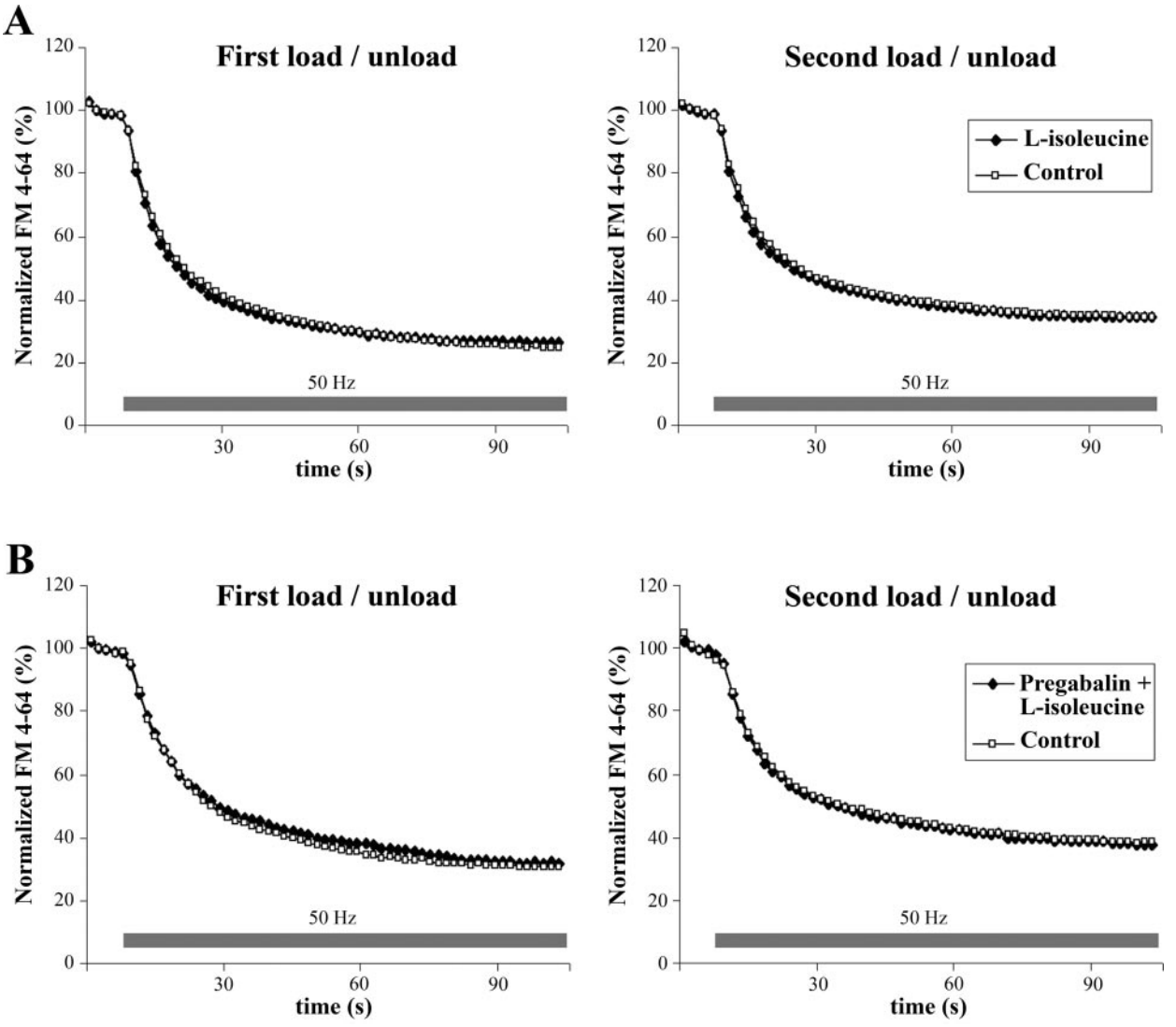

Fig. 6. L-Isoleucine (100 μM) does not have a noticeable effect on presynaptic vesicle release (A), but it blocks the action of pregabalin (B). The experimental protocol is the same as presented in Fig. 2A. Results are from eight L-isoleucine coverslips with at least 150 boutons each and eight control coverslips with at least 150 boutons each (A) and three pregabalin/L-isoleucine coverslips with at least 150 boutons each and three control coverslips with at least 150 boutons each (B).

is noteworthy that several of our findings suggest that pregabalin may be acting on synaptic vesicle release that is independent of a calcium influx via voltage-gated calcium channels. Thus, pregabalin reduces the spontaneous release (without electrical stimulation) of vesicles containing FM dye. This is consistent with previous results that pregabalin reduces the rate of spontaneous glutamate miniature synaptic currents in slices of rat entorhinal cortex (Cunningham et al., 2004). Spontaneous dye release can occur in the absence of extracellular calcium (Erulkar and Rahamimoff, 1978) and seems to require calcium from intracellular stores but not calcium entry via voltage-gated calcium channels (Simkus and Stricker, 2002). Moreover, our results also show that pregabalin reduces the vesicle release evoked by application of hypertonic solution. This release involves the vesicles from the readily releasable pool (i.e., the vesicles that are immediately available for release without trafficking from the cytosol), and it is known to occur in the absence of calcium (Rosenmund and Stevens, 1996). Therefore, it seems likely that pregabalin has effects on neurotransmitter release that are not directly mediated by changes in presynaptic calcium influx. This might occur in response to pregabalin binding at α_2-δ proteins through an allosteric interaction between α_2-δ proteins and other unknown presynaptic proteins involved with vesicle release. Alternatively, this might occur by an allosteric interaction between α_2-δ proteins and α_1 calcium-channel subunits, which are known to interact with other presynaptic proteins, including syntaxin-1, synaptotagmin, syncam, and neurexin. Additional experiments are needed to test these ideas.

A Possible Role for NMDA Receptor Activation in Pregabalin Action. Our finding that an NMDA receptor antagonist prevented the changes in vesicle release from

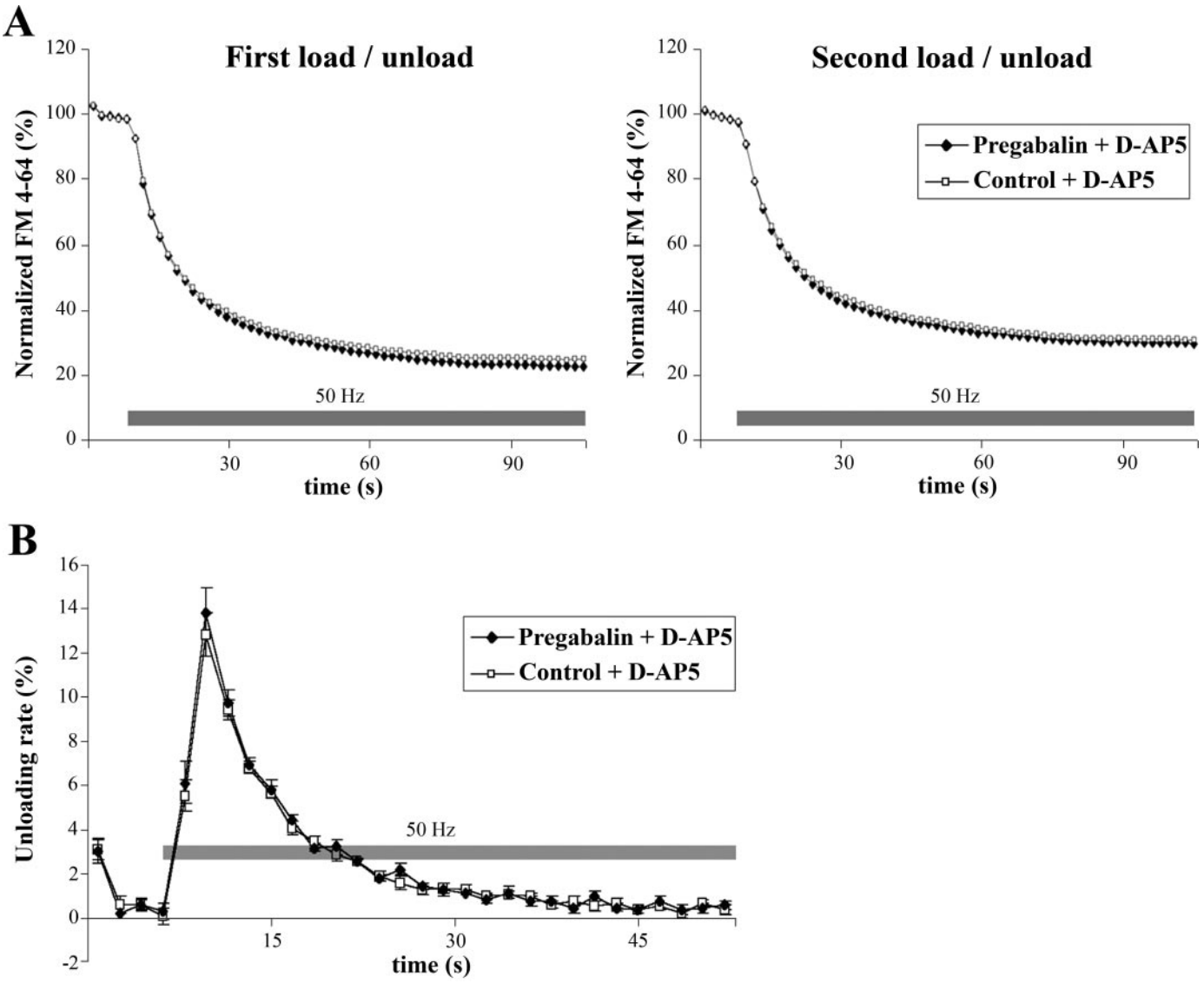

Fig. 7. The effect of pregabalin on synaptic vesicle release is abolished by the application of the NMDA antagonist D-AP5. A, unloading curves in the presence of D-AP5 (50 μM) from control (nine coverslips with at least 150 boutons each) or pregabalin cultures (eight coverslips with at least 150 boutons each). FM 4-64 fluorescence intensities are normalized to the initial resting state of each presynaptic bouton and averaged for each condition. Experimental protocol was as presented in Fig. 2. Standard errors are not presented because they were on the order of 1%. B, the rate of FM 4-64 unloading during electrical stimulation is not affected by pregabalin in the presence of D-AP5. Data are from the same experiments as presented in A.

pregabalin was unexpected. There are several possibilities to explain this finding. The effects of pregabalin may require the activation of postsynaptic NMDA receptors. Previous findings (Micheva et al., 2003) indicate that postsynaptic NMDA receptors can signal in a retrograde manner via activation of postsynaptic nitric-oxide synthase, diffusion of nitric oxide to presynaptic neurons, and subsequent alteration in the trafficking of presynaptic vesicles. It is possible that pregabalin produces its effects on presynaptic vesicles by influencing this signaling pathway. Alternatively, several studies have shown that presynaptic NMDA receptors can alter synaptic action of glutamate synapses, and our findings might be explained by a requirement for activation of presynaptic NMDA receptors (MacDermott et al., 1999). The idea that presynaptic NMDA receptors are involved is supported by a recent study suggesting that gabapentin, which is closely related to pregabalin, reduces presynaptic sodium-channel activation via an NMDA-dependent mechanism (Suarez et al., 2005). However, additional experiments are required to further characterize the NMDA dependence of pregabalin action that our study has suggested.

A Model for Pregabalin Action on Presynaptic Boutons. We propose the following model for pregabalin action, based on the present results and the existing literature. By binding to the α_2-δ type 1 auxiliary subunit of voltage-gated calcium channels, pregabalin modifies the allosteric interactions between the voltage-gated calcium channels and proteins of the presynaptic vesicle-release complex, such as syntaxin 1A, synaptosomal associated protein 25, and synaptotagmin (Jarvis and Zamponi, 2001). This, in turn, changes the interactions between docked synaptic vesicles and the presynaptic membrane and reduces the ability of docked vesicles to spontaneously fuse and release neurotransmitter. Presynaptic NMDA receptor activation may be a prerequisite for the observed pregabalin action because it also can target activity and spontaneous neurotransmission in a calcium-independent manner (Breukel et al., 1998; Suarez et al., 2005). Such a mechanism of action of pregabalin could account for the reduced spontaneous release of neurotransmitter and the reduced release after application or hypertonic solution observed in our study. Because (with few exceptions) the basic vesicle release machinery is the same for glutamatergic and GABAergic synapses, one would expect a similar effect of pregabalin on these two types of synapses, as observed in the present study. A reduction in the spontaneous release of neurotransmitters will change the overall neuronal response properties and, in particular, may decrease the overall neuronal responsiveness (Ho and Destexhe, 2000; Cunningham et al., 2004) and thus reduce the incidence of generation of epileptic discharges and reduce the hyperexcitability of spinal dorsal horn neurons believed to be involved in long-term neuropathic pain states.

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