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Chun Y. Chang

Washington University School of Medicine in St. Louis

Xiaoping Jiang

Washington University School of Medicine in St. Louis

Krista L. Moulder

Washington University School of Medicine in St. Louis

Steven Mennerick

Washington University School of Medicine in St. Louis

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Rapid Activation of Dormant Presynaptic Terminals by Phorbol Esters

Chun Yun Chang,^{1,3} Xiaoping Jiang,¹ Krista L. Moulder,¹ and Steven Mennerick^{1,2}

Departments of ¹Psychiatry and ²Anatomy and Neurobiology, and ³Graduate Program in Developmental Biology, Washington University School of Medicine, St. Louis, Missouri 63110

Presynaptic stimulation stochastically recruits transmission according to the release probability (P_r) of synapses. The majority of central synapses have relatively low P_r , which includes synapses that are completely quiescent presynaptically. The presence of presynaptically dormant versus active terminals presumably increases synaptic malleability when conditions demand synaptic strengthening or weakening, perhaps by triggering second messenger signals. However, whether modulator-mediated potentiation involves recruitment of transmission from dormant terminals remains unclear. Here, by combining electrophysiological and fluorescence imaging approaches, we uncovered rapid presynaptic awakening by select synaptic modulators. A phorbol ester phorbol 12,13-dibutyrate (PDBu) (a diacylglycerol analog), but not forskolin (an adenylyl cyclase activator) or elevated extracellular calcium, recruited neurotransmission from presynaptically dormant synapses. This effect was not dependent on protein kinase C activation. After PDBu-induced awakening, these previously dormant terminals had a synaptic P_r spectrum similar to basally active synapses naive to PDBu treatment. Dormant terminals did not seem to have properties of nascent or immature synapses, judged by NR2B NMDAR (NMDA receptor) receptor subunit contribution after PDBu-stimulated awakening. Strikingly, synapses rendered inactive by prolonged depolarization, unlike basally dormant synapses, were not awakened by PDBu. These results suggest that the initial release competence of synapses can dictate the acute response to second messenger modulation, and the results suggest multiple pathways to presynaptic dormancy and awakening.

Introduction

Presynaptic modulators often act by altering the probability of transmitter release (P_r), which is heterogeneous across the population of presynaptic terminals of a neuron (Hessler et al., 1993; Rosenmund et al., 1993; Murthy et al., 1997). Conventionally, measuring synaptic P_r is accomplished by electrophysiological or optical assessments. Both approaches are biased toward sampling the responses of synapses with higher synaptic P_r . Populations of synapses with extremely low P_r or completely dormant presynaptic terminals are more difficult to detect, despite evidence for such populations (Tong et al., 1996; Murthy et al., 1997; Ma et al., 1999; Moulder et al., 2004; Slutsky et al., 2004). Whether these populations are subject to presynaptic modulation by second messenger systems has only begun to be investigated.

There is good evidence that prolonged activation of cAMP/PKA (protein kinase A) pathways by forskolin (FSK) awakens presynaptically dormant synapses (Chavis et al., 1998; Ma et al., 1999; Moulder et al., 2008). At least in some systems, this presynaptic

activation requires protein synthesis (Ma et al., 1999) and takes several hours. However, rapid activation of cAMP signaling does not seem to increase functional terminals (Trudeau et al., 1996), despite apparent enhancement of synaptic output by electrophysiological assessment (Weisskopf et al., 1994; Trudeau et al., 1996; Lonart et al., 1998). Whether rapid potentiation of transmission by other modulators involves awakening dormant synapses is less clear.

Here, we focus on phorbol ester modulation of glutamate transmission. Classically, phorbol ester stimulation activates protein kinase C (PKC) pathways (Blumberg, 1991). However, phorbol esters also bind to other proteins with diacylglycerol-binding domains, including the vesicle priming protein Munc13; activation of Munc13 by phorbol esters promotes vesicle release through this PKC-independent mechanism (Betz et al., 1998; Rhee et al., 2002). Both PKC-dependent and PKC-independent pathways may participate in acute synaptic modulation by phorbol esters (Wierda et al., 2007; Lou et al., 2008). Although the phenomenon of phorbol ester potentiation and its underlying molecular mechanisms have been intensively studied (Brose and Rosenmund, 2002; Silinsky and Searl, 2003), it remains unknown whether phorbol esters potentiate synaptic function in part by acting on dormant synapses.

Using both electrophysiological and imaging assessments, we show that phorbol esters activate preexisting presynaptically dormant synapses in addition to enhancing transmitter output probability at already active synapses. Interestingly, after activation by phorbol 12,13-dibutyrate (PDBu), presynaptic quiescent synapses appear to have heterogeneous P_r that is similar to basal,

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Correspondence should be addressed to Steven Mennerick, Department of Psychiatry, Washington University School of Medicine, 660 South Euclid Avenue, Campus Box 8134, St. Louis, MO 63110. E-mail: mennerick@psychiatry.wustl.edu.

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untreated active synapses. Despite its effects on basally dormant synapses, PDBu does not reactivate presynaptic terminals rendered inactive by sustained depolarization, suggesting multiple independent routes to presynaptic dormancy. In summary, our work increases understanding of the complex effects of phorbol esters on presynaptic function. Among its rapid effects is a restoration of presynaptic function at terminals that do not normally contribute to synaptic transmission.

Materials and Methods

Cell cultures. Dissociated hippocampal cultures were prepared as previously described (Mennerick et al., 1995; Moulder et al., 2007). Briefly, hippocampi were harvested from Sprague Dawley rats at postnatal days 1–3 and were dissociated enzymatically using 1 mg ml⁻¹ papain, and then mechanically with a glass pipette. Dissociated neurons were then plated at 100 cells mm⁻² for low-density microisland plating (for electrophysiological recording) or ~650 cells mm⁻² for high-density mass cultures (for imaging experiments). For island cultures, culture plates were precoated with 0.15% agarose and were stamped or sprayed with type I collagen (0.5 mg ml⁻¹) as the microdot substrate. For mass cultures, collagen was spread uniformly across a 25-mm-diameter glass coverslip. Culture media consisted of Eagle's minimal essential medium (Invitrogen) supplemented with 5% heat-inactivated horse serum, 5% fetal bovine serum, 17 mM glucose, 400 μM glutamine, 50 U ml⁻¹ penicillin, and 50 μg ml⁻¹ streptomycin. To suppress glial proliferation, 6.7 μM cytosine arabinoside was added to the cultures 3–4 d after plating. One-half of the culture media was removed and replaced with Neurobasal medium plus B27 supplement 1 d after antimetabolic addition. Cells were used for experiments 9–14 d after plating.

Electrophysiology. Whole-cell recordings were performed with a MultiClamp 700B amplifier and Digidata 1440A acquisition system (Molecular Devices); data were acquired in Clampex 10 (Molecular Devices). Recordings were conducted in extracellular solution (bath) consisting of the following (in mM): 138 NaCl, 4 KCl, 2 CaCl₂, 0.01 glycine, 10 glucose, and 10 HEPES (Invitrogen), pH 7.25. AMPA-sensitive receptor (AMPA)-mediated current was isolated by 50–75 μM D-amino-5-phosphonovaleric acid (D-APV) (Tocris); NMDA-sensitive receptor (NMDAR)-mediated currents were isolated by 1–2 μM 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo-[f]quinoxaline-7-sulfonamide (NBQX) (Tocris). The 400 μM kynurenic acid (for AMPAR current) and 250 μM L-amino-5-phosphonovaleric acid (L-APV) (for NMDAR current) (Tocris) were added to reduce receptor desensitization and to minimize access resistance errors associated with the large autaptic currents. During (5S,10R)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*]cyclohept-5,10-imine maleate (MK-801) blocking procedures, L-APV was omitted. Nontransmitter-mediated residual current was evaluated in the presence of 1–2 μM NBQX and 75 μM D-APV and was subtracted from the evoked EPSCs. Solution was perfused by a gravity-based multibarrel perfusion system at the rate of 0.2 ml min⁻¹ with <100 ms complete solution switch. For sucrose-evoked responses, flow rate was increased to 1.6 ml min⁻¹ with <50 ms solution switch. Responses to modulators (PDBu and forskolin) were measured immediately after the 2 min application. Responses to PDBu remained elevated for at least 2 min after modulator removal (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). Electrode pipettes with open tip resistance of 3–5 MΩ were filled with the internal pipette solution containing the following (in mM): 140 K-gluconate, 4 NaCl, 0.5 CaCl₂, 10 EGTA, and 10 HEPES, pH 7.25. After the whole-cell mode was established, cells were voltage clamped at -70 mV, and EPSCs were evoked by a brief (1 ms) depolarization to 0 mV. Only cells with access resistance of <11 MΩ, membrane resistance of >150 MΩ, and leak current of <200 pA were recorded. Series resistance was compensated by 80%. Leak current was subtracted off-line. Signals were sampled at 5 or 10 kHz and filtered at 2 kHz. All recordings were performed at room temperature.

Pharmacological reagents. Unless otherwise specified, reagents were purchased from Sigma-Aldrich. Stock solutions of MK-801 (10 mM; Tocris), NMDA (100 mM), 4-aminopyridine (4-AP) (100 mM), and ifenprodil (3 mM) were dissolved in distilled water and diluted to the indicated concentrations. Stock solutions of bicuculline (50 mM), forskolin (50 mM), 3-[1-(3-

(dimethylamino)propyl]-5-methoxyindol-3-yl]-4-(1H-indol-3-yl)pyrrole-2,5-dione (Gö6983) (2 or 20 mM), 3-[3-[4-(1-methylindol-3-yl)-2,5-dioxopyrrol-3-yl]indol-1-yl]propylcarbamimidothioate (Ro31-8220) (2 or 20 mM), and PDBu (1 or 5 mM) were made in DMSO and diluted in working solutions. The final concentration of DMSO was never higher than 0.1%. This DMSO concentration had no effect on synaptic transmission (supplemental Fig. S3, available at www.jneurosci.org as supplemental material).

Data analysis. Data were analyzed by Clampfit10 (Molecular Devices) or by customized Igor Pro procedures (Wavemetrics). Residual stimulation-associated capacitive and presynaptic currents in the sample traces in all figures were blanked for clarity; sample traces in Figures 3 and 7 were low-pass filtered with a digital eight-pole Bessel filter at 500 Hz for clarity. Data fitting was performed by commercially available fitting routines (Clampfit and Igor Pro). All results are presented as mean ± SEM. Paired or unpaired *t* test or one-way ANOVA were used for statistical analysis.

Imaging experiments, image acquisition, and analysis. Mass cultures prepared under identical conditions and in parallel with autaptic cultures were used for imaging experiments 11–14 d *in vitro*. Mass cultures were used because single synapses could be identified much more reliably in this culture preparation than in autaptic cultures. Autaptic synapses tend to nest near the soma and proximal dendrites, and the extensive synaptic overlap makes sampling individual synapses problematic. Before the experiment, the cultures were rinsed with extracellular solution plus 50 μM D-APV and 1 μM NBQX. Functional synapses were identified by an FM1-43FX dye loading-based assay (Betz and Bewick, 1992). This assay takes advantage of activity-dependent dye loading in presynaptic terminals whose vesicles actively participate in exo-endocytic cycles. Such presynaptic FM dye loading does not occur in presynaptic terminals that are functionally silent (Rosenmund et al., 2002; Altmann et al., 2003; Moulder et al., 2004, 2006).

To label functional synapses, hyperkalemic extracellular solution (45 mM [K⁺]_o, equimolar substitution for Na⁺, with 25 μM D-APV and 1 μM NBQX) was used to trigger transmitter release in the presence of FM1-43FX (10 μM; Invitrogen) for 2 min. This protocol is designed to release all functionally competent vesicles in the recycling pool, regardless of vesicular P_r (Pyle et al., 2000; Harata et al., 2001). The FM1-43FX loading protocol is expected to directly depolarize the membrane potential to approximately -20 mV (Moulder et al., 2006; Crawford et al., 2009) and therefore promote transmitter release from terminals, independent of presynaptic action potentials. After labeling, Advasep-7 (500 μM; CyDex) was added to remove residual dye for another 10 s. Dye labeled cultures were rinsed and fixed in 4% paraformaldehyde and 0.2% glutaraldehyde in PBS for 10 min. Fixed cultures were incubated in blocking solution containing 4% goat serum and 0.05% Triton X-100 for 15 min. For vesicular glutamate transporter 1 (vGluT-1) staining, the fixed cultures were incubated in primary vGluT-1 antisera (Millipore Bioscience Research Reagents) diluted 1:2000 in blocking solution for 3 h. Alexa Fluor 647-conjugated anti-guinea pig antisera (1:500; Invitrogen) were used to visualize vGluT-1 labeling. Cultures on glass were mounted on a microscope slide with Fluoromount-G (Southern Biotechnology). Experimental conditions for each slide were coded before subsequent image acquisition and analysis, and the experimenter was kept naive to experimental conditions through the data acquisition and analysis procedures.

All images were acquired under a 60× oil objective by a C1 scanning laser coupled to a Nikon TE300 inverted microscope (Nikon). Acquisition gain setting, scanning rate, and Z-stack setting were identical across the acquired fields within an experiment. Data analysis was performed in MetaMorph (Molecular Devices). Fluorescence images were acquired sequentially from the vGluT-1 and FM1-43FX channels. Fields were selected on the basis of exhibiting discrete vGluT-1-immunoreactive puncta (Moulder et al., 2010). Ten regions of interest in each field, corresponding to vGluT-1-immunoreactive puncta, were selected without reference to the FM1-43FX image. The FM1-43FX image was then superimposed and independently thresholded. To distinguish active from inactive synapses, we used a relative FM1-43FX thresholded area cutoff. The thresholded FM1-43FX area of active synapses was required to be >12.5% of the thresholded vGluT-1 area of the same punctum. This

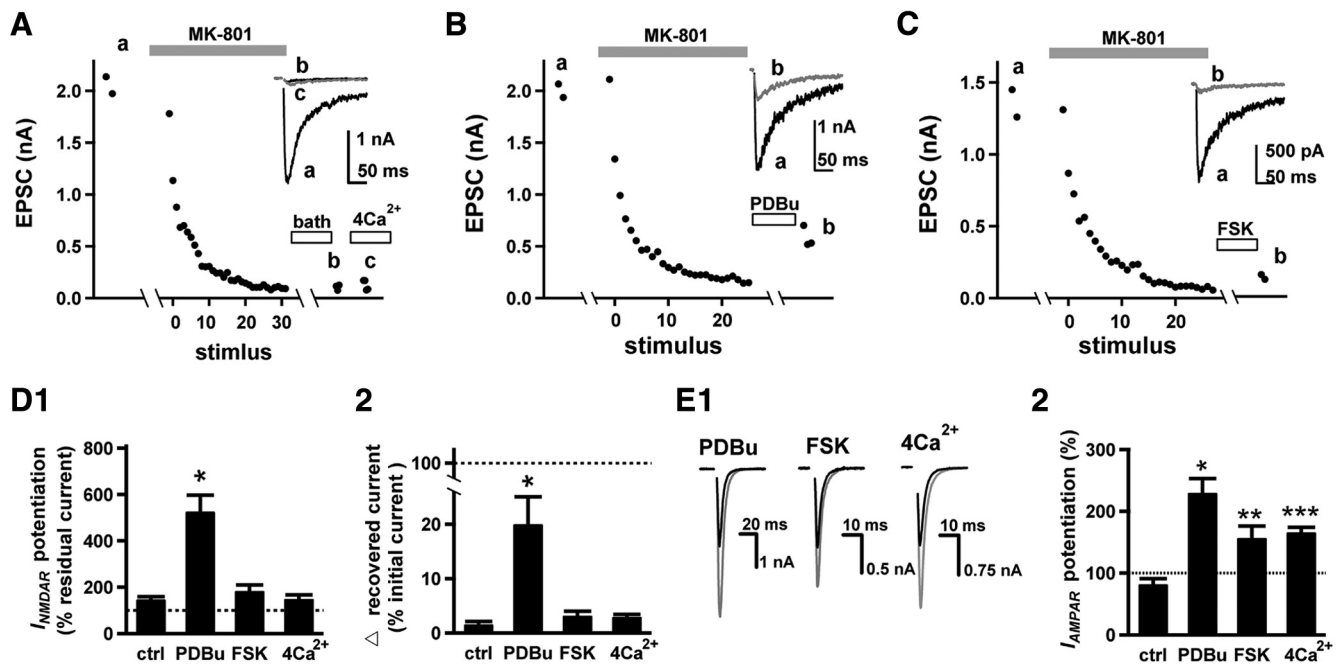


Figure 1. Modulators differentially potentiate NMDAR-mediated EPSCs after MK-801 block of synaptic NMDARs. **A**, Example of progressive MK-801 block during evoked stimulation and subsequent recovery of I_{NMDAR} . Synaptic receptor blockade was accomplished by delivering low-frequency presynaptic stimulation (0.1 Hz) in the presence of MK-801 (5 μ M). The gray bar indicates the time period when MK-801 was applied; the open bars show application of regular extracellular saline solution (bath) or of elevated Ca^{2+} (4Ca²⁺). The recovered I_{NMDAR} in regular external bath or 4 mM Ca^{2+} was sampled 2–3 min after MK-801 wash off. The amplitude of I_{NMDAR} is plotted against the stimulus episode. Inset, Superimposed initial (a), the basal recovered I_{NMDAR} (b), and the recovered current in 4 mM Ca^{2+} (c) shown from the corresponding time point in the amplitude–stimulus plot. **B**, **C**, Similar to **A**, with PDBu (1 μ M) (**B**) or FSK (50 μ M) (**C**) applied during the 2 min interval after MK-801 was wash off. **D1**, Summary of synaptic I_{NMDAR} potentiation by modulators after synaptic receptor blockade. The residual I_{NMDAR} after MK-801 block is set as 100% (PDBu: 526 ± 71%, $n = 10$, * $p < 0.00007$; FSK: 184 ± 25%, $n = 7$; 4 mM Ca^{2+} : 150 ± 17%, $n = 8$; statistical comparison is with the control: 147 ± 12%, $n = 8$). **D2**, Comparison of the percentage I_{NMDAR} recovery (Δ recovered current = recovered current – residual current) after modulator treatment. The Δ recovered I_{NMDAR} is normalized to the initial I_{NMDAR} . The initial I_{NMDAR} is set to 100% (PDBu: 20 ± 5%, $n = 10$, * $p < 0.005$; FSK: 3.2 ± 0.8%, $n = 7$; 4 mM Ca^{2+} : 3.0 ± 0.4%, $n = 8$; statistical comparison is with the control: 1.6 ± 0.5%, $n = 8$). **E**, Synaptic NMDAR blockade did not affect I_{AMPA} potentiation by modulators. **E1**, Sample traces of I_{AMPA} in 4 mM Ca^{2+} , PDBu, and FSK (gray); traces are superimposed on their corresponding initial EPSC (black). The percentage potentiation of EPSCs by modulators is summarized in **E2** (PDBu: 230 ± 23% of initial I_{AMPA} , $n = 10$, * $p < 0.0002$; FSK: 157 ± 19% of the initial I_{AMPA} , $n = 7$, ** $p < 0.02$; 4 mM Ca^{2+} : 163 ± 8% of the initial I_{AMPA} , $n = 7$, *** $p < 0.002$; all statistics are compared with the control: 83 ± 8%, $n = 8$). Error bars indicate SEM.

criterion was chosen because the nonthresholded FM1-43FX labeling intensity of 51 synapses identified as inactive by this criterion in our data set (five experiments) was not significantly different from surrounding cellular background fluorescence near the same synapses ($p > 0.1$). The use of a percentage area criterion guarded against artifactual assignment of small synapses to the inactive group, but an activity criterion based on a fixed-area cutoff of 10 thresholded pixels of FM1-43FX staining (Moulder et al., 2006) yielded similar results (see Results). For the present experiments, 250 vGluT-1-immunoreactive synapses from five independent experiments (10 puncta from each of five fields in each experiment) were analyzed in each condition. Additional evidence for presynaptically inactive synapses is the bimodal distribution of FM1-43FX fluorescence intensity of vGluT-1-positive terminals when hundreds of terminals from a single experiment are examined (Moulder et al., 2006). One component of the distribution is indistinguishable from background fluorescence and thus represents the population of inactive terminals. A second, broader, nonzero component represents active terminals (Moulder et al., 2006).

Results

PDBu recovers postblock NMDAR-mediated current

We first tested whether synaptic modulators could potentiate transmitter output from synapses with basally low release activity. To selectively eliminate neurotransmission from active synapses, we took advantage of an irreversible use-dependent NMDAR antagonist MK-801 (Huettner and Bean, 1988). At low-frequency stimulation (0.1 Hz), MK-801 blocks NMDAR only at synapses that release glutamate in response to the stimulation (Hessler et al., 1993; Rosenmund et al., 1993). Consequently, the

synaptic NMDAR remaining functional after MK-801 blocking trials should represent the receptors residing at synapses that are the least active during the stimulation. We examined whether modulator application after synaptic block of NMDAR current (I_{NMDAR}) causes recovery of synaptic I_{NMDAR} . The recovered I_{NMDAR} is presumably mediated by the receptors that remain functionally intact after MK-801 block and therefore represent the transmitter output from synapses that release glutamate only after modulator treatment.

In the presence of MK-801 (5 μ M), 25–35 action potential stimuli at 0.1 Hz depressed synaptic I_{NMDAR} to ~3% of the initial I_{NMDAR} ($3 \pm 0.4\%$ of the initial I_{NMDAR} ; $n = 8$) (Fig. 1A). Isolated AMPAR current (I_{AMPA}) and the I_{AMPA} paired-pulse ratio (PPR) (second EPSC/first EPSC; 50 ms interpulse interval), however, was minimally affected by MK-801 application (I_{AMPA} amplitude after the MK-801 blocking protocol, 83 ± 7% of the initial I_{AMPA} ; $p = 0.06$; baseline PPR, 0.71 ± 12%; PPR after MK-801 blocking, 0.66 ± 6%; $p = 0.09$; $n = 8$). The insignificant trend toward reduced I_{AMPA} likely resulted from weak, variable time-dependent rundown. These results indicate that MK-801 selectively antagonizes NMDARs; blocking synaptic NMDARs did not detectably change presynaptic release properties. There was very little recovery of I_{NMDAR} 2–3 min after MK-801 removal (Fig. 1A, D2). The slight increase of I_{NMDAR} may reflect minor dissociation of MK-801 from the receptors because of the preceding trials of depolarization applied to sample the residual current after MK-801 block (Huettner and Bean, 1988). Alternatively, the

weak recovery of residual current might represent a small contribution from receptor diffusion from perisynaptic to synaptic sites (Tovar and Westbrook, 2002). Regardless of the causes of the weak recovery, the small fraction of current increase under control conditions indicated that MK-801 block was relatively irreversible within the time interval of our experiments (Huettner and Bean, 1988). Applying similar blocking trials to neurons in 4 mM Ca^{2+} , which was designed to raise vesicular P_r at active terminals, did not further reduce the residual current (blocking in 4 mM Ca^{2+} , $3.2 \pm 1.0\%$ to the initial I_{NMDAR} ; $n = 8$; blocking in 2 mM Ca^{2+} , $4.6 \pm 0.8\%$; $p = 0.32$; $n = 9$), suggesting application of MK-801 in 2 mM Ca^{2+} using our stimulation protocol was sufficient to block virtually all active synapses. Together, these results confirm that our protocol asymptotically blocked NMDARs at the population of presynaptically active terminals.

After similar blocking trials with MK-801, application of PDBu, as previously documented, strongly potentiated I_{AMPA} (Fig. 1E1,E2). PDBu effects on AMPAR-mediated EPSCs have previously been shown to be primarily through presynaptic mechanisms (Malenka et al., 1986; Hori et al., 1999) (but see Carroll et al., 1998). Interestingly, PDBu also promoted a strong recovery of synaptic I_{NMDAR} after MK-801 block (Fig. 1B,D1,D2). PDBu resulted in approximately fivefold potentiation of the residual I_{NMDAR} after MK-801 block (Fig. 1D1), and the net current recovery was $\sim 20\%$ of the initial current before receptor block (Fig. 1D2).

cAMP increases awakening of dormant presynaptic terminals, but the effect is slow to develop (Ma et al., 1999; Yao et al., 2006). We used acutely applied FSK to determine whether rapid cAMP increases, similar to rapid PDBu effects, recruit release from synapses that are not blocked by MK-801. As a presynaptic comparator, we also examined the effect of elevated extracellular Ca^{2+} on residual I_{NMDAR} . As expected, FSK application robustly potentiated I_{AMPA} (Fig. 1E1,E2). However, in contrast to PDBu, acute FSK application after MK-801 block resulted in only a minimal recovery of postblock synaptic I_{NMDAR} (Fig. 1C,D1,D2). Evoked EPSCs in 4 mM Ca^{2+} , which is expected to increase vesicular P_r at active terminals but not to awaken dormant synapses, increased I_{AMPA} to ~ 1.5 -fold (Fig. 1E1,E2), but again this manipulation caused only a minimal increase of I_{NMDAR} after MK-801 block (Fig. 1A,D1,D2). The residual current potentiation and the ratio of current recovery to the initial current before MK-801 block were similar in FSK and 4 mM Ca^{2+} (Fig. 1D1,D2), suggesting that acute FSK, similar to elevated Ca^{2+} , did not significantly recruit transmission from synapses protected from MK-801 block. The minimal recovery of I_{NMDAR} in 4 mM Ca^{2+} after MK-801 block, which potentiated I_{AMPA} , also indicates that potentiation of residual I_{NMDAR} did not result from transmitter spillover to the receptor domains that were not previously exposed to transmitter.

To confirm further that the recovered I_{NMDAR} after PDBu potentiation was not mediated by residual receptors that were not completely blocked at the initially active terminals, we also examined the effect of PDBu on I_{NMDAR} recovery after MK-801 block of synaptic current in elevated Ca^{2+} (4 mM). Blocking receptors with MK-801 in higher Ca^{2+} , which raises overall vesicular and synaptic P_r at active terminals while block is proceeding, ensures even more complete receptor block, including extrasynaptic receptors that might be recruited only under high vesicular P_r conditions. After receptor block in elevated Ca^{2+} , PDBu still significantly promoted I_{NMDAR} recovery (PDBu potentiation, $515 \pm 52\%$ of residual current; $n = 8$). This result confirms that PDBu-dependent recovery of I_{NMDAR} was not caused by incomplete

receptor block at the terminals in combination with transmitter spillover after potentiation. We hypothesized that PDBu-induced I_{NMDAR} recovery after MK-801 block represents a unique presynaptic effect of PDBu potentiation at initially dormant synapses.

Although these experiments help exclude incomplete MK-801 block because of low vesicular P_r , they do not account for the possibility that NMDA receptors remain unblocked because of action potential propagation failures into terminals during stimulation. PDBu might recover synaptic I_{NMDAR} as a result of increased excitability in axon branches that normally fail to transmit action potentials. We have previously shown that action potential invasion is reliable at autaptic synapses under normal conditions, and manipulations designed to increase axon excitability do not significantly alter autaptic output (He et al., 2002). Nevertheless, we performed additional tests for the present work. First, we tested whether increasing presynaptic stimulus duration recruits larger synaptic responses. We found no significant change in EPSC amplitude with presynaptic stimulus durations of 1, 2, or 3 ms (supplemental Fig. S1A, available at www.jneurosci.org as supplemental material). Furthermore, wider stimuli did not recover more synaptic I_{NMDAR} after the MK-801 blocking protocol (supplemental Fig. S1B, available at www.jneurosci.org as supplemental material). As another test of complete MK-801 block, we applied 4-AP (100 μM) after the MK-801 blocking protocol to artificially enhance excitability of unclamped axonal regions. We observed no significant I_{NMDAR} recovery after MK-801 block (supplemental Fig. S1C, available at www.jneurosci.org as supplemental material), unlike our observations with PDBu. Additional evidence that changes in excitability do not explain the PDBu effect come in experiments below in which we used secretagogues (e.g., hypertonic challenge and direct depolarization) designed to circumvent action potential propagation entirely. Together, these results all suggest that PDBu-induced excitability changes do not account for the observation of I_{NMDAR} recovery after MK-801 block.

Modulators equally enhance I_{AMPA} and I_{NMDAR}

To test our hypothesized explanation of PDBu induced current recovery after MK-801 block, we next performed various tests of a postsynaptic explanation for the PDBu-induced I_{NMDAR} recovery. Differential effects of PDBu and FSK on post-MK-801, residual I_{NMDAR} may result from preferential potentiation of specific postsynaptic receptors (i.e., PDBu preferentially potentiated I_{NMDAR} , or FSK preferentially potentiated I_{AMPA}). In mature hippocampal neurons, AMPARs and NMDARs are colocalized in excitatory terminals (Forsythe and Westbrook, 1988; Bekkers and Stevens, 1989); pure presynaptic potentiation typically affects AMPAR- and NMDAR-mediated EPSCs in parallel (Perkel and Nicoll, 1993; Tong and Jahr, 1994). If PDBu and FSK preferentially modulate NMDARs or AMPARs, we would expect that PDBu and FSK should differentially affect EPSCs mediated by NMDARs versus EPSCs mediated by AMPARs. We measured dual-component EPSCs before and after modulator application in the absence of MK-801 (Fig. 2A). We defined the amplitude of the AMPAR component of the EPSC as the current peak within 10 ms after stimulation was initiated, whereas the amplitude of I_{NMDAR} was denoted as the averaged current within a 5 ms bin 35 ms after stimulation initiation (Tsien et al., 1996; Myme et al., 2003).

Application of PDBu potentiated I_{AMPA} and I_{NMDAR} in parallel, with no distinguishable difference in the degree of potentiation (Fig. 2A, left; B, left panel). This result is consistent with the

idea that acute PDBu treatment has primarily a presynaptic action. After acute incubation with FSK, potentiation in I_{AMPA} was also indistinguishable from I_{NMDAR} potentiation (Fig. 2A, right; B, right panel). Thus, FSK treatment also resulted in an equal enhancement of I_{AMPA} and I_{NMDAR} . These results showed that, with brief incubation, PDBu and FSK did not preferentially augment current mediated by specific postsynaptic receptors and therefore suggest a presynaptic effect of EPSC potentiation.

Increases in open probability (P_o) of NMDARs have been reported with phorbol ester stimulation and/or PKC activation (Durand et al., 1992; Xiong et al., 1998; Lan et al., 2001). An increase in P_o might be expected to result in nonparallel modulation of AMPAR and NMDAR EPSC components. Therefore, the results in Figure 2 argue against a PDBu effect on P_o under our conditions. In addition, if PDBu increases P_o of NMDARs and thereby contributes to the I_{NMDAR} recovery after MK-801 block, we would also expect that PDBu should retard the decay of NMDAR-mediated EPSCs (Jahr, 1992). However, we found that, in the presence of PDBu, the weighted time constant of NMDAR-mediated EPSCs was 178 ± 10 ms, which was not significantly different from the weighted decay constant in the baseline (164 ± 28 ms; $n = 7$; $p = 0.86$). Therefore, PDBu-mediated recovery of I_{NMDAR} does not result from increased P_o of NMDARs under our conditions.

PDBu does not increase surface NMDARs

Work from others has shown that prolonged treatment with phorbol esters promotes surface NMDAR insertion and receptor assembly in hippocampal neurons (Lan et al., 2001; Scott et al., 2001). We next explored whether acute treatment with PDBu in our system leads to detectable increases in surface NMDARs that might cause I_{NMDAR} recovery after MK-801 block. To test whether PDBu increased surface NMDARs, we blocked whole-cell NMDARs by applying MK-801 ($20 \mu\text{M}$) in the presence of the exogenous agonist NMDA (1 mM) and examined the increases of the whole-cell I_{NMDAR} after PDBu treatment. Whole-cell blockade of NMDARs depressed NMDA-evoked current to $\sim 4\%$ of the initial current ($4.3 \pm 1.8\%$; $n = 6$) (Fig. 3A, top panel). Whole-cell blockade of NMDARs affected neither evoked synaptic I_{AMPA} ($104 \pm 10\%$ of baseline; $n = 6$) nor PPR measured by I_{AMPA} (baseline PPR, $0.89 \pm 15\%$; PPR after whole-cell block, $0.78 \pm 17\%$; $p = 0.32$), suggesting that blocking whole-cell NMDARs did not affect presynaptic transmitter output. Two minutes after MK-801 wash-off, residual whole-cell responses to NMDA were slightly increased (Fig. 3A, top panel; C, left). The slight increase of whole-cell I_{NMDAR} may result from weak unblocking of antagonist from the receptors during the preceding trial of NMDA application, which measured the residual current after whole-cell MK-801 block (Huettner and Bean, 1988). Acute PDBu application after whole-cell receptor blockade resulted in minimal increases in the NMDA-evoked whole-cell current (Fig. 3A, bottom panel). The

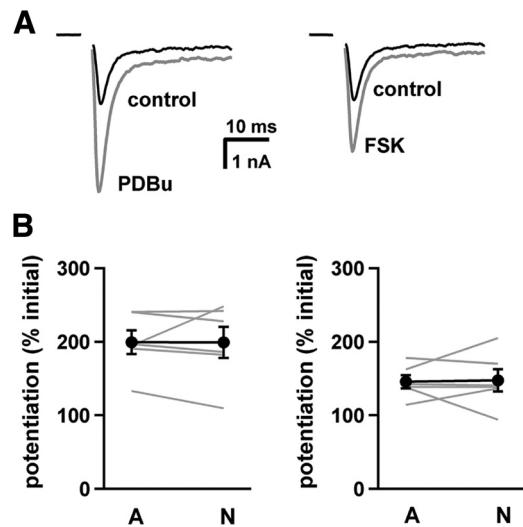


Figure 2. Modulators equally potentiate AMPAR- and NMDAR-mediated EPSCs. *A*, The effect of modulators on AMPAR and NMDAR EPSCs was examined by comparing AMPAR and NMDAR components of EPSCs before and after 2 min of modulator application. Examples of EPSC in PDBu (left panel; gray) or FSK (right panel; gray) are superimposed on the respective baseline EPSCs (black). *B*, Summary of potentiation of AMPAR (A) or NMDAR (N) components by PDBu (left) and FSK (right). The gray lines represent responses from individual cells. For PDBu, I_{AMPA} , $199 \pm 16\%$ of baseline; I_{NMDAR} , $199 \pm 21\%$ of baseline; $n = 6$; $p = 0.99$. For FSK, I_{AMPA} , $145 \pm 9\%$ of baseline; I_{NMDAR} , $147 \pm 15\%$ of baseline; $n = 6$; $p = 0.88$. Error bars indicate SEM.

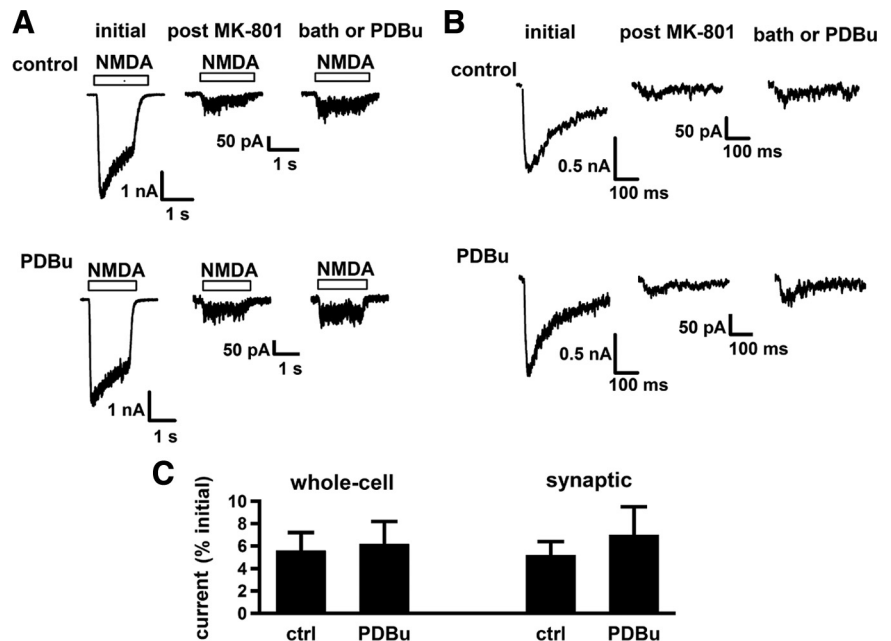


Figure 3. Acute PDBu application does not increase surface NMDARs. *A*, After whole-cell blockade of NMDARs, PDBu caused minimal recovery of whole-cell I_{NMDAR} . Sample traces of time-dependent (*A*, top) or PDBu-dependent (*A*, bottom) effect on whole-cell I_{NMDAR} after blocking surface NMDARs. *B*, Similar to *A*. The comparison of time-dependent effect and the effect of PDBu on synaptic I_{NMDAR} after whole-cell NMDAR blockade. *C*, Quantification of residual whole-cell and synaptic I_{NMDAR} after control or PDBu treatment after surface receptor blockade. The peak currents are normalized to the initial current before MK-801 block (whole-cell current: $5.8 \pm 2.5\%$ in control, $n = 6$; $6.2 \pm 2.2\%$ in PDBu, $n = 6$; $p = 0.58$; synaptic current: $5.2 \pm 1.2\%$ in control, $n = 6$; $7 \pm 2.5\%$ in PDBu, $n = 6$; $p = 0.59$). Error bars indicate SEM.

mild increases in NMDA-evoked current in PDBu was indistinguishable from that in control (Fig. 3C, left), suggesting that acute application of PDBu did not significantly increase total NMDARs at the cell surface.

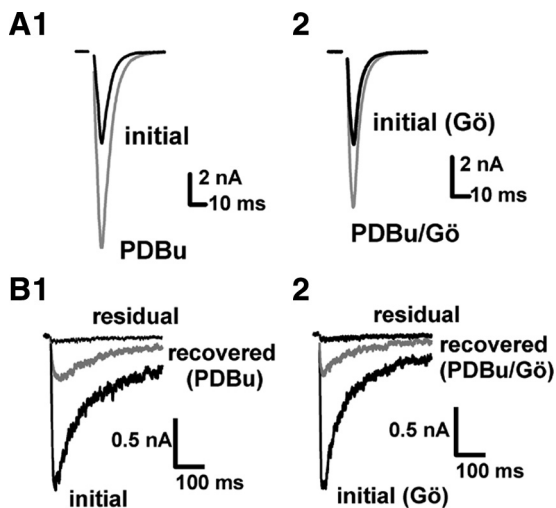


Figure 4. PKC inhibition does not prevent I_{NMDAR} recovery by PDBu. **A**, PDBu effectively potentiated I_{AMPA} (**A1**) and robustly recovered I_{NMDAR} (**B1**) after MK-801 block. **A1**, Sample trace of I_{AMPA} potentiation after PDBu treatment from the same cell is superimposed on the initial current. **B1**, PDBu-recovered I_{NMDAR} is superimposed on the initial (before blocking) and the postblock residual current. **A2**, **B2**, Similar to **A1** and **B1**, PKC inhibitor Gö6983 ($2 \mu\text{M}$) was present before and during the experiments. Average PDBu effect on I_{AMPA} potentiation in the absence (PDBu) or presence (PDBu/Gö) of PKC inhibitor: $192.3 \pm 23.5\%$ in PDBu, $n = 9$; $147.2 \pm 19.1\%$ in PDBu/Gö6983, $n = 11$; $p = 0.15$. Average effects of PDBu from the experiment in **B** are given as percentage of initial current before MK-801 block: $17.6 \pm 4.7\%$ in PDBu, $n = 9$; $15.4 \pm 2.9\%$ in PDBu/Gö6983, $n = 11$; $p = 0.7$.

To test whether PDBu selectively increases synaptic NMDARs, we examined the effect of PDBu on the synaptic I_{NMDAR} after whole-cell blockade of NMDARs. Consistent with the results from sampling whole-cell NMDA responses, we found that, after whole-cell blockade of NMDARs, PDBu application did not significantly increase synaptic I_{NMDAR} compared with control cells (Fig. 3B, C, right), despite strong potentiation measured in I_{AMPA} ($225 \pm 32\%$ of baseline; $n = 6$; $p < 0.04$). These results therefore suggest that acute treatment of PDBu did not lead to significant increases of NMDARs in postsynaptic sites. Together, our results suggest that acute application of PDBu does not detectably increase surface NMDARs.

PDBu-mediated recovery of I_{NMDAR} is PKC independent

Under certain conditions, phorbol esters enhance the clustering, movement, and channel gating of NMDARs (Durand et al., 1992; Kutsuwada et al., 1992; Lu et al., 2000; Lan et al., 2001; Scott et al., 2001; Groc et al., 2004), and these effects are PKC dependent (Durand et al., 1992; Xiong et al., 1998; Lu et al., 2000; Lan et al., 2001; Scott et al., 2001; Fong et al., 2002). Presynaptically, phorbol esters act at least partly through PKC-independent mechanisms to potentiate transmission (Searl and Silinsky, 1998; Rhee et al., 2002; Lou et al., 2008). To distinguish whether postsynaptic PKC activation by PDBu results in NMDAR modification, which in turn leads to the current recovery after MK-801 block, we evaluated the action of PDBu after pharmacological inhibition of PKC activity. Because PKC activity is present in both presynaptic and postsynaptic compartments, we first tested PKC-independent presynaptic potentiation of PDBu. We measured PDBu potentiation of I_{AMPA} in the presence a PKC inhibitor. We used the broad-spectrum PKC inhibitor Gö6983 to block PKC activation by PDBu. The pharmacological activity of Gö6983 was verified by two independent assays in hippocampal excitatory autapses (Chang and Mennerick, 2010). These control experi-

ments were performed concurrently with experiments in the present work and showed that Gö6983 inhibits two previously documented PKC-dependent phorbol ester effects: phorbol ester-induced speeding of vesicle replenishment after depletion (Stevens and Sullivan, 1998) and phorbol ester-induced occlusion of $G_{\text{I/O}}$ G-protein-mediated synaptic depression (Zamponi et al., 1997).

Application of Gö6983 ($2 \mu\text{M}$) alone did not alter evoked AMPAR-mediated EPSCs (control, $5.06 \pm 0.98 \text{ nA}$, $n = 9$; Gö6983, $4.82 \pm 0.78 \text{ nA}$, $n = 10$; $p = 0.85$). Preincubation of Gö6983 did not prevent subsequent PDBu potentiation of I_{AMPA} (Fig. 4A1,A2), consistent with an important role of PKC-independent mechanisms in phorbol ester potentiation (Rhee et al., 2002).

Having confirmed that PDBu effectively potentiated presynaptic neurotransmission in the absence of PKC activation, we next asked whether PKC inhibition, which should also prevent postsynaptic modification of NMDARs, compromises current recovery by PDBu. After MK-801 block of synaptic NMDARs, PKC inhibition did not prevent PDBu-mediated recovery of synaptic I_{NMDAR} (Fig. 4B2) compared with PDBu alone. Inhibiting PKC activity by another class of PKC inhibitor, Ro31-8220 ($2 \mu\text{M}$), yielded a similar result (I_{AMPA} potentiation: $172.1 \pm 12.8\%$ in PDBu/Ro31-8220, $n = 11$; $246.1 \pm 36.1\%$ in PDBu, $n = 10$; $p = 0.07$; I_{NMDAR} recovery: $20.5 \pm 4.7\%$ in PDBu/Ro31-8220, $n = 11$; $32.5 \pm 7.1\%$ in PDBu, $n = 10$; $p = 0.17$). These results suggest that PKC-dependent modulation of postsynaptic NMDARs does not contribute strongly to the recovery of I_{NMDAR} . Together, these results suggest that the enhancement by PDBu of synaptic strength at terminals with very low synaptic P_{T} or dormant terminals is mainly PKC independent.

PDBu potentiation and the readily releasable vesicle pool

If our hypothesis is correct that PDBu recovers I_{NMDAR} by presynaptically resurrecting functionally dormant synapses, this effect should also be evident as increases in the number of readily releasable vesicles, the vesicle pool that is docked and primed at presynaptic active zones. Readily releasable vesicle pool (RRP) size is often measured as hypertonic sucrose-evoked AMPA EPSCs (Rosenmund and Stevens, 1996; Schikorski and Stevens, 2001). Hypertonicity-evoked release also circumvents potential PDBu effects on action potential propagation or waveform, since this secretagogue bypasses action potential propagation and Ca^{2+} influx (Rosenmund and Stevens, 1996). We used 0.75 M sucrose to ensure a saturated rate of vesicle release (Rosenmund and Stevens, 1996; Basu et al., 2007; Wierda et al., 2007). In our cultures, PDBu treatment led to an $\sim 25\%$ increase in the RRP (Fig. 5A), quantitatively consistent with the degree of I_{NMDAR} recovery observed in Figure 1D2. Therefore, results from sucrose-evoked EPSCs help exclude both excitability changes and changes in NMDA receptor function as explanations for the PDBu-induced recovery of I_{NMDAR} .

We also performed similar experiments in mass cultures to ensure that activation of dormant synapses is not unique to autaptic synapses. In conventional mass cultures that form more complex and divergent synaptic interconnections, we examined sucrose responses as in the autaptic recordings in Figure 5A. Similar to autaptic responses, sucrose-evoked synaptic responses in mass culture were mildly but significantly potentiated after PDBu application (Fig. 5B). These observations from conventional synaptic connections suggest that the effects of PDBu are not confined to autaptic synapses.

In mass cultures, we also tested for PDBu-mediated recovery from dormancy using an NMDAR-based assay. We triggered

transmitter release by incubating cells for 10 min in 200 mM sucrose in the presence of 10 μ M MK-801 (with NBQX and bicuculline present throughout). Because sucrose-induced release is not dependent on action potentials or calcium, our protocol helped ensure that even NMDARs at synapses that were infrequently activated by spontaneous activity would be blocked. After incubation, we measured synaptic NMDAR responses to spontaneous network activity. After MK-801 block, we never saw synaptic events within 150–210 s continual recording from any of four cells, even during elevated K^+ application to stimulate network activity and glutamate release ($n = 4$). By contrast, during PDBu application, synaptic NMDAR events became more evident (12 burst-like events in 150 s of recording from four cells) (supplemental Fig. S4, available at www.jneurosci.org as supplemental material). In all cells, we verified that these post-PDBu currents were sensitive to D-APV. The burst-like nature of the currents could be associated with NMDAR resurrection by PDBu throughout the network, with the long-lived NMDAR EPSPs promoting bursts of firing in pre-synaptic cells. Thus, this experiment is generally consistent with the idea that presynaptic dormancy also applies to more complex networks.

Synaptic release properties and PDBu-mediated potentiation

Our results thus far exclude postsynaptic explanations for PDBu-mediated revival of I_{NMDAR} after synaptic MK-801 block and support the idea that PKC-independent presynaptic actions of PDBu recruit release from dormant terminals in simple and complex networks. In the literature, phorbol esters enhance vesicle release efficiency, increase synaptic P_r , and hence increase synaptic strength (Malenka et al., 1986; Shapira et al., 1987; Basu et al., 2007). In many studies, the effect of phorbol ester on vesicular release efficiency is evaluated by paired-pulse modulation (Shapira et al., 1987; Muller et al., 1988; Yawo, 1999), or by the ratio of charge integral from an isolated evoked EPSC relative to the release from the entire release-ready vesicle pool (Basu et al., 2007; Wierda et al., 2007). These estimates, however, evaluate the conglomerate of changes in release from the entire population vesicles distributed across all synapses (Branco and Staras, 2009). Therefore, nothing is known about the synaptic P_r profile of the initially dormant synapses once their release activity is restored.

The P_r profiles of a population of synapses can be revealed by the kinetics of MK-801 progressive block of I_{NMDAR} (Hessler et al., 1993; Rosenmund et al., 1993). We used the kinetic changes in the progressive block after PDBu potentiation to probe changes of synaptic P_r distribution in the activated synapses after potentiation. We also examined the synaptic P_r profile from those basally quiescent terminals, once they were activated by PDBu, with similar MK-801 progressive block.

In control cells, the decay of the first 25 peak NMDAR EPSCs during MK-801 progressive block was best described by a sum of two exponentials (Fig. 6A2, open circle), suggesting that synaptic transmission was composed of fast and slow components, which represent at least two populations of synapses with high and low P_r (Hessler et al., 1993). Note that this observation does not ex-

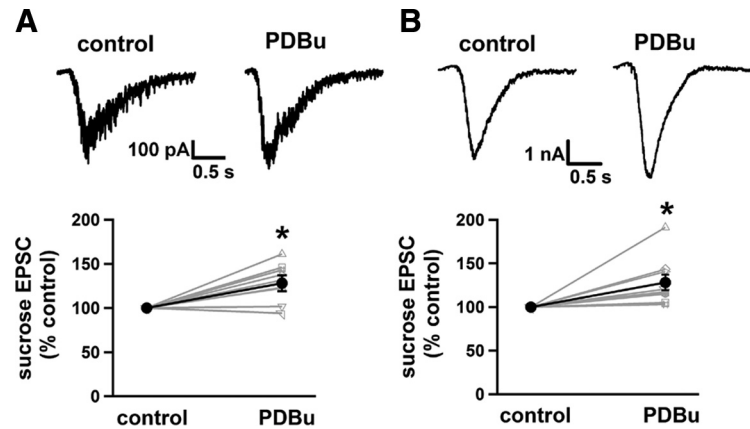


Figure 5. Sucrose-evoked EPSCs are increased by PDBu. **A**, Hypertonic sucrose solution (0.75 M) was used to evoke release from autaptic neurons as a measure of the RRP size before and after PDBu administration for 2 min. Sucrose was applied during the entire trace shown. After PDBu challenge, RRP, measured as sucrose-evoked postsynaptic charge integral, was slightly but significantly increased [$128 \pm 9\%$ to control (100%); $n = 9$; $*p < 0.02$]. The percentage increase was consistent with the degree of I_{NMDAR} recovery. The gray traces represent individual examples of normalized postsynaptic charge measured before and immediately after PDBu treatment. **B**, Similar to **A**, EPSC response to hypertonic sucrose challenge in mass cultures. PDBu increased the EPSC to $128 \pm 8\%$ of baseline (100%) ($n = 9$; $*p < 0.03$). Bath solution included 25 μ M bicuculline and 25 μ M D-APV. Error bars indicate SEM.

clude the idea of a continuous distribution of synaptic P_r values (Rosenmund et al., 1993; Murthy et al., 1997).

PDBu treatment before any MK-801 application accelerated the subsequent MK-801 progressive block of the current (Fig. 6A1; A2, solid circle). Because we have previously ruled out the effect of PDBu on P_o of NMDARs (Fig. 2), the most plausible explanation for the faster EPSC block by MK-801 is that PDBu elevated P_r across synapses. The accelerated decay during progressive current block also yielded a greater proportion of the fast component compared with the control (23.8 ± 9.8 vs $64 \pm 3\%$) (for full fit parameters, see Fig. 6A2, legend), suggesting that PDBu increased the average synaptic P_r , as well as increasing the proportion of high P_r terminals (Rosenmund et al., 1993).

Blocking synaptic NMDARs at untreated synapses eliminated I_{NMDAR} from synapses that respond to our stimulus protocol. After receptor blockade, PDBu promoted recovery of I_{NMDAR} as described previously. The MK-801 progressive block of PDBu-recovered I_{NMDAR} also consisted of biexponential components with significantly slower decay (Fig. 6A2, gray circle) compared with the basal, unblocked population of terminals that were potentiated by PDBu before progressive block. The slower decay of the EPSC block in the recovered current suggests that the recovered current arises from a subset of terminals that exhibited significantly lower synaptic P_r than the PDBu-potentiated population of initially active synapses.

When comparing the rate of progressive block, the contrast in synaptic P_r profiles between PDBu-recovered current (slow MK-801 block) (Fig. 6A2, gray) and the current that was derived from all synapses in the presence of PDBu (fast MK-801 block) (Fig. 6A, solid) suggests that PDBu did not potentiate synaptic output uniformly across terminals. Release from initially dormant synapses was restored by PDBu. However, this restoration was not accompanied by the high synaptic P_r profile and fast MK-801 block observed at initially active terminals potentiated by PDBu. Instead, the MK-801 block profile from the awakened terminals was closer to the slow MK-801 block profile of naive active synapses before PDBu application (control, without PDBu) (Fig. 6A2, open). The difference in synaptic P_r distributions induced by PDBu from initially active terminals versus quiescent termi-

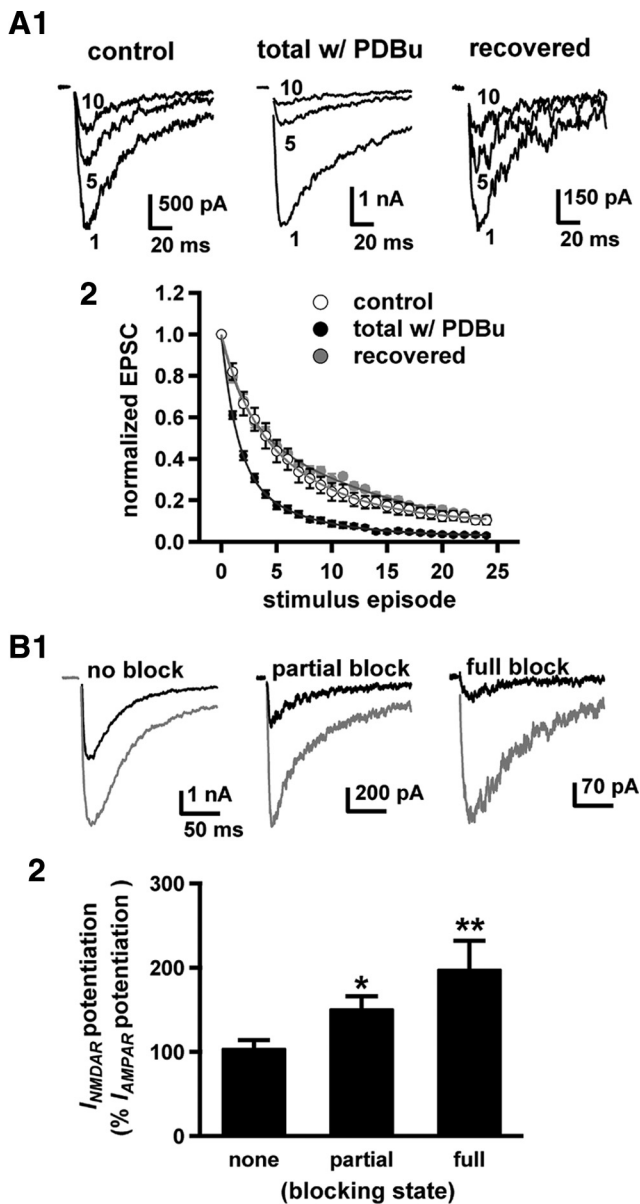


Figure 6. Synaptic P_r differs at active versus dormant synapses after PDBu potentiation. **A**, PDBu-activated quiescent synapses show lower overall synaptic P_r . **A1**, Examples of graded depression of I_{NMDAR} during MK-801 progressive block in control synapses (left), synapses that were potentiated by PDBu without previous MK-801 block (middle), and PDBu-recovered synapses (right). EPSCs from the indicated stimulus episodes in the presence of MK-801 are superimposed. **A2**, Peak I_{NMDAR} during MK-801 is normalized to the initial I_{NMDAR} and plotted against stimulus episode. The plot, averaged from eight to nine cells, is best fitted by a sum of two exponentials (gray lines) in all three conditions. The kinetics of progressive block (25 stimuli) has $\tau_{fast} = 2.0 \pm 0.6$ (23.8 \pm 9.8%) and $\tau_{slow} = 7.6 \pm 0.9$ (67.3 \pm 9.2%) in control (open; $n = 8$), $\tau_{fast} = 1.38 \pm 0.04$ (64 \pm 3%) and $\tau_{slow} = 5.99 \pm 0.59$ (32 \pm 3%) in PDBu-potentiated current (solid; $n = 9$), and $\tau_{fast} = 1.98 \pm 0.35$ (38 \pm 6%) and $\tau_{slow} = 14.58 \pm 3.8$ (62.6 \pm 2%) in PDBu-recovered current (gray; $n = 8$). **B**, PDBu more strongly potentiated I_{NMDAR} at synapse with low P_r . **B1**, Examples of PDBu-dependent potentiation from a neuron without receptor blockade (left), a neuron with synaptic I_{NMDAR} that was partially blocked (\sim 15% of initial current; middle), and a neuron with synaptic I_{NMDAR} that was fully blocked (right). The EPSCs after PDBu potentiation (gray) are superimposed on the EPSC before PDBu treatment (black). **B2**, Summary of PDBu-mediated potentiation in all three conditions. Level of I_{NMDAR} potentiation by PDBu is normalized to I_{AMPAR} potentiation from the same cells (no block, $n = 12$; partial block, $n = 10$; full block, $n = 11$; $p < 0.00003$ by one-way ANOVA; * $p < 0.003$, compared with no block; ** $p < 0.05$, compared with partial block by paired t test). Error bars indicate SEM.

nals may suggest that initial release competence of the terminal affects the nature of PDBu potentiation.

To test further whether the initial release properties of synapses affects their response to PDBu, we examined whether PDBu differentially potentiates synaptic output from terminals with different P_r . We reasoned that a single episode of brief depolarization should evoke transmitter release from the terminals of highest synaptic P_r , the synaptic output from the high P_r terminals should dominate the isolated EPSC at baseline. Likewise, after partially (achieved with MK-801 during a limited number of stimulus trials) or fully blocking the synaptic I_{NMDAR} , the residual current should represent the synaptic output from the remaining low P_r terminals. If the initial P_r state affects synaptic response to PDBu potentiation, we would expect differential effects of PDBu potentiation on the residual current from partially or fully blocked cells compared with that in the control. We compared the level of PDBu potentiation in the control current, the residual current from partially blocked cells, and the residual current from fully blocked cells.

In the control cells without any preceding receptor block, PDBu, as previously shown, increased baseline I_{NMDAR} approximately twofold (Fig. 6B1, left). Partial elimination of I_{NMDAR} to \sim 15% of the initial NMDAR-mediated current did not affect I_{AMPAR} (102 \pm 10% of the initial I_{AMPAR} before MK-801 block; $n = 10$). Interestingly, PDBu treatment in partially blocked cells resulted in approximately threefold potentiation from the residual I_{NMDAR} (Fig. 6B1, middle). When compared with the cells in which the synaptic I_{NMDAR} was fully eliminated, PDBu potentiation of the residual current was, as previously shown, close to fivefold (Fig. 6B1, right).

Because there is considerable variability in the degree of PDBu potentiation across cells, we used PDBu potentiation of AMPAR-mediated EPSCs as the internal potentiation reference and normalized the level of PDBu potentiation in I_{NMDAR} to I_{AMPAR} potentiation from the same cell. This normalization should represent the true PDBu potentiation of I_{NMDAR} from synapses with different P_r . After normalizing to the level of I_{AMPAR} potentiation, PDBu treatment in control, unblocked cells yielded the expected relative potentiation value near 100% (resulting from parallel NMDAR and AMPAR EPSC potentiation) (Fig. 6B2). When similar normalization was applied to the current potentiation from partially blocked and from fully blocked cells, the ratio of I_{NMDAR} versus I_{AMPAR} potentiation increased from \sim 100% in control to \sim 150% in the partially blocked cells and to \sim 200% in fully blocked cells (Fig. 6B2). The graded increases of PDBu potentiation in I_{NMDAR} suggest that PDBu more strongly potentiates low P_r terminals. Thus, the initial synaptic P_r may influence synaptic response to PDBu potentiation. This result, combined with the progressive MK-801 block experiment, is consistent with the idea that level of potentiation might be negatively correlated to the initial release activity (Stevens and Wang, 1994; Gerber et al., 2008).

NR2B contribution of the recovered current does not differ from baseline

The results thus far suggest that PDBu potentiated postblock I_{NMDAR} by presynaptic mechanisms; the recovered currents arise from a subset of dormant synapses, or synapses with very low P_r , that are not recruited by Ca^{2+} or by FSK. Studies have suggested that synaptic NMDAR composition correlates with synaptic activity as well as with developmental stage. Synapses with lower functionality or immature (nascent) synapses possess greater NR2B subunit postsynaptically (Williams et al., 1993; Monyer et

al., 1994; Kirson and Yaari, 1996; Tovar and Westbrook, 1999; Ehlers, 2003). Therefore, PDBu-recovered synapses may be dominated by NR2B-containing receptors. We used the NR2B-specific antagonist ifenprodil to probe NR2B contribution to the recovered I_{NMDAR} . We compared the ifenprodil sensitivity of the initial I_{NMDAR} and of PDBu-recovered I_{NMDAR} . In the control condition, the peak of the initial (untreated, unblocked) I_{NMDAR} was reduced by ~60% in the presence of ifenprodil (3 μM) (Fig. 7A, left panel). After MK-801 block, PDBu-recovered I_{NMDAR} showed similar ifenprodil sensitivity (Fig. 7A, right panel), suggesting that PDBu-recovered I_{NMDAR} consisted of a similar NR2B contribution compared with the initial EPSC.

In addition, we also examined the deactivation kinetics of the initial and the recovered I_{NMDAR} . EPSCs mediated by NR2B-containing receptors feature slower deactivation kinetics compared with EPSCs mediated by NR2A-containing receptors (Cull-Candy and Leszkiewicz, 2004). The deactivation current of NMDAR-mediated EPSC can be fitted by a sum of two exponentials, which yields a weighted $\tau = 132 \pm 13$ ms ($n = 6$) in the initial current (Fig. 7B). In the recovered current, we found a slightly but not significantly slower decay with weighted $\tau = 181 \pm 24$ ms (Fig. 7B). Together, the pharmacological and kinetic results suggest that there is no significant difference in the level of NR2B component in the initial current and the recovered current. Thus, the synapses that give rise to the recovered current do not preferentially harbor NR2B-containing receptors.

PDBu promotes transmitter release from presynaptically silent synapses

Our results suggest that PDBu promotes synaptic output from synapses that contribute very little to baseline transmission. These synapses could represent synapses with very low P_r and/or synapses that are completely dormant. Work from our group and others has shown that a fraction of glutamate terminals appear to be completely dormant presynaptically (Rosenmund et al., 2002; Altmann et al., 2003; Moulder et al., 2004; Ting et al., 2007). These latent synapses are considered presynaptically inactive because they contain presynaptic vesicles, identified by immunoreactivity for vGluT-1 or other vesicle markers, but fail to detectably recycle vesicles, defined by FM1-43FX uptake on strong depolarization designed to trigger recycling of all release-competent vesicles. We next asked whether PDBu-recovered EPSCs represent, in part, restoration of synaptic activity at these presynaptically inactive terminals. We examined the percentage of inactive synapses from the cultures that were acutely exposed to FSK or to PDBu for 2 min. The synaptic activity of individual terminals was assessed by FM1-43FX labeling with hyperkalemic depolarization (45 mM $[\text{K}^+]_o$ for 2 min) (Pyle et al., 2000; Harata et al., 2001); excitatory terminals were then identified by positive immunoreactivity for vGluT-1. Inactive excitatory synapses were those that were immunoreactive for vGluT-1 but lacking FM1-43FX labeling (i.e., failed to meet a threshold that exceeded background fluorescence

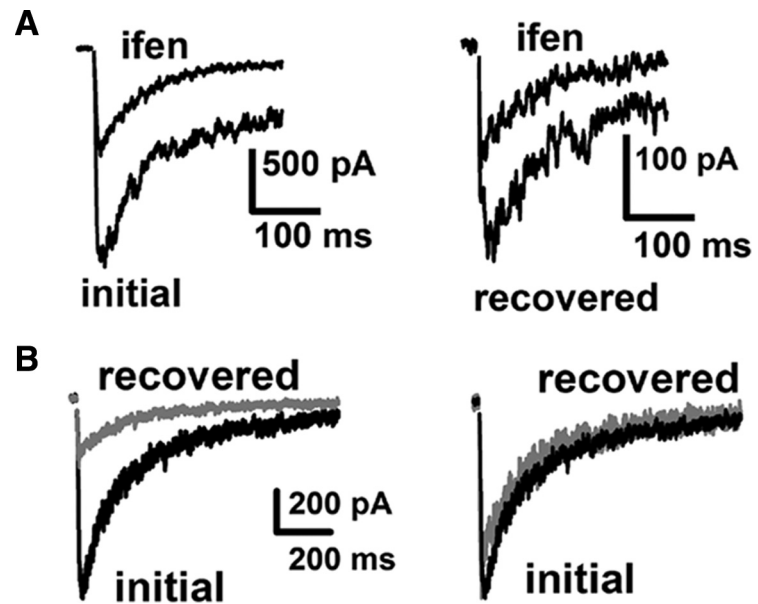


Figure 7. The initial I_{NMDAR} and PDBu-recovered I_{NMDAR} have similar NR2B contribution. **A**, The initial (left) and the recovered I_{NMDAR} (right) exhibited similar sensitivity to ifenprodil (3 μM). The sample traces of the initial (left) and the recovered (right) I_{NMDAR} in the presence of ifenprodil are superimposed on the current in the absence of ifenprodil. Average ifenprodil sensitivity in the initial current and in PDBu-recovered current was $59.3 \pm 4.1\%$ at baseline, $n = 9$; $51.8 \pm 4.9\%$ in PDBu-recovered current, $n = 9$; $p = 0.06$. **B**, The initial and the recovered I_{NMDAR} have similar deactivation kinetics. The decay of the recovered I_{NMDAR} is indistinguishable from that of the initial current. Example of recovered I_{NMDAR} superimposed on the initial current (left). Right, The recovered I_{NMDAR} is scaled and superimposed on the peak of the initial current. The average weighted τ of the baseline NMDAR-mediated EPSCs and the recovered EPSCs from 6 cells (weighted $\tau = 132 \pm 13$ ms in baseline; 181 ± 24 ms in PDBu-recovered current; $p = 0.07$).

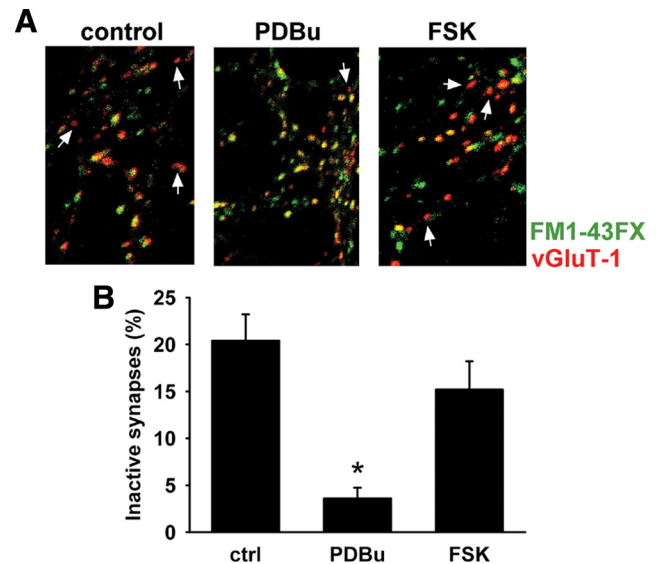


Figure 8. PDBu reduces the percentage of inactive excitatory synapses. **A**, Example images from control (left), PDBu (middle)-, and FSK (right)-treated synapses. Active synapses were labeled with FM1-43FX (green); glutamatergic terminals were identified by immunoreactivity to vGluT-1 antisera (red). Puncta that are positive for vGluT-1 antisera but devoid of FM1-43FX labeling are inactive excitatory synapses (arrows). **B**, Quantification of the percentage of inactive glutamatergic terminals in control ($20.4 \pm 2.8\%$), or PDBu ($3.6 \pm 1.1\%$), and FSK ($15.2 \pm 3\%$)-treated synapses ($*p < 0.00005$ compared with control; $n = 5$). Summary results represent 250 puncta in each condition sampled in 50 fields from five independent experiments. Fields were selected and analyzed by an observer naive to experimental condition. Error bars indicate SEM.

intensity) (see Materials and Methods). In the control condition, the percentage of inactive synapses from the identified excitatory terminals was ~20% (Fig. 8A, left; B). Acute application of PDBu prominently reduced the percentage of inactive synapses (Fig.

8A, middle; B), indicating restored vesicle cycling at initially dormant terminals. In contrast to PDBu, acute (2 min) application of FSK did not change the percentage of inactive synapses (Fig. 8A, right; B).

We have previously used a fixed area threshold to identify inactive synapses, whereby active synapses must exceed a thresholded area of 10 pixels in the FM1-43 channel (Moulder et al., 2006, 2010). By this criterion, a somewhat higher percentage of inactive synapses was observed in all conditions, but similar comparative results were obtained ($27.2 \pm 3.0\%$ control; $7.2 \pm 1.8\%$ PDBu; $25.6 \pm 3.4\%$ FSK).

Our previous work has shown that prolonged treatment (4 h) with FSK reduces the percentage of inactive synapses (Moulder et al., 2008). In sibling cultures of those represented in Figure 8, we repeated the experiments with 4 h FSK treatment and independently verified the reduction in dormant terminals with prolonged FSK treatment ($13.6 \pm 2.3\%$ presynaptically inactive glutamatergic terminals compared with $27.2 \pm 2.9\%$ in control; $n = 5$; $p < 0.0008$). Therefore, the effects of prolonged activation of cAMP-dependent pathways are distinguishable from acute potentiation by FSK. In summary, acute treatment of PDBu, but not FSK, effectively activated transmitter release from the preexisting presynaptically silent synapses.

PDBu potentiation in depolarization-silenced terminals

We previously reported that increasing network activity by prolonged (4 h) depolarization with 35 mM extracellular potassium ($[K^+]_o$) depresses synaptic output (Moulder et al., 2004). The synaptic output is depressed as a result of an increase in presynaptically silent synapses (Moulder et al., 2004). We next asked whether depolarization-inactivated presynaptic terminals, like the dormant synapses in baseline conditions, can be reactivated by PDBu. If PDBu restores release from depolarization-inactivated terminals, we would expect a greater I_{AMPA} potentiation and a greater I_{NMDAR} recovery in depolarized cultures, relative to the depressed baseline in these cultures. We depolarized neurons with 35 mM $[K^+]_o$ for 4 h and examined whether PDBu treatment more strongly potentiates I_{AMPA} and more strongly recovers postblock I_{NMDAR} . Depolarization reduced the average I_{AMPA} by 70% (Fig. 9A1,A2), as previously reported (Moulder et al., 2004). In the depolarized neurons, PPR did not change while the evoked EPSC was depressed (Fig. 9A, legend), suggesting that vesicle P_r from the remaining active terminals did not change. These results are consistent with the idea of binary inactivation of terminals (Moulder et al., 2004). In depolarized neurons, PDBu treatment did not cause more potentiation of I_{AMPA} compared with the control (nondepolarized) (Fig. 9B). Similarly, PDBu resulted in a similar level of recovery in postblock I_{NMDAR} (Fig. 9C). These results suggest that PDBu does not potentiate synaptic output from the synapses that are inactivated by prolonged depolarization.

Discussion

In this study, we used pharmacology, physiology, and activity-dependent presynaptic labeling to probe the properties of quiescent (mute) synapses after synaptic potentiator treatment. These multiple approaches showed that presynaptically quiescent synapses are rapidly activated by PDBu, but not by FSK or by elevated extracellular Ca^{2+} . Both basally functional and basally dormant, refractory terminals are potentiated by PDBu. After PDBu activation, initially quiescent terminals display heterogeneous synaptic P_r reminiscent of the P_r of basally active synapses. Quiescent synapses contain NMDA receptors pharmacologically

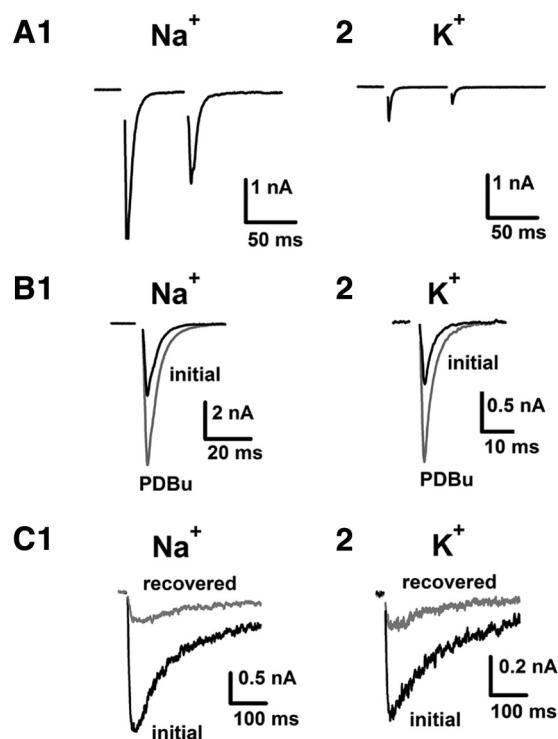


Figure 9. PDBu does not reactivate depolarization-inactivated synapses. **A**, Prolonged depolarization (35 mM $[K^+]_o$ for 4 h) reduced synaptic current but did not affect PPR. Sample paired AMPAR-mediated EPSCs (50 ms interpulse interval) in control (**A1**) and in previously depolarized neurons (**A2**). EPSC measurements here and in other panels were all performed in physiological $[K^+]_o$ (4 mM) (see Materials and Methods). The average amplitude of I_{AMPA} was 3.80 ± 0.94 nA in control (Na^+ ; $n = 9$), and 1.07 ± 0.34 nA in depolarized neurons (K^+ ; $n = 10$; $p < 0.03$). The paired-pulse ratio ($EPSC_{second}/EPSC_{first}$) in control was 0.69 ± 0.05 ($n = 9$) and in sibling depolarized neurons was 0.70 ± 0.06 ($n = 10$; $p = 0.84$). **B**, I_{AMPA} potentiation by PDBu was similar in control and in depolarized neurons. Examples of PDBu potentiated I_{AMPA} in control (**B1**) and in depolarized neurons (**B2**). Average PDBu potentiation in I_{AMPA} in control was $264.3 \pm 70\%$ relative to baseline ($n = 9$) and in depolarized cultures it was $266 \pm 37.7\%$ relative to baseline ($n = 10$; $p = 0.98$). **C**, Sample traces of PDBu-mediated recovered I_{NMDAR} in control (**C1**) and in depolarized neurons (**C2**). Recovered currents (gray) are superimposed on the initial currents (black). Average effect of PDBu on the recovered synaptic I_{NMDAR} in control was $21.6 \pm 6.1\%$ relative to the initial current ($n = 9$) and in depolarized neurons it was $31.3 \pm 6.1\%$ to the initial current ($n = 10$; $p = 0.27$).

and kinetically indistinguishable from those of active synapses. Last, PDBu restores release at basally quiescent synapses, but not at depolarization-inactivated synapses. These observations highlight different mechanisms underlying seemingly similar second messenger-mediated presynaptic potentiation; whereas acute FSK-mediated potentiation mainly enhances synaptic output from the preexisting active terminals, PDBu increases P_r at the active terminals and restores release competence at basally dormant synapses. These observations may help to elucidate the roles of different signaling pathways in temporal or spatial changes of neuronal connectivity.

Presynaptic causes of PDBu-mediated I_{NMDAR} recovery

Several alternative mechanisms could potentially cause the potentiation-associated revival of the synaptic I_{NMDAR} after MK-801 block. First, transmitter could spill out from synapses as a result of transmission enhanced by conventional increases in vesicular P_r . This transmitter overlap from active terminals may taint receptors not previously exposed to transmitter during MK-801 application and could cause PDBu-induced recovery of I_{NMDAR} . Enhanced presynaptic release increases the likelihood of

transmitter spillover, especially when glutamate transporter are compromised (Mennerick and Zorumski, 1995; Asztely et al., 1997; Diamond, 2001; Arnth-Jensen et al., 2002; Christie and Jahr, 2006). In the present study, however, neither of two additional presynaptic potentiators produced significant I_{NMDAR} recovery, suggesting that increasing transmission alone, with any accompanying spillover, is not sufficient to cause current recovery by PDBu after MK-801 block.

A second alternative presynaptic explanation for PDBu-induced recovery of I_{NMDAR} is that PDBu enhances excitability at axon branches where action potential invasion fails before PDBu incubation. Several lines of evidence argue against this possibility. First, artificially enhancing excitability does not recover I_{NMDAR} (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). Second, two experimental protocols designed to circumvent action potentials for evoking release, hypertonicity-evoked EPSCs and hyperkalemia-induced FM1-43FX labeling, both suggest that terminals that do not initially participate in release are recruited by PDBu.

A third alternative explanation for the effects of PDBu after MK-801 block is postsynaptic modification of NMDARs. Several studies showed that phorbol esters increase surface expression (Lan et al., 2001; Scott et al., 2001), alter gating properties (Durand et al., 1992; Lan et al., 2001; Lin et al., 2006), and enhance lateral movement (Fong et al., 2002; Groc et al., 2004) of NMDARs; inhibiting PKC activity abolishes the modification by phorbol ester of the receptors (Lan et al., 2001; Fong et al., 2002; Lin et al., 2006). Several experiments distinguish PDBu-dependent revival of I_{NMDAR} from postsynaptic receptor modification. First, with short treatment of PDBu, we failed to detect significant whole-cell or synaptic current recovery after blocking all surface receptors, despite robust presynaptic potentiation assessed by I_{AMPA} . Second, although not all presynaptic effects of PDBu are PKC dependent, the postsynaptic effects on NMDARs are PKC dependent (Durand et al., 1992; Lan et al., 2001; Scott et al., 2001; Fong et al., 2002; Lin et al., 2006). Preventing PKC activation in the presence of PDBu did not hamper I_{NMDAR} recovery. Third, the slower progressive NMDAR block of PDBu-recovered I_{NMDAR} compared with PDBu-speeded progressive block in active synapses excludes simple lateral movement of NMDARs into active terminals. Fourth, sucrose-evoked AMPAR EPSCs are potentiated by the amount expected from I_{NMDAR} recovery experiments (Fig. 5). We cannot fully explain why this effect of PDBu has not been observed in all previous experiments (Basu et al., 2007; Wierda et al., 2007), although the small effect size (25%) relative to effects on evoked EPSCs may partially explain the discrepancy. Finally, results from presynaptic, activity-dependent FM dye labeling (Fig. 8) directly support a presynaptic explanation for PDBu-induced I_{NMDAR} recovery.

Presynaptically dormant synapses

Synapses with low functional connectivity, caused either presynaptically or postsynaptically, serve as a reservoir that increases synaptic malleability (Voronin and Cherubini, 2004; Kerchner and Nicoll, 2008). Presynaptically silent synapses occur in developing and mature hippocampal cultures (Tong et al., 1996; Ma et al., 1999), slices (Kullmann et al., 1996; Gasparini et al., 2000), spinal cord (Jack et al., 1981), and cultured sensory to motor neurons in *Aplysia* (Kim et al., 2003). In hippocampal cultures, release at quiescent synapses can be recruited by repetitive stimulation (Shen et al., 2006; Yao et al., 2006), application of the neurotrophic factor BDNF (Collin et al., 2001; Slutsky et al., 2004), or by prolonged activation of cAMP signaling (Ma et al.,

1999; Moulder et al., 2008), as opposed to the acute cAMP elevation in the present study. The neurotrophic or activity-dependent activation of dormant synapses may involve cytoskeletal reorganization at the presynaptic terminals, which facilitates the maturation of release apparatus (Slutsky et al., 2004; Shen et al., 2006; Yao et al., 2006).

The contrast between immediate actions of FSK and PDBu may reflect distinct downstream targets of the modulators. The need for slow, cAMP-dependent unsilencing pathways, nonetheless, makes the rapidity of PDBu unsilencing quite surprising and may suggest multiple routes to synaptic unsilencing. Our results show that the rapid unsilencing by PDBu is PKC independent. Because PKC-independent effects of phorbol esters involve the vesicle priming protein Munc13 (Betz et al., 1998; Rhee et al., 2002), PDBu-induced synaptic awakening likely involves Munc13-induced vesicle priming at dormant terminals.

Our previous work has demonstrated a form of activity-dependent presynaptic adaptation of synaptic output; chronic depolarization presynaptically silences glutamate transmission (Moulder et al., 2004, 2006). Depolarization-silenced terminals are apparently distinct from basally quiescent terminals, as PDBu restores release at basally quiescent synapses but not at depolarization-silenced synapses. We have demonstrated this both with imaging (Moulder et al., 2008) and with electrophysiological assessments (present work). Although reduced levels of the priming protein Munc13-1 appears to be a commonality between basally silent and depolarization-silenced synapses (Jiang et al., 2010), other components contributing to release competence must differ to explain the selective PDBu sensitivity of basally dormant synapses. Therefore, there may be at least two partially independent molecular pathways that lead to presynaptic silencing. Additional study is required to delineate the molecular distinction between the two classes of presynaptically silent synapses.

PDBu-mediated potentiation and synaptic P_r

An increased average P_r from a population of synapses can be achieved by increasing P_r at each terminal, and/or by increasing the proportion of high P_r terminals. According to the accelerated fast decay and the increased fast component in MK-801 progressive block after PDBu potentiation, PDBu seems to increase the proportion of high P_r terminals. This interpretation is consistent with the increases of vesicle turnover at active terminals by PDBu observed by others (Waters and Smith, 2000) and echoes the idea that PDBu acts on Munc13 to enhance fusion efficiency of vesicles (Basu et al., 2007). A similar synaptic P_r shift is observed with FSK treatment at the calyx of Held (Kaneko and Takahashi, 2004), suggesting that both FSK and PDBu increase synaptic P_r of functional terminals.

Interestingly, after PDBu potentiation, the synaptic P_r profile of initially quiescent synapses does not match the synaptic P_r profile of the entire population of PDBu-potentiated synapses (Fig. 6A2). This could represent a deficiency in the quiescent synapses that renders these synapses unable to exhibit PDBu-induced P_r potentiation. By this view, PDBu has two entirely independent effects. One effect increases synaptic P_r at already active synapses, and the other effect restores dormant synapses to an active state with a baseline synaptic P_r distribution. Alternatively, the dormant synapses represent synapses at the extreme low end of the synaptic P_r spectrum. By this view, the PDBu effect on the dormant synapses is an extension of the observation that the lowest P_r terminals are most sensitive to PDBu stimulation (Fig. 6B). This latter view is more parsimonious since it does not

postulate different molecular mechanisms for the synaptic P_r increases and unsilencing effects. However, this interpretation is difficult to reconcile with the observations that elevated Ca^{2+} , acute FSK treatment, and high potassium depolarization (during FM1-43FX labeling) are all ineffective in recruiting the PDBu-sensitive terminals containing low P_r vesicles.

Together, our observations provide evidence for differential potentiation by synaptic modulators and suggest that presynaptically dormant synapses can be rapidly recovered. Our results suggest multiple molecular and physiological pathways, including those that target vesicle priming, can trigger presynaptic unsilencing. Our studies also provide new insight into the multiple presynaptic actions of phorbol esters. Rapid awakening of dormant terminals is a form of plasticity that may be particularly well suited for altering the functional connectivity of neural networks.

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