β-Phorbol ester-induced enhancement of exocytosis in large mossy fiber boutons of mouse hippocampus

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Abstract β-Phorbol esters (BPE), synthetic analogues of diacylglycerol (DAG), induce the potentiation of transmission in many kinds of synapses through activating the C₁ domain-containing receptors. However, their effects on synaptic vesicle exocytosis have not yet been investigated. Here, we evaluated the vesicular exocytosis directly from individual large mossy fiber boutons (LMFBs) in hippocampal slices from transgenic mice that selectively express synaptopHluorin (SpH). We found that the activity-dependent increment of SpH fluorescence (ΔSpH) was enhanced by 4β-phorbol 12,13-diacetate (PDAc), one of the BPEs, without influencing the recycled component of SpH. These PDAc effects on ΔSpH were almost completely inhibited by staurosporine, a non-selective antagonist of protein kinases. However, intermittent synaptic transmission was still potentiated through a staurosporine-resistant mechanism. The staurosporine-sensitive cascade may facilitate the vesicle replenishment, thus maintaining the fidelity of transmission at a high level during repetitive firing of the presynaptic neuron.

Keywords Exocytosis · Synaptic transmission · Presynaptic mechanism · PKC · Munc13-1 · Synaptic plasticity

Introduction

In the central nervous system (CNS) signals are transmitted from neuron to neuron at synapses. Synapses are also the principal sites of short- and long-term changes of neuronal networks. In the hippocampus, the axon of a dentate granule cell (mossy fiber, MF) provides robust excitatory inputs on 11–18 CA3 pyramidal cells at their proximal dendrites [1, 2]. Each transmission is mediated by a large MF bouton (LMFB), which forms a complex of tens of excitatory synapses. The MF-CA3 transmission is also highly dynamic over a large range during short- and long-term plasticity. These peculiar morphological and physiological features led to the proposal that the MF input might be involved in filtering out context for building the complete episodic memory [3]. Pharmacological studies using β-phorbol esters (BPE), synthetic analogues of diacylglycerol (DAG), one of the signaling messengers produced by phospholipases, showed that they induced the potentiation of transmission in many kinds of synapses through activating the C₁ domain-containing receptors [4, 5]. The BPEs are amongst the most potent in up-regulating the transmission at the MF-CA3 synapse, suggesting that the DAG/BPE-dependent cascade is involved in the plasticity of this synapse [6–10]. However, their effects on synaptic vesicle exocytosis have not yet been investigated.

The inside of a secretory vesicle is acidic (pH 5.6), whereas it becomes neutral (pH 7.4) instantaneously upon exocytosis [11]. The intravesicular change of pH is optically detected by a fluorescence change of a pH-sensitive
derivative of green fluorescent protein (pHluorin) when it is expressed inside the secretory vesicles by fusing to the luminal domain of a vSNARE-type vesicular membrane protein synaptobrevin/VAMP-2 [11–13]. In this paper we
optically evaluated the exocytosis-dependent changes of fluorescence from individual LMFBs in acute hippocampal
slices from transgenic mice that express this fusion protein
(synaptopHluorin, SpH) only in the MF boutons [14]. We
found that one of the BPEs, 4β-phorbol 12,13-diacetate
(PDAc) enhanced the activity-dependent SpH response in a
single LMFB without influencing the SpH recycling rate.
This effect of PDAc was almost completely blocked by
staurosporine, which inhibits a broad spectrum of protein
kinases at their ATP-binding sites, but not the non-PKC C1
group-II metabotropic glutamate receptors, which are
selectively expressed in the MF presynaptic terminals [16,
17]. Although the MF synaptic transmission is only slightly
attenuated by these drugs after β-phorbol ester-induced
potentiation [18], a reduction of fEPSPs was actually
observed in the range of 10–60%. Recordings were made
with a Gene Clamp amplifier (Axon Instruments, Foster
City, CA) and amplified and filtered at 10 kHz with an
FLA-01 amplifier (Cygnus Technology, Inc., Delaware
Water Gap, PA), digitized at 20 kHz with a DigiData 1320
A/D converter (Axon Instruments). Data were analyzed
with Clampfit 9.2 software (Axon Instruments).

Optical imaging of exocytosis

SpH was excited with an argon laser at 488 nm, and fluo-
rescence was collected through a 505-nm long-pass filter
under conventional confocal microscopy equipped with a
63× 0.95 NA objective (LSM 510 META, Carl Zeiss,
Oberkochen, Germany). Individual MF boutons were iden-
tified in the stratum lucidum of acute slices of hippocampus
by SpH fluorescence (Fig. 1a, b) as some SpH molecules are
distributed in the plasma membrane [13, 14, 19]. Through-
out every experiment the fluorescence intensity of a region of
interest (ROI) was measured with fixed sensitivity and
was expressed in arbitrary fluorescence units (AFU), while
the laser power was also set at a fixed intensity. The sam-
ppling frequency of images was set at 2 Hz (Figs. 1, 2, 3, 4c,
and 5) or 1 Hz (Fig. 4a). Since only a small subset of LMFBs
was responsive to the MF stimulation, they were detected by
the following protocol. The LMFBs were electrically stimu-
lated at 10 Hz for 10 s, while confocal images (512 × 512
pixels) were sampled (Fig. 1c, d). To identify the LMFBs,
three bright fluorescent spots in the same focal plane were
used as landmarks. Once one of the landmarks went out of
focus, the experiment was no longer included in the analysis.
Both baseline and responsive images were median-filtered at
5 pixels, and the difference image was calculated by sub-
tracting the baseline image from the response image
(Supplementary movie). Subsequently, the difference image
was median-filtered at 8 pixels, and the signals derived from
small-sized boutons or non-specific intrinsic fluorescence
was largely removed. Each spot with brightness greater than
a threshold of 10 AFU was defined as a responsive LMFB
(Fig. 1e). Circular ROIs of 2.24-μm diameter were set at the
responsive LMFBs, and the time series data of ROIs were
acquired from raw image stacks. At least ten other ROIs
were also set at non-responsive MF regions of the same
image, and the background fluorescence changes were
recorded. The background fluorescence changes, which

Methods

Hippocampal slice preparation

The experiments were carried out using 14–21-day-old
heterozygous mice from one of the thy-1 promotor-syna-
ptobfluorin (SpH) transgenic lines with the background of
C57BL/6, TV-42, RIKEN BRC, acc. no. 01519 (http://
www.brc.riken.jp/lab/animal/en/), which express SpH
selectively in the mossy fiber (MF) boutons of the hippo-
campus [14]. The mice were decapitated under ether-

anaesthesia, and hippocampal slices (300–400 μm) were
prepared as described [15]. For the dissection, a cutting
solution containing (in mM) 229 mannitol, 3 KCl, 26
NaHCO3, 1 H3PO4, 7 MgCl2, 0.2 lidocaine HCl, pH 7.4
(0°C) with 95% O2 and 5% CO2 mixed gas was used.
Experiments were done at 23–25°C, while the slices were
superfused (2 ml/min) with artificial cerebrospinal fluid
(ACSF) containing (in mM) 114 NaCl, 2.5 KCl, 26
NaHCO3, 1 NaH2PO4, 10 mannitol, 2.5 CaCl2, 1.3 MgCl2,
10 glucose (pH 7.4 with 95% O2 and 5% CO2 mixed gas).
All animal procedures were conducted in accordance with
the guiding principles of the Physiological Society of Japan
and NIH.

Extracellular recordings

Field excitatory postsynaptic potentials (fEPSPs) were
recorded in the stratum lucidum of the CA3 region using
glass microelectrodes with a pipette resistance of 0.7–
2 MΩ filled with 1.75% Na2SO4 solution. A tungsten
bipolar stimulating electrode was placed in the dentate
hilus, and two electrical pulses (200 μs duration, 150–250
μA intensity, 100 ms inter-stimulus interval) were
delivered intermittently every 30 s. At the end of the
experiments, we examined the sensitivity of fEPSP to
either 1 μM DCG-IV or 10 μM L-CCG-1, an agonist for
the group-II metabotropic glutamate receptors, which are
selectively expressed in the MF presynaptic terminals [16,
17]. Although the MF synaptic transmission is only slightly
attenuated by these drugs after β-phorbol ester-induced
potentiation [18], a reduction of fEPSPs was actually
observed in the range of 10–60%. Recordings were made
with a Gene Clamp amplifier (Axon Instruments, Foster
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A/D converter (Axon Instruments). Data were analyzed
with Clampfit 9.2 software (Axon Instruments).
were mainly derived from the fluorescence bleaching, were averaged, normalized to the value at time 0 (the normalized background fluorescence). For each ROI the effect of fluorescence bleaching was removed by dividing the SpH fluorescence value by the normalized background fluorescence value of the same time point. The time-dependent change of the SpH fluorescence intensity was thus obtained for each ROI (Fig. 1f, thin lines). These ratio data were also digitally filtered by applying a weighted moving-average protocol as described [15] (Fig. 1f, thick lines). The weights, 0.080251, 0.137137, 0.182615, 0.2, 0.182615, 0.137137 and 0.080251, were designed by the Kaiser window finite impulse response (FIR) algorithm so that the sum of the squares of errors was minimized, while the sum of the weights was equal to 1. The mean noise amplitude was calculated as the average of the absolute difference between the raw ratio data and the filtered ratio data. The signal range was calculated by subtracting the minimal filtered ratio value during another 10 s just before stimulation from the maximal filtered ratio value during 10-s repetitive stimulation. The signal-to-noise ratio (S/N) was thus obtained by dividing this signal range by the mean noise amplitude. If $S/N < 5$, the data were not employed for the subsequent analyses. The time-dependent change of the ratio data ($\Delta SpH$) was obtained by subtracting the minimal filtered ratio value from the ratio data. In the following figures showing the $\Delta SpH$, the thin lines represent ratio data before filtering and the thick lines those after filtering. Averaged $\Delta SpH$ are also shown as in Fig. 1g. Image analysis was performed with ImageJ software (http://rsb.info.nih.gov/ij/), and the time series data of ROIs were sampled in digits and analyzed with Excel software (Microsoft, USA) and R software (http://cran.r-project.org/).

Chemicals

Pharmacological reagents were bath-applied in the recording chamber (2 ml) at a constant flow rate (2 ml/min). Reagents used in this study and their sources were as follows: kynurenic acid (Sigma-Aldrich, St. Louis, MO), lidocaine HCl (Sigma-Aldrich), (2S,20R,30R)-2-(2-carboxycyclopropyl)glycine (DCG-IV, Tocris Cookson, Bristol, UK); (2S,10S,20S)-2-(2-carboxycyclopropyl)glycine (L-CCG-1, Tocris), bafilomycin A1 (Wako, Osaka, Japan); 4b-phorbol 12,13-diacetate (PDAc, Wako); 4a-phorbol (Sigma-Aldrich); staurosporine (Sigma-Aldrich). Bafilomycin A1 was dissolved in DMSO containing 20% pluronic acid, then diluted. PDAc, 4a-phorbol and staurosporine were dissolved in DMSO, then diluted. Stocks of these were preserved at $-20^\circ$C.

Statistical analysis

Values are expressed as mean ± SEM (number of experiments) unless otherwise noted. Statistical significance was tested by the Wilcoxon signed-ranks test for paired data.
and by the Mann–Whitney U-test or the Kolmogorov–Smirnov test for unpaired data. The significance limit was set at $P = 0.05$ in any test.

**Results**

**Ca$^{2+}$-sensitivity of Δ$SpH$**

In this study we measured the basal synaptic transmission using the first fEPSPs of paired stimuli at 0.033 Hz. On the other hand, the Δ$SpH$ is the cumulative response to the repetitive stimulation (10 Hz for 10 s); [Ca$^{2+}$]o at 2.5 mM (top trace), 5 mM (middle trace) or 0 mM (bottom trace). [Ca$^{2+}$]o dependence of fEPSP ($n = 8$ slices). c Sample records of Δ$SpH$ from a LMFB by a train of repetitive stimulation (10 Hz for 10 s); [Ca$^{2+}$]o at 2.5 mM (top trace), 5 mM (middle trace) or 0 mM (bottom trace). d [Ca$^{2+}$]o dependence of Δ$SpH$ ($n = 12$ boutons, 3 slices).

Previously, it was shown that the fEPSP is augmented by PDAC at the stratum lucidum of the hippocampus at the concentration of 0.5–10 μM [6, 7, 9, 10, 18]. This was also the case in the present study, and the fEPSP was potentiated by 10 μM PDAC to, on average, 533 ± 64% ($n = 8$ slices) of control (Fig. 3a, filled circles). On the other hand, the PDAC treatment did not affect the amplitude of the fiber volley response preceding the fEPSP, which is an indication of centripetal transmission.
of the number of stimulated axons (open diamonds, mean 110 ± 4%, n = 3 slices). As shown in the Fig. 3a insets, the PDAc treatment significantly reduced the paired-pulse ratio from 2.1 ± 0.14 to 1.2 ± 0.03 (n = 8 slices, \( P < 0.01 \), Wilcoxon signed-ranks test), suggesting the involvement of presynaptic mechanisms [10]. The SpH images were sampled twice before (Fig. 3a, red double line no. 1) and after PDAc treatment (red double line no. 2). Figure 3b shows representative \( \Delta SpH \) traces during MF stimulation of three LMFBs in b (indicated by white arrows) are compared before (sampling 1, black) and after PDAc (sampling 2, blue). In some LMFBs, the negligibly small \( \Delta SpH \) before (sampling 1, black) became obvious after PDAc (sampling 2, blue). Each LMFB was plotted two-dimensionally to the sampling-1 (control) and the sampling-2 (test) values of \( \Delta SpH \); vehicle alone (open circles, \( n = 31 \) boutons, 5 slices), 4α-phorbol (yellow squares, \( n = 30 \) boutons, 6 slices) and PDAc (blue diamonds, \( n = 65 \) boutons, 10 slices). The magenta line shows that both are equal. Cumulative probability plots of the ratio value of the \( \Delta SpH \) at sampling 2 divided by that at sampling 1 (ratio-2/1 of \( \Delta SpH \); vehicle alone (black line), 4α-phorbol (gray line) and PDAc (blue line) comparing the ratio value of the \( \Delta SpH \) at sampling 2 divided by that at sampling 1 (ratio-2/1 of \( \Delta SpH \)) in cumulative probability plots (Fig. 3f). Although the PDAc significantly enhanced the \( \Delta SpH \) (\( P < 0.0005 \), Kolmogorov–Smirnov test), the effects of 4α-phorbol were indistinguishable from the vehicle alone control. This is consistent with the notion that the 4α-phorbol esters are biologically inactive [4] and that the PDAc-dependent enhancement of the \( \Delta SpH \) appeared to be specific to the C1 domain-containing receptors such as PKC and Munc13s [4, 5, 25].

Evaluation of SpH recycling

After fusion, the SpH molecules in the vesicular membrane are exposed to the extracellular space (pH 7.4) and...
de-protonated to become fluorescent [11–13]. The synaptic vesicles are then recycled into a vesicular pool, refilled with transmitter and reutilized for the following transmission [22, 26]. The SpH molecules are retrieved from the
plasma membrane with the new vesicles during the process of endocytosis and are protonated to become non-fluorescent with the progress of the reacidification of the intravesicular space by the activity of V-type H$^{+}$ ATPase [19, 27]. Therefore, the rising phase of the $\Delta SpH$ represents the difference between the exocytosis and the molecule’s recycling through the process of endocytosis-reacidification, whereas the falling phase kinetics are solely dependent on the recycling [15, 27, 28]. The PDAc can enhance the $\Delta SpH$ either through increasing the exocytosis rate or through decreasing the recycling rate.

When the intravesicular reacidification is completely blocked by bafilomycin A1, a potent inhibitor of the V-type H$^{+}$ ATPase [13, 15], the recycling vesicles are expected to be trapped in alkaline. To evaluate the contribution of the recycled SpH, the $\Delta SpH$ was normalized to the value at the end of the train stimulation (10 Hz for 10 s) in the absence of bafilomycin A1, and the effects of bafilomycin A1 were examined as shown in Fig. 4a. As expected the falling rate of the SpH response was almost null in the presence of 5 µM bafilomycin A1. Therefore, the contribution of recycled SpH was estimated by subtraction of the $\Delta SpH$ before bafilomycin A1 from that after (Fig. 4b). This bafilomycin A1-sensitive component steadily increased during the train stimulation, and was 10 ± 6% ($n = 30$ boutons, 7 slices) at the end of the train stimulation. The bafilomycin A1-sensitive component was again examined in the presence of PDAc, in the absence of bafilomycin A1, and the effects of bafilomycin A1 were also examined as shown in Fig. 4a. As expected the falling rate of the SpH response was almost null in the presence of 5 µM bafilomycin A1. Therefore, the contribution of recycled SpH was estimated by subtraction of the $\Delta SpH$ before bafilomycin A1 from that after (Fig. 4b). This bafilomycin A1-sensitive component steadily increased during the train stimulation, and was 10 ± 6% ($n = 30$ boutons, 7 slices) at the end of the train stimulation. The bafilomycin A1-sensitive component was again examined in the presence of PDAc, was 16 ± 8% ($n = 26$ boutons, 3 slices), and was insignificantly different from the value in the absence ($P > 0.5$, Mann–Whitney U-test).

The recycling rate of SpH during the train stimulation can also be approximated by the initial rate of the fluorescence reduction after the last stimulation (Fig. 4b) [28]. As the first step, the filtered $\Delta SpH$ was normalized to the value at the end of the train stimulation (10 Hz for 10 s). Next, a regression line was fitted to the data of 0–5 s after the last stimulation, and its slope was adopted as the SpH recycling rate (Fig. 4c). As shown in Fig. 4d, the effects of PDAc on the SpH recycling rate were almost negligible and were similar to the effects of vehicle alone ($P > 0.5$, Mann–Whitney U-test).

Taken together, there was no evidence that PDAc decreased the SpH recycling rate. The PDAc-dependent enhancement of the SpH response is well attributable to the change of the exocytosis rate.

Effects of staurosporine

Since the SpH response was specifically enhanced by PDAc, but not by 4β-phorbol, the PDAc-dependent enhancement of the SpH response appeared to be specific to the C$_1$ domain-containing receptors such as PKC and Munc13s [4, 5, 25]. To investigate the downstream mechanisms of BPE, we examined the effects of staurosporine, which inhibits a broad spectrum of protein kinases at their ATP-binding sites [29, 30]. As shown in Fig. 5a, the PDAc potentiated the fEPSP to a mean 232 ± 20% ($n = 12$ slices) of control in the presence of staurosporine (1 µM). Although the magnitude of this potentiation was significantly smaller than in the absence of staurosporine ($P < 0.0001$, Mann–Whitney $U$-test), it was still significantly greater than the control (vehicle alone and 4β-phorbol treatments, $P < 0.01$, Mann–Whitney $U$-test) (Fig. 5b). During the PDAc-induced fEPSP potentiation, the paired-pulse ratio was always decreased from its initial value. However, this was not the case in the presence of staurosporine (Fig. 5c), and the paired-pulse ratio was on average 2.3 ± 0.09 ($n = 12$ slices), which is insignificantly different from the control (2.4 ± 0.17, Wilcoxon signed-ranks test).

As shown in Fig. 5d, the average profiles of the $\Delta SpH$ ($n = 65$ boutons, 15 slices) were compared before and after PDAc (10 µM), while the slices were pretreated with staurosporine (1 µM). The maximal $\Delta SpH$ at the end of a train of stimulation was 26.7 ± 1.3 AFU at sampling 1 and 26.4 ± 1.4 AFU at sampling 2, and the difference was insignificant ($P > 0.7$, Wilcoxon signed-ranks test). The subtracted curve (difference) indicates that PDAc was almost ineffective on the $\Delta SpH$ in the presence of staurosporine. Figure 5e shows the cumulative probability plots of the ratio-2/1 values of $\Delta SpH$. The effects of PDAc were almost completely blocked by staurosporine ($P < 0.0001$, Kolmogorov–Smirnov test) and indistinguishable from the vehicle alone control. The staurosporine-sensitive cascade appears to be one of the major downstream reactions of PDAc in the case of exocytosis during repetitive activation of the LMFBs. This is in contrast to the case of intermittent synaptic transmission (e.g., 0.033 Hz) where a significant potentiation remained even in the presence of staurosporine.

Discussion

The BPEs, such as 4β-phorbol-12,13-dibutyrate (PDBu, 10 µM) and PDAc (2 µM), increased the MF-dependent population spike with negligible effects on the glutamate sensitivity [6]. The PDAc increased the quantum content with little changes in the quantum size [7], and its potentiation was accompanied by a reduction of the paired-pulse ratio [10]. In the present study the PDAc-dependent enhancement of fEPSP was not accompanied by an enhancement of the fiber volley response as previously noted [18], suggesting the up-regulation of synaptic transmission. These lines of electrophysiological evidence suggest that the BPEs would enhance the quantal
transmitter release from the LMFBs. However, the validity of this interpretation has to be re-investigated taking into consideration the nonuniform probability of release, the concentration of glutamate in the synaptic cleft, the effects of the rapid desensitization of the glutamate AMPA receptors and the unsilencing of postsynaptic responsiveness [31–36]. This paper presents additional evidence in a more direct way that the BPEs enhance the exocytosis in a single LMFB in acute hippocampal slices using the SpH transgenic mice. The ∆SpH was enhanced on average 2.2-fold by the PDAc (10 μM), although the magnitude of potentiation was variable from bouton to bouton. On the other hand, its effect on the recycled component of ∆SpH, which is regulated by the endocytosis and the subsequent readification of the vesicles, was negligible. Therefore, the PDAc-dependent change of the ∆SpH could be attributed to the change of the exocytosis rate. However, its effects on the endocytosis have to be further investigated since the synaptobrevin/VAMP-2, which can be detected by the SpH fluorescence, was retrieved from the plasma membrane in a way kinetically differentiated from other vesicle marker proteins, such as the synaptophysin and the vesicular glutamate transporters [37, 38].

It should be noted that our ∆SpH measurement evaluated a different aspect of exocytosis from the previous electrophysiological measurements of synaptic transmission. Since the ∆SpH showed small sensitivity to [Ca²⁺]₀ between 2.5 and 5 mM, the contribution of the vesicle fusion probability is small. Rather, it could be dependent on the magnitude of the vesicle replenishment [15]. However, in the CA3-CA1 synapses of hippocampus, the SpH fluorescence change evoked by a train of repetitive stimulation (10 Hz for 5 s) is dependent on [Ca²⁺]₀ (0.5–5 mM) like fEPSP [39]. It is possible that these two synapses are different in the [Ca²⁺]₀-∆SpH relationship, but the underlying mechanisms of this difference should be investigated in future.

Staurosporine-sensitive and -resistant mechanisms

DAG is a key messenger regulating the efficacy of synaptic transmission. The effects of DAG and its analogues, BPEs, are partly mediated by the activation of protein kinase C (PKC), which facilitates exocytosis in a wide variety of cells and presynaptic terminals [40–42]. They also regulate the efficacy of synaptic transmission through activating non-PKC C₁ domain-containing receptor proteins, such as Munc13s, which are presynaptic vesicle priming proteins [43–45]. It has been suggested that the PKC-dependent and Munc13-1-dependent pathways synergistically modulate the exocytosis [4, 5]. In this study the PDAc-dependent enhancement of the ∆SpH was almost completely blocked by staurosporine, one of the broad spectrum protein kinase inhibitors. This suggests the involvement of some protein kinases in a cascade downstream of the C₁ domain-containing receptors. However, the effects of more specific inhibitors have to be studied to identify the key molecules. A previous study described that the PDAc-induced potentiation of MF synaptic transmission is partially antagonized by a PKC-selective inhibitor, bisindolylmaleimide I (BIS-I), but a BIS-I resistant component of potentiation remained [10]. However, we found that BIS-I and its derivatives, which differentiate PKC from other kinases, were strongly fluorescent at the SpH emission spectra when included in the cell. Therefore, it will be necessary in the future to study the possible involvement of PKC using mice in which one of PKC isoforms is knocked out [46] or RNAi-dependent knockdown of one of the PKC isoforms [47].

We also found that the basal synaptic transmission (e.g., 0.033 Hz) was still potentiated in the presence of staurosporine, whereas the effects on the ∆SpH, which is induced by a train of repetitive stimulation, were completely inhibited. Therefore, during a train of repetitive stimulation of LMFB, the response to the first stimulation, which is detectable by fEPSP, and the integrated exocytosis measured by the ∆SpH would be different in the sensitivity to staurosporine. The effects of staurosporine were almost quantitatively equivalent to those of BIS-I in the case of fEPSP. The PDAc (10 μM) treatment potentiated the fEPSP to 232 ± 20% of control in the presence of staurosporine, but to 533 ± 64% of control in the absence. The same treatment potentiated the fEPSP to 258 ± 34% in the presence of BIS-I [10]. Since the staurosporine-resistant component of potentiation was not accompanied by a reduction of the paired-pulse ratio, an indication of increased vesicle fusion probability (see Appendix), other mechanisms are suggested to be involved. The possibility should be investigated that the staurosporine-resistant fEPSP potentiation is accompanied by the enhancement of postsynaptic sensitivity to glutamate. The spatio-temporal changes of glutamate concentration are also under the regulation of the amount of transmitter release per vesicle, the speed of glutamate release, the synaptic cleft morphology and the speed of glutamate clearance from the synaptic cleft [48, 49]. The BPEs have been demonstrated to increase the RRP size in other synapses [50–52] as well as in the slow exocytosis of endocrine cells [53], although it remains undetermined whether these effects are actually PKC-dependent or not. Since the RRP is fractionally small in an LMFB [15, 24] and would be depleted in 10–20 action potentials if it is not replenished, its increase would hardly be detectable in our SpH study.

The presence of a staurosporine-resistant component of fEPSP potentiation suggests that the signaling cascade via the non-PKC C₁ domain-containing receptors is involved.
in the facilitation of synaptic transmission through these mechanisms. Recently, it has been suggested that Munc13-1, one of the non-PKC C1 domain-containing receptors, localized to active zones in the presynaptic terminal, may also be involved in the regulation of synaptic efficacy by BPEs [54–56]. When the DAG/BPE-binding site of Munc13-1 is genetically deficient or non-functional, the effects of BPEs are largely impaired [56, 57]. Although the present study did not prove whether BPEs potentiate MF synaptic transmission through Munc13-1, it does not conflict with the notion that the PKC-dependent and Munc13-1-dependent pathways synergistically modulate the exocytosis [4, 5].

The $\Delta SpH$ unsilencing

We found that the $\Delta SpH$, which was negligibly small at sampling 1, become obvious at sampling 2 after the PDAc in some LMFBs. These synapses are possibly presynaptically silent or weak in the release ability before PDAc [34]. Alternatively, the transmitter release might be rapidly depressed during repetitive stimulation. These possibilities should be clarified in future studies, e.g., the simultaneous recordings of EPSC and SpH fluorescence. Recent studies using cultured networks of developing hippocampal neurons have described the presence of presynaptically silent synapses, which become transmissible through mechanisms dependent on cAMP-protein kinase A (PKA) or BDNF-Cdc signaling cascades [58–62]. At the CA3-CA1 synapses of the mouse hippocampus, the slow presynaptic component of long-term potentiation (LTP), which is detected by the change of SpH fluorescence, is mediated by the PKA and the L-type Ca$^{2+}$ channels [39]. It is possible that some boutons are unsilenced in the $\Delta SpH$. Since MF-LTP is dependent on the AC-PKA cascade [63, 64], it is probably accompanied with the increased $\Delta SpH$. The unsilencing of $\Delta SpH$ might be related to the accumulation and organization of the large arrays of vesicular and non-vesicular molecules required for exocytosis as they are during synaptogenesis [65, 66]. PKC is one of the protein kinases involved in these processes. For example, PKC phosphorylates GAP-43, one of major proteins of the presynaptic and growth cone membrane, during synaptic potentiation [67]. Another PKC substrate is myristoylated alanine-rich C kinase substrate (MARCKS), which is involved in both synaptic maturation and the synaptic plasticity [68, 69].

In the hippocampus of a living animal the signals are usually a train of impulses of variable frequencies [70], sometimes at around 10 Hz (a theta rhythm) [71, 72]. When DAG/BPEs facilitate the fusion probability by increasing the Ca$^{2+}$ influx and the Ca$^{2+}$ sensitivity of exocytosis [10], they up-regulate the transmission efficacy for impulses arriving early in a train, but down-regulate it for impulses arriving later because of the depletion of RRP. This effect could explain the reduction of the paired-pulse ratio. Even if the RRP were to be increased, the potentiation should be transient if it is not replenished. On the other hand, the staurosporine-sensitive enhancement of exocytosis followed a more prolonged time course. Therefore, the DAG/BPE-dependent signaling cascade is suggested to be involved in the facilitation of the vesicle replenishment through a staurosporine-sensitive mechanism and to maintain the fidelity of transmission at a high level during a train of repetitive firings of the presynaptic neuron.

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Appendix

Reduction of the paired-pulse ratio

According to the quantal hypothesis [36, 73], a postsynaptic response ($E$) is related to the following relationship. 

$$E = Npq,$$

(1)

where $q$ is the postsynaptic response by a single quantum (quantal size). The meanings of $N$ and $p$ are definition-dependent. If $p$ is regarded as the probability of vesicle fusion to the plasma membrane, then $N$ refers to the number of vesicles drawn from the next action potential, the readily releasable pool (RRP) or the release-ready pool [20–22]. On the other hand, if $p$ is regarded as the release probability of a release site, $N$ should be the number of release sites, the morphological correlates of which are the number of active zones of a presynaptic terminal. When a presynaptic axon is stimulated by two pulses of a short interval, the vesicle fusion probability by the second action potential ($p'$) is generally increased by some mechanisms dependent on the residual Ca$^{2+}$ [74–78]. On the analogy of the Eq. 1, the second postsynaptic response ($E'$) will be expressed as,

$$E' = (N(1-p)+R)p'q'.$$

(2)

Here, $R$ is the number of vesicles replenished to the RRP during the interval between the first and the second action potentials, and $q'$ is the quantal size of the second response.
The paired-pulse ratio \( (r) \) is thus the function of \( p \) with the relationship,
\[
r = E_0/E = (1 - p + R/N)(p'/p)(q'/q). \quad (3)
\]
The value \( R/N \) is dependent on the vesicle recycling mechanisms. The value \( p'/p \) is dependent on the underlying mechanisms of facilitation. The value \( q'/q \) is mainly influenced by the postsynaptic factors, such as the desensitization of transmitter receptors. It is also influenced by the synaptic geometry, such as the narrowness of the synaptic cleft, the transmitter clearance activities, such as the glutamate uptake by the astrocytes as well as the presynaptic factors, such as the transmitter density in the vesicle [48, 49]. Although these values appear to be less variable than \( p \), their effects have to be taken into consideration. Since \( r \) is negatively related to \( p \), the enhancement of the vesicle fusion probability at the first action potential \( (p) \) is expected to be accompanied with the reduction of \( r \).

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