chapter 4

Phosphorylation of synaptotagmin-1 is essential for phorbol ester-induced potentiation of synaptic vesicle release

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Abstract
Presynaptic vesicle release can be acutely upregulated by activation of the diacylglycerol (DAG) / protein kinase C (PKC) pathway. Munc13 and Munc18-1 were previously identified as essential substrates in this pathway. Synaptotagmin-1 is phosphorylated by PKC, which increases binding of synaptotagmin-1 to SNARE proteins, but previous research suggested that this does not influence secretory granule secretion in neuroendocrine cells. We show that a non-phosphorylatable mutant of synaptotagmin-1 completely inhibits phorbol ester-induced potentiation of evoked release in hippocampal neurons. This mutation also strongly reduced potentiation of spontaneous release, but only if alternative Ca²⁺ sensors, doc2 proteins, were absent. Importantly, replenishment of the readily releasable vesicle pool after intense stimulation was unaffected by the mutation, suggesting that synaptotagmin-1 phosphorylation acts downstream of priming. Thus, phosphorylation of synaptotagmin-1 is essential for a late step in PKC-dependent potentiation of vesicle release.
Introduction

Precise regulation of synaptic transmission is essential for information processing and storage in the brain. The strength of presynaptic terminals is swiftly adapted upon increases in action potential (AP) firing (short-term plasticity) and the activation of presynaptic receptors, which both activate a cascade of intracellular signal transduction pathways [1,2]. The diacylglycerol (DAG)/ protein kinase C (PKC) pathway is one of the most dominant pathways in this respect. Activation of this pathway with synthetic analogs of DAG (phorbol esters) leads to a 50-100% potentiation of spontaneous and AP evoked release within tens of seconds at a variety of synapses [3-5]. Furthermore, activation of PKC is critical for multiple forms of presynaptic plasticity and recovery after intense stimulation [6-9]. DAG directly activates the vesicle priming factor Munc13 [10-12] and indirectly acts on many other downstream effectors via the activation of PKC. The activation of both Munc13 and PKC is essential for phorbol ester-induced potentiation of release [11,13].

PKC phosphorylates a range of presynaptic active zone proteins, including (but not limited to) voltage-gated Ca2+ channels (VGCCs) [14], GAP43 [15], Munc18-1 [16,17], SNAP25 [18], NSF [19] and synaptotagmin-1 (syt1) [20] (see ref [2] for review). The phosphorylation of VGCCs and SNAP25 increases the influx of Ca2+ and the refilling rate of the readily releasable vesicle pool (RRP), respectively [14,21]. We previously identified Munc18-1 as an essential PKC substrate, since a non-phosphorylatable Munc18-1 mutant completely inhibits PKC-dependent potentiation of vesicle release, and severely affects short-term plasticity [13]. The role of other PKC substrates is much less understood, and it is not known to what extent these substrates contribute to PKC-induced synaptic plasticity.

The phosphorylation of syt1 by PKC is of particular interest. Syt1 is the vesicular Ca2+ sensor that mediates fast AP evoked release hippocampus [22] and drives a large fraction of spontaneous release [23]. In this latter form of release, syt1 competes with alternative sensors, in particular doc2s, for SNARE binding and the initiation of vesicle fusion [24,25]. Syt1 bears a highly conserved phosphorylation site (threonine 112) in a putative α-helix in the linker between the transmembrane and C2A domain, that can be phosphorylated by both PKC and Ca2+/Calmodulin (CaM) -dependent protein kinase (CaMK) [20,26,27]. Phosphorylation of this site markedly increases the binding of syt1 to SNARE proteins in synaptosomal preparations [28]. Most mutations that interfere with the interaction between syt1 and SNARE proteins dramatically affect synaptic efficacy (see for instance [29-31]), and thus phosphorylation of T112 is likely to regulate synaptic transmission. Unexpectedly, in mouse embryonic chromaffin cells, interference with phosphorylation of T112 did not have an effect on the size and refilling of the RRP, as well as phorbol-ester induced potentiation [32]. However, we previously observed only limited contribution of PKC in the regulation of vesicle release in mouse embryonic chromaffin cells compared to adult bovine chromaffin cells and neurons [13,33]. It is therefore not unlikely that syt1 phosphorylation affects vesicle release in neuronal preparations.

In the current study, we investigated the effect of PKC-mediated phosphorylation of syt1 on synaptic transmission in mouse hippocampal neurons. Based on the high
Figure 4.1. syt\textsuperscript{T112A} supports normal synaptic transmission. A. Schematic representations of syt\textsuperscript{WT} and syt\textsuperscript{T112A} constructs. TM is the transmembrane domain. B. Typical examples of syt1 KO neurons infected with syt\textsuperscript{WT}, syt\textsuperscript{T112A} or GFP. Neurons were stained for GFP (blue), vGlut (red) and syt1 (green). Scale bars represent 5 µm. C. Average synaptic vGlut intensity (syt\textsuperscript{WT} n = 24 cells, syt\textsuperscript{T112A} n = 25, GFP n = 23; N = 2). D. Average synaptic syt1 intensity. *** p < 0.001 with Kruskal Wallis ANOVA, n.s.: not significant. E. Average synapse surface area. F. Average number of synapses per cell. G. Typical examples of EPSCs evoked by a single AP. The stimulation artifact was blanked out. H. Average EPSC amplitude (syt\textsuperscript{WT} n = 47, syt\textsuperscript{T112A} n = 50, N = 7). I typical examples of mEPSCs. J. average mEPSC frequency per cell, (syt\textsuperscript{WT} n = 31, syt\textsuperscript{T112A} n = 21, N = 4). K. average mEPSC amplitude per cell. All data is displayed at mean ± S.E.M.
conservation of this site across species [20], the striking increased SNARE binding upon phosphorylation [28] and the apparent difference in efficacy of PKC in vesicle release in various preparations [33], we hypothesized that phosphorylation of syt1 contributes to PKC-induced synaptic plasticity. We found that the non-phosphorylatable mutant (T112A) supports normal synaptic transmission and has no effect on RRP size. However, this mutation completely abolished phorbol-ester induced potentiation of evoked release, and, in the absence of doc2 proteins, severely reduced the potentiation of spontaneous release. Surprisingly, other forms of PKC-dependent plasticity, including paired-pulse facilitation and RRP replenishment, were unaffected in the non-phosphorylatable mutant. Based on these results we conclude that PKC-mediated phosphorylation of syt-1 is essential for specific forms of PKC-dependent presynaptic plasticity.

Results

sytT112A supports basal synaptic transmission

To test if phosphorylation of syt1 by PKC affects synaptic transmission, we rescued syt1 knockout (syt1-KO) autaptic neurons with Semliki forest viral particles encoding for wild-type syt1 (sytWT) or nonphosphorylatable syt1 (sytT112A Figure 4.1A). We first tested synaptic expression levels of both constructs with immunofluorescence, since proteins

![Figure 4.1A](image1)

**Figure 4.1A.** Typical examples of AP evoked release in control and after superfusion with 1 µM PMA. Stimulation artifacts have been blanked out. B. Change in average normalized evoked EPSC amplitude induced by PMA. APs were evoked at 0.02 Hz. (sytWT n = 13, sytT112A n = 14, N = 3). C. Typical examples of mEPSCs in control and after superfusion with PMA. D. Change in average normalized mEPSC frequency induced by PMA (sytWT n = 11, sytT112A n = 12, N = 3).
levels of syt1 affect RRP size [32]. sytWT and sytT112A were expressed at comparable levels in presynaptic terminals (Figure 4.1B-D). In addition, both constructs had no effect on the synaptic vGlut levels, a marker for the number of presynaptic vesicles [34], on synapse surface area, or on the number of synapses per neuron (Figure 4.1 B-F). We then tested the effect of the T112A mutation on basal synaptic transmission in autaptic glutamatergic neurons. Under resting conditions, PKC activity is too low to affect presynaptic strength [11,13], and thus sytT112A is not expected to have an effect basal synaptic transmission. Indeed, the amplitude of excitatory postsynaptic currents (EPSC) evoked by a single AP stimulation did not differ between sytWT and sytT112A (sytWT 3.6 ± 0.4 nA, n = 47; sytT112A 3.8 ± 0.4 nA, n = 50; N = 7 Figure 4.1 G-H). In addition, no differences were observed in the frequency (sytWT 9.5 ± 2.1 Hz, n = 28; sytT112A 12.5 ± 2.9 Hz, n = 26; N = 5) and amplitude (sytWT 27.6 ± 1.2 pA; sytT112A 28.5 ± 1.0 pA) of spontaneous release (mEPSCs, Figure 4.1 I-K). Thus, sytT112A is indistinguishable from sytWT in terms of synaptic protein expression levels and basal synaptic transmission.

Phosphorylation of T112 is essential for PMA-induced potentiation of release
Phorbol esters are widely used to activate the DAG/PKC pathway in synapses [3-5], and critically depend on the activation of PKC to potentiate vesicle release in hippocampal

Figure 4.3. sytT112A inhibits potentiation of both spontaneous and evoked release in syt1/doc2a/doc2b TKO neurons. A. Typical examples of AP evoked release in TKO neurons rescued with sytWT and sytT112A in control and after superfusion with 1 μM PMA. Stimulation artifacts have been blanked out. B. Change in average normalized evoked EPSC amplitude induced by PMA. APs were evoked at 0.02 Hz. (sytWT n = 23, sytT112A n = 21, N = 4). C. Typical examples of mEPSCs in TKO cells rescued with sytWT or sytT112A in control and after addition of PMA. D. Changes in average normalized mEPSC frequency induced by PMA (sytWT n = 15, sytT112A n = 14, N = 4).
neurons [13]. To test if syt1 phosphorylation affects the response to phorbol esters, we measured the effect of phorbol 12-myristate 13-acetate (PMA) in syt1 KO neurons rescued with syt\textsuperscript{WT} or syt\textsuperscript{T112A} on spontaneous and AP-evoked release (Figure 4.2). PMA potently increased AP evoked release (normalized amplitude in PMA = 1.53 ± 0.3, n = 13, N = 3) and spontaneous release (normalized frequency in PMA = 1.7 ± 0.3, n = 11) in syt\textsuperscript{WT} expressing cells (Figure 4.2A-D); in line with previous studies [4,10,11,13]. In contrast, PMA had no effect on EPSC amplitude in syt\textsuperscript{T112A} expressing cells (normalized amplitude in PMA = 0.88 ± 0.3, n = 14, Figure 4.2A-B), indicating that, like Munc18-1, phosphorylation of syt1 is essential in this form of plasticity. Strikingly, potentiation of spontaneous release was still observed in neurons expressing syt\textsuperscript{T112A}. Although potentiation was slightly delayed compared to syt\textsuperscript{WT}, PMA-application increased spontaneous release in syt\textsuperscript{T112A} expressing cells to frequencies comparable to syt\textsuperscript{WT} (normalized frequency in PMA = 1.44 ± 0.3, n = 12, Figure 4.2C-D). This suggests that phosphorylation of syt1 is dispensable for PKC-dependent potentiation of spontaneous release.

Previous studies showed that spontaneous release may be initiated by multiple Ca2+ sensors, including syt1 [23] and doc2s [24,25]. We therefore hypothesized that in the absence of syt1 phosphorylation, the increased spontaneous release is mediated by doc2 proteins. To test this, we repeated the PMA experiments in neurons obtained from doc2a/doc2b/syt1 triple knockout (TKO) mice (Figure 4.3). As expected, syt\textsuperscript{T112A} did not show potentiation of evoked release upon addition of PMA (syt\textsuperscript{WT} 1.3 ± 1.0, n = 23; syt\textsuperscript{T112A} 1.0 ± 0.1, n = 21; N = 4. Figure 4.3 A-B). Importantly, the potentiation of spontaneous release was greatly reduced in TKO cells rescued with syt\textsuperscript{T112A}, although some remaining potentiation was observed (syt\textsuperscript{WT} 2.5 ± 0.5, n = 15; syt\textsuperscript{T112A} 1.5 ± 0.2, n = 14. Figure 4.3 C-D). Thus, phosphorylation of syt1 at T112 potentiates both spontaneous and evoked release, but the effect on spontaneous release can be (partly) substituted by doc2s and a fourth unidentified Ca2+ sensor.

\textbf{syt\textsuperscript{T112A} does not affect short-term plasticity and RRP size}

The phorbol ester experiments suggest that, like Munc18-1 [13], syt1 is an essential substrate for PKC to potentiate synaptic transmission. We previously found that phosphorylation of Munc18-1 is critical for multiple steps of PKC-dependent plasticity, including paired-pulse plasticity, recovery after high-frequency stimulation, and

\textbf{Figure 4.4. PKC mediated phosphorylation of syt1 does not affect short-term plasticity.} A. Typical example images of a paired pulse protocol with 20, 50, 100, 200, 500 and 1000 ms interval. Stimulation artifacts have been blanked out. B. Average paired pulse ratio for each interval. Ratio was calculated as EPSC2/EPSC1. syt\textsuperscript{WT} n = 20, syt\textsuperscript{T112A} n = 23, N = 3. C. Typical examples of EPSCs evoked by 100 AP at 40 Hz. Stimulation artifacts have been blanked out. D. Normalized EPSC amplitude during 100 AP at 40 Hz and subsequent stimulation at 0.2 Hz. Black error bars represent SEM. E. Cumulative charge of the synchronous EPSC during 100 AP at 40Hz. Shaded area represents S.E.M., dotted lines denote a linear fit back-extrapolated from the last 40 stimuli. F. Average RRP size per cell, estimated by back-extrapolation of the cumulative synchronous charge in E. syt\textsuperscript{WT} n = 16, syt\textsuperscript{T112A} n = 22, N = 3. G. RRP refilling rate during 40 Hz stimulation, estimated by the slope of back-extrapolation. H. Average vesicular release probability per cell, calculated as charge of first evoked divided by the RRP size.
increased release willingness from the RRP [13]. To test if syt\textsuperscript{T112A} produces a phenocopy of non-phosphorylatable Munc18-1, and thus also affects short-term plasticity, we applied paired pulse and high frequency stimulation protocols to syt\textsuperscript{WT} and syt\textsuperscript{T112A} rescued KO cells (Figure 4.4). No significant differences were observed in paired pulse ratio at all intervals tested (20 to 1000 ms) indicating that syt\textsuperscript{T112A} has no effect on paired pulse facilitation. (Figure 4.4A-B). In addition, we did not observe an effect on the EPSC rundown during intense stimulation (100 AP at 40 Hz), or the recovery after this AP train (Figure 4.4C-D). Thus, in contrast to Munc18-1, phosphorylation of syt1 by PKC is not required for paired-pulse plasticity or Ca\textsuperscript{2+}-dependent RRP refilling.

We furthermore tested if phosphorylation of syt1 affects the size of the RRP. Back-extrapolation of the cumulative charge measured during 100 AP at 40Hz is commonly used to estimate the RRP size and refilling [6,35,36]. With this method, we did not find significant differences in RRP size (syt\textsuperscript{WT} 215.30 ± 32.3 pC, n = 16; syt\textsuperscript{T112A} 252.9 ± 38.4 pC, n = 22, N = 4Figure 4.4E). This is in line with previous studies, which reported that DAG/PKC activation does not affect the size of the RRP [5,12,13]. The RRP refilling rate (syt\textsuperscript{WT} 62.0 ± 13.9 pC/s; syt\textsuperscript{T112A} 74.2 ± 20.3 pC/s Figure 4.4F), vesicular release probability (syt\textsuperscript{WT} 0.13 ± 0.02; syt\textsuperscript{T112A} 0.15 ± 0.03; see Figure 4.4G) and the amount of asynchronous release during the 40 Hz train (syt\textsuperscript{WT} 2468.2 ± 468.4 pC; syt\textsuperscript{T112A} 3020.2 ± 580.7 pC) were also similar in syt\textsuperscript{WT} and syt\textsuperscript{T112A} rescued neurons. These results demonstrate that syt\textsuperscript{T112A} does not affect the size and replenishment of the RRP during and after high frequency stimulation.

Discussion
Diacylglycerol and its synthetic analog, phorbol ester, are well known to acutely potentiate presynaptic strength [3-5], and operate via the activation of both Munc13 and PKC [10-13]. We have previously identified Munc18-1 as an essential substrate for PKC-dependent plasticity [13]. In the current study, we discovered that phosphorylation of syt1 is also essential for potentiation of evoked release induced by DAG.

Phosphorylation of syt1 is essential for the DAG/PKC pathway in nerve terminals
Expression of non-phosphorylatable syt-1 (syt\textsuperscript{T112A}) completely abolished potentiation of evoked release induced by PMA, demonstrating that, like Munc18-1, phosphorylation of syt1 is essential for PMA-induced potentiation (Figure 4.2). In addition, potentiation of spontaneous release was severely reduced, but only when doc2a and doc2b were absent (Figure 4.3). Surprisingly, other forms of PKC-dependent plasticity were unaffected by syt\textsuperscript{T112A}. In particular recovery after intense stimulation, which critically depends on activation of the DAG/PKC pathway [10,13], was unaffected by this mutation (Figure 4.4). Thus, syt1 phosphorylation mediates a distinct step of PKC-dependent plasticity, most likely downstream of vesicle priming. The high intracellular Ca\textsuperscript{2+} concentration during and after intense stimulation probably increases release probability sufficiently to bypass the need for syt1 phosphorylation.

We only observed an effect of syt\textsuperscript{T112A} on spontaneous release in neurons lacking expression of doc2s (Figure 4.3). Syt1, doc2a and doc2b all drive spontaneous release, and compete with each other for binding to the SNARE complex [23-25]. Our data
indicate that potentiation of spontaneous release is independently supported by multiple Ca2+-sensors and underscores the molecular redundancy in this form of vesicle fusion (reviewed in [37]). Doc2a and doc2b are not known to be PKC substrates, and thus the increased spontaneous release is likely to be caused by the increased priming rate upon activation Munc13 and Munc18-1 [10,13].

**Contribution of PKC is cell-type specific**

Previous studies demonstrated that phosphorylation of syt1 at T112 does not affect vesicle release in embryonic chromaffin cells [32]. Our results underscore that, despite many similarities, key differences exist between the release machineries of chromaffin cells and synapses. Indeed, previous studies observed that manipulation of vesicle docking and fusion proteins have strikingly dissimilar effects when comparing both model systems [38-41]. Most importantly for the current study, PKC seems to affect release to a much larger extent in adult bovine chromaffin cells as compared to murine embryonic chromaffin cells [21,33]. PMA strongly potentiates release from embryonic chromaffin cells [32,42], but potentiation persists after inhibition of PKC [33], deletion of Munc18 [42] or rescue of syt1-KO with sytT112A [32]. In addition, the relative contribution of PKC to DAG-induced potentiation appears to be different in specific types of synapses. In hippocampal synapses, PKC activation is essential [8,13], while in the Calyx of Held, PKC is not strictly required, but strongly amplifies the effect of DAG [6,11]. Surprisingly, PKC

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<th>Vesicle priming stages</th>
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<tr>
<td>unprimed vesicle pool</td>
<td>Munc18 Munc13</td>
<td>Munc13</td>
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<td>replenishment</td>
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<td>Readily releasable pool (RRP)</td>
<td>Munc18 Munc13 Syt1</td>
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<td>Fusion</td>
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**Figure 4.5.** Working model for DAG-induced potentiation of presynaptic strength. Activation of the DAG/PKC pathway increases RRP replenishment by activation of Munc13 and phosphorylation of Munc18-1. Furthermore, it increases the fusion willingness of primed vesicles, which besides Munc13 and Munc18-1, requires the phosphorylation of syt1. Interference with one of these three effectors will inhibit or severely reduce the effect of phorbol ester on synaptic vesicle release. On the right, two substrates of CaM and CaMK are depicted, that are shared with the DAG/PKC pathway. It interplay between different CaM/CaMK substrates, and the role of syt1 in this pathway, is unknown.
does not seem to contribute to DAG-induced potentiation in the neuromuscular junction [43]. The nature behind this discrepancy remains an important open question.

**Molecular mechanism of DAG-induced potentiation**

What could be the molecular mechanism of phorbol ester-induced potentiation of release? Three aspects of synaptic transmission are potentiated by the DAG/PKC pathway: (a) spontaneous release, (b) RRP refilling, and (c) the vesicular release probability of primed vesicles. All three hallmarks depend on Munc13, PKC and PKC-dependent phosphorylation of Munc18-1. [10-13]. Most studies agree that DAG/phorbol esters do not change the size of the RRP [5,11-13,44], although small increases in RRP size have been observed under some circumstances [45,46] (see ref [12] for a detailed discussion). Phosphorylation of Munc18-1 decreases its affinity for syntaxin [16,47], while DAG binding to Munc13 is thought to disinhibit its catalytic MUN domain [12]. The MUN domain facilitates the transition from the syntaxin-Munc18 to Munc18-SNARE complex [48]. DAG and PKC might further accelerate this transition, by both decreasing syntaxin-Munc18 binding and increasing MUN-domain availability, which increases vesicle priming. This potentiates both spontaneous release (DAG/PKC hallmark a) and RRP refilling (b). DAG production also lowers the energy barrier for fusion of primed vesicles by binding to Munc13 [12], and increasing Ca2+ channel conductance [7,14] and Ca2+ sensitivity for release via PKC [7,44] (hallmark c). Phosphorylation of Munc18-1 is essential for this step to occur [13].

Since phosphorylation of syt1 does not affect RRP replenishment (Figure 4.4), it is likely to contribute to DAG-dependent potentiation in a manner that is distinct in molecular terms from how Munc13 and Munc18 contribute to potentiation. PKC-dependent phosphorylation probably potentiates synaptic transmission at a step after the assembly of the Munc-18-SNARE complex, presumably by lowering the energy barrier for fusion (Figure 4.5).

**PKC and CaMK share their effectors?**

Syt1 residue T112 can be phosphorylated both by PKC and by CaMK [20], suggesting that phosphorylation of this site also occurs independently of DAG/PKC. Interestingly, Munc13 can bind to CaM, which increases its priming activity in a similar fashion as DAG binding [49,50]. Thus, the DAG/PKC and CaM/CaMK pathways converge on two specific substrates, Munc13 and syt1, and thus might (partly) operate in similar fashion. It is not known whether Munc18-1 can be phosphorylated by CaMK, or whether another CaMK substrate could substitute Munc18-1 in this pathway. The extent to which CaMK modulates presynaptic strength is incompletely understood, but the shared substrates may provide important clues on how this kinase operates at the synapse.

In conclusion, a large body of evidence has identified PKC as one of the most prominent kinases regulating vesicle release at the presynapse. In hippocampal synapses, phosphorylation of syt1 is essential for a late step of the DAG/PKC pathway to potentiate synaptic transmission.
References


44. Wu XS, Wu LG: Protein kinase c increases the apparent affinity of the release machinery to Ca2+ by enhancing the release machinery downstream of the Ca2+ sensor. J Neurosci 2001, 21:7928-7936.


