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Tyrosine phosphorylation of synaptophysin in synaptic vesicle recycling

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Abstract

The integral synaptic vesicle (SV) protein synaptophysin was one of the first nerve terminal proteins identified. However its role, if any, in the SV life cycle remains undetermined. One of the most prominent features of synaptophysin is that its cytoplasmic C-terminus largely consists of pentapeptide repeats initiated by a tyrosine residue. Synaptophysin is heavily phosphorylated by tyrosine kinases in the nerve terminal, suggesting that this phosphorylation is central to its function. This review will cover the evidence for tyrosine phosphorylation of synaptophysin and how this phosphorylation may control its function in the SV life cycle.

Keywords

synaptophysin; phosphorylation; tyrosine; exocytosis; neurone; plasticity

Introduction

Nerve communication or neurotransmission is dependent on the release of neurotransmitter from the nerve terminal of a neurone. Neurotransmitter release results from the fusion of neurotransmitter-containing synaptic vesicles (SVs) with the plasma membrane of the nerve terminal. Maintenance of neurotransmission is dependent on the subsequent endocytosis and recycling of SVs. Early studies that examined the molecular mechanism of neurotransmitter release originally focussed on the proteomic content of the SVs, since they were assumed to contain the essential machinery for vesicle fusion. One of the most abundant proteins in SVs is the integral membrane protein synaptophysin (comprising 8% of total SV protein). Synaptophysin was one of the first SV proteins to be identified and subsequent homology screening studies identified synaptophysin II (synaptoporin) as a neuronal homologue [1]. Later other related proteins such as the neuronal synaptogyrin and non-neuronal pantophysin and mitsugumin 29 were also identified [2-4].

Considering its abundance it is surprising that the role of synaptophysin in the SV life cycle is still relatively unknown. Early studies in *Xenopus* reconstitution systems suggested a central role for synaptophysin in neurotransmitter release using either its overexpression or injection of antisense or antibodies to perturb its function [5-8]. However when synaptophysin knockout mice were generated, they were viable and had no obvious defects in neurotransmitter release [9,10]. The lack of an obvious phenotype was attributed to possible redundancy with other isoforms of synaptophysin, such as synaptophysin II and / or synaptogyrin. In agreement, mice lacking both synaptophysin and synaptogyrin displayed defects in synaptic plasticity, however evoked neurotransmitter release was still normal [11].

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Since even the synaptophysin / synaptogyrin knockout mouse failed to exhibit a detectable phenotype for evoked neurotransmitter release, it has been proposed that synaptophysin may play a role at a different stage of the SV life cycle. Evidence for such a role is mounting. For example, in synaptophysin knockout mice examination of the morphology of retinal rod photoreceptors (which do not express synaptophysin II) suggest a defect in SV endocytosis. This is because SV numbers are reduced and deformations in the plasma membrane are observed [12]. In support of this, injection of a GST fusion protein of the cytoplasmic C-terminus of synaptophysin into the squid giant synapse results in a rundown in SV availability and an increase in the number of clathrin-coated vesicles [13]. This was interpreted as a selective inhibition of a clathrin-independent form of endocytosis that leaves clathrin-dependent endocytosis unaffected. Interestingly the major binding partner of synaptophysin in nerve terminals is the integral SV protein synaptobrevin [14,15], which has recently been implicated in a form of endocytosis that rapidly reuses SVs that have just undergone exocytosis [16].

Synaptophysin and its other family members also share a calcium-dependent interaction with the GTPase dynamin I [17] whose activity is essential for SV endocytosis [18]. This provides a possible mechanism through which it may regulate endocytosis, since SV endocytosis is also a calcium-dependent process [19]. Synaptophysin also binds cholesterol and this interaction has been proposed to be essential for the biogenesis of synaptic-like microvesicles in neuroendocrine cells [20]. A role for synaptophysin in either SV endocytosis or a subsequent recycling step would be compatible with a role in synaptic plasticity inferred from experiments in knockout mice, since SVs have to recycle to maintain SV supply.

In vitro tyrosine phosphorylation of synaptophysin

Synaptophysin has a C-terminal cytoplasmic domain that contains 10 pentapeptide repeats, 9 of which are initiated by a tyrosine residue, suggesting synaptophysin may be a target for tyrosine kinases within the nerve terminal. In fact synaptophysin is the major tyrosine phosphoprotein on SVs [21] and is phosphorylated by the non-receptor tyrosine kinase src *in vitro* [22] and by both src and fyn when overexpressed in a COS cell line [11]. Incredibly the tyrosine phosphorylation sites on synaptophysin are still unidentified almost 20 years after its discovery as a phosphoprotein. This is most likely due to the absence of trypsin cleavage sites in its C-terminus, which would greatly aid phosphorylation site identification. It is likely that a high proportion of the 9 putative sites on the C-terminus (Figure 1) are phosphorylated, since it is common for src substrates to be multiply phosphorylated in tyrosine rich domains. For example, the src substrate p130^{Cas} is hyperphosphorylated in a highly efficient processive manner, where the kinase binds to a proline-rich recognition sequence via its SH3 domain [23]. This hyperphosphorylation results in at least one tyrosine phosphorylation site becoming a binding site for the SH2 domain of src. The fact that src is found in a complex with synaptophysin suggests that it may also use a processive mechanism to phosphorylate synaptophysin [22]. The tyrosine phosphatase SH-PTP1 also immunoprecipitates in a complex with synaptophysin and src, and could thus provide compensatory dephosphorylation of synaptophysin [24]. As yet, there are no reports for a direct interaction of SH-PTP1 with synaptophysin while it has been previously shown that SH-PTP1 binds to src [25].

In vivo tyrosine phosphorylation of synaptophysin

There is ample evidence that synaptophysin can be tyrosine phosphorylated *in vitro* however the evidence for its *in vivo* phosphorylation is less well characterised. Under resting conditions, synaptophysin immunoprecipitated from brain homogenates is tyrosine

phosphorylated [24,26]. However, metabolic radiolabelling of brain slices with $^{32}\text{P}_i$ shows no incorporation of isotope into synaptophysin on tyrosine residues [27]. Furthermore, exposure of brain slices to KCl produces no tyrosine labelling, suggesting that synaptophysin phosphorylation may not be regulated by depolarising stimuli [27]. The lack of labelling of synaptophysin is not due to a lack of tyrosine kinase activity *per se*, since there are multiple substrates phosphorylated by stimulation-dependent tyrosine kinase activity in synaptosomes [28,29], brain slices [30] and cultured neurones [31,32]. A small amount of tyrosine labelling is found on synaptophysin that was purified from synaptosomes after metabolic labelling [21,27]. However, these results have to be treated with caution, since proteins recovered immediately after synaptosome preparation may be hyperphosphorylated due to increased basal kinase activity resulting from the preparation procedure (personal communication Phillip Robinson, Australia). Thus synaptophysin is tyrosine phosphorylated in nerve terminals but these phosphate groups do not seem to turn over rapidly and may not be stimulation-dependent.

Tyrosine phosphorylation of synaptophysin in SV recycling

The lack of stimulation-dependent tyrosine phosphorylation of synaptophysin *in vivo* is in agreement with a lack of convincing evidence for a direct and essential role for tyrosine phosphorylation in neurotransmitter release [33]. It is generally accepted that if tyrosine phosphorylation has any role in neurotransmitter release it is a modulatory one via channels and receptors [32,34]. Similarly, there is very little evidence to support a role for tyrosine phosphorylation in SV endocytosis. In adrenal chromaffin cells the tyrosine kinase antagonist tyrphostin A23 inhibited a form of clathrin-independent endocytosis that involved excess retrieval of membrane [35]. However the same inhibitor also interferes with the binding of the adaptor protein AP-2 to tyrosine-based internalisation motifs on cargo [36], therefore any role for tyrosine phosphorylation in SV endocytosis remains undetermined.

Tyrosine phosphorylation of synaptophysin in synaptic plasticity

In contrast to the lack of an essential role for tyrosine phosphorylation in neurotransmitter release, there is convincing evidence that tyrosine phosphorylation is required for long-term potentiation (LTP) [37,38]. Tyrosine phosphorylation has been proposed to have a predominantly postsynaptic role in LTP, however the requirement for tyrosine phosphorylation parallels the proposed role(s) of synaptophysin in synaptic plasticity. A direct link between the two has been suggested, where tyrosine phosphorylation of synaptophysin was increased in parallel with glutamate release from synaptosomes prepared from LTP-treated hippocampal slices [26]. Tyrosine kinase inhibitors prevented LTP, the evoked increase in synaptophysin phosphorylation and glutamate release. Inhibition of NMDA receptors with AP5 gave identical results, suggesting the observed presynaptic effect was dependent on signalling from the postsynapse. Furthermore in a study of src in the hippocampus of rats trained in a spatial memory task, presynaptic src activity was transiently increased after training and a long term increase in the src / synaptophysin interaction occurred [39]. It would be predicted therefore that synaptophysin would be more phosphorylated in the presence of increased src and increased enzyme activity. Thus under conditions that evoke changes in synaptic strength, tyrosine phosphorylation of synaptophysin may be stimulation-dependent.

Control of protein interactions by tyrosine phosphorylation of synaptophysin

Synaptophysin shares a number of interactions with nerve terminal proteins, the best characterised being the formation of a heterodimer with synaptobrevin [14,15].

Synaptobrevin is a v-SNARE that is essential for SV fusion [40] but may also perform an essential role in retrieving SVs to be used for the readily releasable pool [16]. The amount of heterodimer formed has been proposed to be stimulation-dependent, however it is still unclear whether stimulation increases and decreases complex formation [41-44]. This interaction has been proposed to control the availability of synaptobrevin to form SNARE complexes for SV fusion since it is disrupted by calcium *in vitro* and *in vivo* [41-43]. However recent *in vivo* cross-linking studies in synaptosomes have challenged these findings, showing an increase in heterodimer formation on stimulation [44]. In that study the synaptophysin / synaptobrevin heterodimer is unaffected by the application of various inhibitors of serine/threonine and tyrosine kinases to synaptosomes [44]. These findings are unsurprising since the synaptophysin / synaptobrevin interaction involves the membrane spanning domains of the two proteins, and therefore it is unlikely that binding will be affected by phosphorylation of the cytoplasmic tail of synaptophysin [45].

The interaction of synaptophysin with dynamin I may be phosphorylation-dependent however, since the tyrosine-based pentapeptide motifs on the synaptophysin C-terminus are the binding sites for dynamin I [17]. Src also directly interacts with dynamin I *in vitro* and *in vivo* [46] suggesting that a tripartite complex of dynamin / src / synaptophysin exists that may be regulated by synaptophysin phosphorylation.

Phosphorylation of synaptophysin by other protein kinases

Synaptophysin can be phosphorylated in a stimulation-dependent manner in nerve terminals, but on serine not tyrosine residues. There is good evidence to suggest that synaptophysin is a substrate for calcium / calmodulin dependent protein kinase II (CaMKII), since it is phosphorylated on SVs by an endogenous protein kinase that is stimulated by calcium and calmodulin and sensitive to inhibitors of CaMKII [27]. Furthermore, the *in vitro* phosphorylation of purified synaptophysin by CaMKII produces an identical phosphopeptide pattern to that obtained by endogenous calcium-dependent synaptophysin phosphorylation where peptides were generated using chymotrypsin digestion. The putative phosphoserines are located in the C-terminus of synaptophysin but have not been identified. However, based on the predicted digestion by chymotrypsin it is likely that 3 of the 4 serines present in the C-terminus are phosphorylated by CaMKII [27]. Intriguingly, none of the serine containing sequences conform to the CaMKII phosphorylation consensus sequence of R-X-X-S (Figure 1).

Perspectives

The role of synaptophysin in the SV life cycle is still shrouded in uncertainty. It is now almost universally agreed that the protein has no essential role in SV exocytosis and but may have a role in determining synaptic strength. It would seem therefore that the most likely point of action for synaptophysin in the SV life cycle would be somewhere that could regulate the availability of SVs, such as SV endocytosis or subsequent recycling steps. With the advent of a new generation of fluorescent techniques that can visualise these processes in individual nerve terminals [47,48] it will be possible to elucidate any role through a series of targeted experiments in either synaptophysin or synaptophysin / synaptogyrin knockout mice.

The major question regarding the tyrosine phosphorylation of synaptophysin is, what function does it perform? The lack of an essential role for tyrosine phosphorylation in SV exocytosis parallels the lack of a role for synaptophysin in the process and the absence of stimulation-dependent tyrosine phosphorylation. However tyrosine phosphorylation of synaptophysin may regulate synaptic strength. For example, src activity and the src / synaptophysin interaction is increased *in vivo* on learning, and tyrosine phosphorylation of

synaptophysin is increased during LTP [26,39]. This suggests that tyrosine phosphorylation of synaptophysin is stimulation-dependent *in vivo*, but only when coupled to specific stimuli. Stimuli that evoke LTP are typically high frequency trains of action potentials which cause a large increase in the concentration of residual intracellular calcium within the nerve terminal [49]. Since the synaptophysin / dynamin I interaction is only stimulated by calcium in the high μM range [17] it may be that synaptophysin is only tyrosine phosphorylated when calcium is elevated to a certain level by src attached to dynamin I.

For the role of tyrosine phosphorylation of synaptophysin to be fully understood its phosphorylation sites have to be identified. Since tryptic peptide analysis cannot be used other enzymes will have to be employed. One of these is chymotrypsin, which cleaves on the C-terminal side of tyrosine residues and would thus permit a thorough phospho-peptide analysis of the synaptophysin C-terminus. However the best hope may be a systematic mutagenesis study to examine the effect of point mutating candidate tyrosine residues and determining which sites are phosphorylated *in vitro* by src. This approach is not without its drawbacks, since removal of the major tyrosine phosphorylation sites may lead to the promiscuous phosphorylation of normally unphosphorylated tyrosines. However an extreme approach may be to mutate all nine tyrosine residues and determine the effect of this mutant on SV recycling by its overexpression on a background of the synaptophysin knockout mouse. Once these types of experiments have been completed we may begin to have a better idea of why synaptophysin is tyrosine phosphorylated.

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Abbreviations

SV	synaptic vesicle
LTP	long-term potentiation
CaMKII	calcium calmodulin-dependent kinase II

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Figure 1.

Alignment of the amino acid sequence of the C-termini of synaptophysin (Syp I), synaptophysin II (Syp II) and synaptogyrin (Syp I). Synaptophysin has nine putative tyrosine residues for phosphorylation whereas synaptophysin II contains only five and is not tyrosine phosphorylated (unpublished observations [11]). Synaptogyrin has six possible sites and is tyrosine phosphorylated. Black bars indicate shared amino acid homology between at least 2 protein sequences.