Use of phospho-specific antibodies to demonstrate phosphorylation of Munc18/nSec1 in chromaffin cells.

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In most regulated secretory cells, including chromaffin cells, the signal for exocytosis is an increase in the cytosolic free Ca\(^{2+}\) concentration\(^1\). Another general characteristic of regulated exocytosis is its acute regulation by protein phosphorylation. Many studies over the past 20 years have shown that exocytosis is modulated by protein kinases (PKs) in almost all regulated secretory cell types, including neurons and chromaffin cells\(^2\)\(^-\)\(^4\). Pharmacological approaches using cell permeable activators or inhibitors have implicated a wide range of serine/threonine and tyrosine kinases, including Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII), casein kinase II (CK2), mitogen activated protein kinase, myosin light chain kinase and src family kinases\(^5\). However, one short fall of this approach is that the modulation of exocytosis may be indirect. Thus, application of pharmacological modulators of kinase activity (or better still, purified kinases themselves) to permeabilized cells, where receptors and ion channels are bypassed, is a much more rigorous demonstration of a role for kinases in the direct modulation of the exocytotic machinery. A review of the literature reveals that only PKA (cAMP-dependent protein kinase) and PKC (phospholipid/diacylglycerol-dependent protein kinase), or their pharmacological effectors, modulate exocytosis in almost all permeabilized regulatory secretory cell types examined, including exocrine, endocrine and neuronal cells. The primary action of both PKA and PKC in neurons has been shown to be downstream of Ca\(^{2+}\) entry, and indeed purified PKA and PKC enzymes enhance exocytosis in permeabilised chromaffin cells\(^6\)\(^,\)\(^7\). Abundant evidence therefore suggests that the effects of PKA and PKC on regulated secretion are due to phosphorylation of components of the exocytotic machinery.

In order to identify the functionally important phosphoproteins that mediate these effects of protein kinases on exocytosis, various labs (including ours) have screened exocytotic proteins for in vitro phosphorylation by purified kinases\(^4\)\(^,\)\(^5\). Although this has revealed many in vitro substrates, relatively few of these have been shown to be phosphorylated in cellular preparations and to be functionally altered in terms of their biochemical characteristics and effect on exocytosis in cells. One such protein is Munc18/nSec1, which is a member of the Sec1 protein family that has been implicated in most membrane fusion events. Indeed Munc18 is essential for synaptic vesicle exocytosis, as indicated
by the complete absence of neurotransmitter release in Munc18-1 knockout mice. As these animals contain a normal complement of docked synaptic vesicles, this suggests a post-docking role for Munc18 in the late stages of exocytosis. Such a role is consistent with the observed effects of Munc18 mutants on the kinetics of catecholamine release in chromaffin cells. However, in chromaffin cells from the above knockout mice, there is a profound impairment of vesicle docking, suggesting an additional, early role for Munc18 upstream of tethering. Clearly, the functional role(s) of Munc18 remains elusive, although it is generally agreed that its high-affinity interaction with syntaxin is important for at least one function. Munc18 is phosphorylated in vitro by PKC on Ser-306 and Ser-313, and this phosphorylation reduces the affinity of Munc18 for its binding partner, syntaxin, in vitro binding assays. A recent study has demonstrated that mutation of Ser-306 and Ser-313 to glutamate (to mimic the negative charge imposed by phosphorylation of these residues) causes a similar reduction in affinity for syntaxin.

Importantly, overexpression of this phosphomimetic mutant in chromaffin cells had the same effect on catecholamine release kinetics as the PKC activator, PMA: decreasing the half-width, rise-time and quantal size of amperometric spikes. Furthermore, as PMA was unable to elicit further changes to spike kinetics upon expression of the phosphomimetic mutant, this strongly suggests that the mechanism by which PKC activation alters exocytotic release kinetics is via phosphorylation of Munc18 on Ser-306 and/or Ser-313.

In light of these recent data, we investigated whether the phosphorylation of Munc18 on Ser-306 and Ser-313 seen in vitro also occurred in vivo in response to physiological stimulation. To do this, antisera were raised against phosphorylated peptides surrounding these phosphorylation sites. Phospho-specific antisera to both sites were generated, as defined by their ability to detect Munc18 that had been pre-phosphorylated in vitro by PKC but not unphosphorylated protein. Specificity of the antisera was further shown by the fact that neither antiserum detected recombinant proteins mutated at Ser-306 and Ser-313 after the proteins had been incubated with PKC. No specific signal could be detected in secretagogue- or PMA-treated chromaffin cells or synaptosomes using the phospho-Ser306-specific
antibody. This suggests either that the antibody cannot detect endogenous Ser-306-phosphorylated Munc18 or that this site is not phosphorylated in vivo under the conditions examined here. In contrast, Munc18 phosphorylation at Ser-313 was readily observed in PMA-treated chromaffin cells and synaptosomes using the phospho-Ser313-specific antibody. In addition, Ser-313 phosphorylation also occurred in permeabilized chromaffin cells in response to free \( \text{Ca}^{2+} \) concentrations that trigger exocytosis. Maximal phosphorylation was observed at 10 \( \mu \text{M} \) \( \text{Ca}^{2+} \), the optimum for exocytosis. This phosphorylation is likely caused by PKC, as the PKC inhibitor, bisindolylmaleimide, inhibited the \( \text{Ca}^{2+} \)-induced increase in phosphorylation observed in permeabilized cells. Furthermore, Ser-313 phosphorylation was also induced in intact cells by histamine, which causes \( \text{Ca}^{2+} \)-dependent exocytosis in chromaffin cells. Therefore, Munc18 is phosphorylated in adrenal chromaffin cells on secretagogue stimulation, and not only in response to PMA treatment. Interestingly, the phosphorylation of Ser-313 observed with combined \( \text{Ca}^{2+} \) and PMA treatment was greater than that seen with either treatment individually. It may be that \( \text{Ca}^{2+} \)-induced membrane fusion frees Munc18 from syntaxin, as when these two proteins are bound Munc18 cannot be phosphorylated by PKC.

In summary, we have shown that Ser-313 of Munc18 is phosphorylated in chromaffin cells response to secretagogue stimulation. As we have also found this to be true for rat brain synaptosomes, this suggests that activity-dependent phosphorylation of this residue may be a general phenomenon. Intriguingly, both presynaptic protein phosphorylation and modulation of neurotransmitter release kinetics have been implicated in synaptic plasticity. It is conceivable that phosphorylation of Munc18 at Ser-313 - a residue essential for the modulation of catecholamine release kinetics in chromaffin cells - could be a mechanism linking these processes.

REFERENCES
