Phosphorylation of Munc18 by Protein Kinase C Regulates the Kinetics of Exocytosis*

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Protein phosphorylation by protein kinase C (PKC) has been implicated in the control of neurotransmitter release and various forms of synaptic plasticity. The PKC substrates responsible for phosphorylation-dependent changes in regulated exocytosis in vivo have not been identified. Munc18a is essential for neurotransmitter release by exocytosis and can be phosphorylated by PKC in vitro on Ser-306 and Ser-313. We demonstrate that it is phosphorylated on Ser-313 in response to phorbol ester treatment in adrenal chromaffin cells. Mutation of both phosphorylation sites to glutamate reduces its affinity for syntaxin and so acts as a phosphomimetic mutation. Unlike phorbol ester treatment, expression of Munc18 with this phosphomimetic mutation in PKC phosphorylation sites did not affect the number of exocytotic events. The mutant did, however, produce changes in single vesicle release kinetics, assayed by amperometry, which were identical to those induced by phorbol ester treatment. Furthermore, the effects of phorbol ester treatment on release kinetics were occluded in cells expressing phosphomimetic Munc18. These results suggest that the dynamics of vesicle release events during exocytosis are controlled by PKC directly through phosphorylation of Munc18 on Ser-313. Phosphorylation of Munc18 by PKC may provide a mechanism for the control of exocytosis and thereby synaptic plasticity.

Protein phosphorylation has been long known as an important mechanism for the regulation of exocytosis although, with only a few exceptions such as the synapsins (1), the targets for regulation by phosphorylation in vivo are unknown. Treatment with phorbol esters modifies regulated exocytosis in many different neuronal and non-neuronal (2, 3) cell types leading to increased vesicle recruitment into the ready releasable pool (4–6), acceleration of fusion pore expansion (7), or changes in the kinetics of exocytosis (8, 9). PKC also has a key role in synaptic plasticity (10). The effects of phorbol ester were originally attributed to activation of PKC although the PKC substrates responsible had not been identified, and it is not known if the same target regulates all of the parameters modified by phorbol esters. The SNARE proteins, syntaxin 1, SNAP-25, and VAMP play key roles in exocytosis (11–13), and formation of the SNAP-25 complex has been suggested to be a driving force for membrane fusion (14). The syntaxin-binding protein Munc18a (15) (also known as nSec1, Ref. 16) is also essential for neurotransmitter release (17–19). Other key proteins in regulated exocytosis include rab3 and its effectors (20) and synaptotagmin the likely Ca2+ sensor (21, 22). Among these proteins, SNAP-25 (23), VAMP (24), synaptotagmin I (25), rab3 (26), and Munc18 (27, 28) have been shown to be PKC substrates in vivo. PKC phosphorylation of specific residues in intact cells has only been demonstrated for SNAP-25 (29) and synaptotagmin I (25). In no case has the functional consequences of these phosphorylation events for exocytosis been established. Indeed, the phosphorylation of SNAP-25 by PKC in PC12 cells lagged well behind the effects of phorbol ester on the extent of exocytosis (29). In that study, it was also shown that the phorbol ester effects had both a PKC-dependent and a PKC-independent component. The synaptic protein Munc13 has been identified as an alternative phorbol ester-binding protein (30, 31), and recently it has been suggested that the effects of phorbol ester on synaptic transmission are mediated entirely by Munc13 (32). A PKC-dependent component of phorbol ester stimulation of exocytosis was shown to involve vesicle recruitment to the plasma membrane in PC12 cells (33). Phorbol ester-stimulated recruitment of vesicles into the ready releasable pool in adrenal chromaffin cells was inhibited by bisindolylmaleimide in chromaffin cells (4, 34) consistent with a role for PKC. In addition, we have shown that another aspect of exocytosis, the kinetics of single vesicle release events, is modified by phorbol esters in a PKC-dependent (bisindolylmaleimide-sensitive) fashion in adrenal chromaffin cells (9). It is clear that alternative approaches are needed to establish whether PKC-mediated phosphorylation of any identified substrate directly regulates one or more aspect of exocytosis.

Munc18 binds tightly to syntaxin holding it in a closed conformation that prevents its assembly into a SNARE complex (35–37). In some as yet uncharacterized way, Munc18 is required to donate syntaxin ready for its interaction with the other SNARE proteins and in its absence membrane fusion is abolished. Modification of the interaction between Munc18 and

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1 The abbreviations used are: PKC, protein kinase C; SNARE, soluble NSF attachment protein receptors; NSF, 4-morpholineethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; PMA, phorbol 12-myristate 13-acetate; GST, glutathione S-transferase; PBS, phosphate-buffered saline; EGFP, enhanced green fluorescent protein; SNAP, soluble NSF attachment protein; dGlu, double mutations to Glu; dAla, double mutations to Ala.
syntaxin could, therefore, be an important mechanism for the regulation of membrane fusion, and we have shown that mutations in Munc18, which modify the affinity of this interaction, change the kinetics of single vesicle release events in chromaffin cells (38). Interestingly, Munc18a is phosphorylated by PKC on Ser-306 and Ser-313 in vitro, and this reduces the amount of Munc18 that binds to syntaxin (26). We have investigated whether phosphorylation on these sites occurs in intact cells and demonstrate that phosphorylation of Ser-313 of Munc18 in response to PKC activation leads to changes in the kinetics of vesicle fusion and release.

MATERIALS AND METHODS

Phosphospecific Antiser—Rabbit polyclonal phosphospecific antibodies were produced by AbCam Ltd (Cambridge, UK) using the peptides CQETTRqLKDFS for Ser-306 and CDFSqSPSRMKNTG for Ser-313. Cysteine was included at the N terminus of each peptide for conjugation to carrier proteins and for use in affinity purification. Rabbit polyclonal antibodies were raised to these peptides, affinity-purified using a Sulfolink kit (Pierce), and characterized by Western blotting of phosphorylated and non-phosphorylated Munc18. For phosphorylation, His-tagged proteins were incubated in MES buffer, pH 6.9 (50 mM MES, 10 mM MgCl₂, 1 mM dithiothreitol, 0.5 mM EDTA) with 10 μM Munc18a or Munc18b, 5 μM calcium, and 5 μM phosphatidylserine. Samples were separated by SDS-PAGE, transferred to nitrocellulose, and probed with monoclonal antibody against Munc18 (BD Biosciences).

Amperometric Recording—Electrophysiological recording conditions were as described previously (9, 38). Briefly, cells were incubated in bath buffer (139 mM potassium glutamate, 0.2 mM EGTA, 20 mM PIPES, 2 mM ATP, and 2 mM MgCl₂, pH 6.5) and a 5-μm diameter carbon fiber electrode was positioned in contact with a cell. For stimulation, a cell permeabilization/stimulation buffer (139 mM potassium glutamate, 20 mM PIPES, 5 mM EGTA, 2 mM ATP, 2 mM MgCl₂, 20 μM dGlu, pH 6.5) was pressure-ejected from a glass pipette on the opposite side of the cell, and amperometric responses were monitored with a VA-10 amplifier (NPI Electronic, Tamm, Germany). For examination of the effects of phorbol ester treatment, recordings were taken of untreated control cells, and then 100 nM PMA added to the bath for at least 10 min before recording from treated cells.

For the comparison of control- and mutant-expressing cells, analysis of untransfected and transfected cells before and after PMA treatment was carried out in parallel on the same batch of cells. To rule out any variability between cell batches and carbon fibers, transfected cells and untransfected cells as controls were recorded alternately in the same dishes and with the same carbon fibers. Data from transfected cells was always compared with the respective control cells. For all treatments, cells were derived from multiple cell preparations. The data were subsequently analyzed using Origin (9). All of the data are shown as mean ± S.E., and statistical differences were assessed using the non-parametric Mann Whitney test.

Analysis of Catecholamine Release from Cell Populations—Chromaffin cells in culture were washed in a Krebs-Ringer buffer (145 mM NaCl, 5 mM KCl, 1.3 mM MgCl₂, 1.2 mM NaH₂PO₄, 10 mM glucose, and 20 mM HEPES, pH 7.4), and then incubated for 10 min in the absence or presence of 100 nM PMA. The cells were then stimulated and permeabilized by incubation in 139 mM potassium glutamate, 20 mM PIPES, 0.2 mM EGTA, 2 mM MgCl₂, 20 μM dGlu, pH 6.5 with either freshly prepared Ricin A or CaCl₂ added to give a free Ca²⁺ concentration of 10 μM. After 10 min, the supernatant and the cells were taken for analysis of catecholamine using a fluorimetric assay (40).

RESULTS

Use of Phosphospecific Antiser to Demonstrate Phosphorylation of Ser-306 and Ser-313 of Munc18 in Intact Cells—To test whether PKC phosphorylated residues Ser-306 and Ser-313 of Munc18, phosphospecific antisera were generated using phosphorylated peptides surrounding each phosphorylation site. These antisera specifically recognized recombinant Munc18a phosphorylated by PKC in vitro but not the non-phosphorylated protein (Fig. 1A). In these experiments the bacterially expressed Munc18 often ran as a doublet, presumably due to some limited proteolysis, but the two forms behaved essentially identically in all assays. Recombinant His₆-tagged Munc18 was also prepared with double mutations to either alanine (dAla) or glutamate (dGlu) at Ser-306 and Ser-313. Specificity of the antiser was further shown by the fact that neither antiser detected the proteins mutated at Ser-306 and Ser-313 after the proteins had been incubated with PKC (Fig. 1B). Adrenal chromaffin cells are widely used in the study of regulated exocytosis, and phorbol ester treatment modifies the extent (4, 6) and kinetics of exocytosis (8, 9) from them. Phosphorylation of Munc18, therefore, was examined in these cells using the phos-
phosphospecific antisera. A specific signal from the antiphospho-
Ser-306 antiserum was not obtained from either control or
PMA-treated cells and therefore, it is unclear if this residue is
phosphorylated in vivo. In contrast, a specific signal for Ser-313
phosphorylation was obtained based on competition with the
immunizing peptide (Fig. 1, C and D). Phosphorylation of Ser-
313 was low in control cells but was markedly increased by
PMA treatment, showing that this residue is phosphorylated
following PKC activation in intact cells (Fig. 1 C).

Effect of Mutations in Ser-306 and Ser-313 of Munc18 on
Binding to Syntaxin—It has been shown that phosphorylation
of Munc18 by PKC reduced the amount of the protein recovered
with syntaxin in an in vitro binding assay (28). As shown in
Fig. 2A, in vitro phosphorylation of Munc18 by PKC signifi-
cantly reduced the affinity of its binding to syntaxin 1A (39)
from a $K_D$ of 56 to 390 nM. In the study identifying Ser-306 and
Ser-313 as phosphorylation sites, two additional phosphoryl-
ated peptides were not characterized. In order to determine
whether phosphorylation of only the two identified residues
was responsible for the effect on syntaxin binding, they were
both mutated to alanines (dAla) or glutamate (dGlu) in vitro.
phosphorylated conditions with PKC, and then the separated proteins were probed with anti-Munc18 or the phos-
specific antisera. C, adrenal chromaffin cells in culture were treated
under control conditions or with PMA for 15 min. Separated proteins
were then probed with anti-Munc18 or the phosphospecific antisera as
indicated. D, the same blots were reprobed with the phosphospecific antisera but in the presence of excess of the immunizing peptide.

Fig. 2. Phosphorylation of Munc18 or mutation of Ser-306 and
Ser-316 to glutamate reduces its affinity for syntaxin. A, recom-
binant His-tagged wild-type Munc18 was incubated with (phospho,
open circles) or without (mock, closed circles) PKC under phosphory-
lating conditions, and then binding to GST-syntaxin (200 nM) was as-
sayed at the indicated Munc18 concentrations. Binding is shown as a
percentage if the maximum observed with wild-type protein. B, wild-
type Munc18 or recombinant proteins with Ser-306 or Ser-313 mutated
to alanine or glutamate were expressed as GST fusion proteins, incu-
bated with syntaxin at the indicated concentrations, and syntaxin bind-
ing assayed. Data from wild-type protein are indicated by closed circles,
dAla by open circles, and dGlu by triangles. C, binding of $^{35}$S-labeled
Munc18 and the dGlu mutant to syntaxin and competition by the
indicated concentration of unlabeled His-tagged wild-type Munc18.

to confirm the reduced affinity of the dGlu mutant for binding
to syntaxin and to rule out any potential problems resulting
from bacterial expression of the mutant, binding assays were
carried out using radiolabeled Munc18a prepared by in vitro
transcription and translation. Binding of $^{35}$S-labeled proteins
to syntaxin was carried out in the absence or presence of
competing unlabeled wild-type Munc18. Binding of the dGlu
form of Munc18 was much more efficiently competed than that
of wild-type protein, showing the reduced affinity of the dGlu
mutant (Fig. 2C).
Munc18 Phosphorylation and Exocytosis

Fig. 3. PMA treatment but not expression of a double glutamate mutant of Munc18 increases the extent of exocytosis. A, effect of PMA on release of catecholamine in a cell population experiment. The cells were pretreated with PMA and then permeabilized with digitonin in the absence or presence of 10 μM Ca2+ as indicated. Catecholamine released over a 20-min period was assayed and expressed as a percentage of total cellular content. B, time course of amperometric spikes after stimulation with digitonin/Ca2+ in control (n = 20) and PMA-treated (n = 23) cells. C, time course of amperometric spikes after control (n = 32) and dGlu Munc18-expressing (n = 35) cells from the same plates.

Effect of Phorbol Ester or the Phosphomimetic Mutation of Ser-306 and Ser-313 of Munc18 on the Extent of Vesicle Fusion—In order to test the effect of phosphorylation in vivo we expressed Munc18 with Ser-306 and Ser-313 mutated to glutamate as this mutant was phosphomimetic in the syntaxin binding assay. Use was made of a well characterized assay (8, 9, 42, 43) based on the direct stimulation of exocytosis with local application of digitonin and Ca2+ to permeabilize the cells and allow Ca2+ entry, and use of carbon-fiber amperometry to analyze the extent and kinetics of single vesicle release events from adrenal chromaffin cells (44, 45). Treatment of chromaffin cells with phorbol esters results in an increase in catecholamine release when measured in populations of intact or permeabilized cells (6, 46, 47) most likely due to an increase in the recruitment of secretory vesicles (6). The extent of this overall increase in catecholamine release for a cell population experiment (around 40%) is shown by the example in Fig. 3A for digitonin-permeabilized cells challenged with 0 and 10 μM Ca2+. We examined whether phorbol ester treatment or expression of the dGlu Munc18 mutant would increase the number of exocytotic events detected by amperometry. In a series of independent experiments, PMA-treatment increased the number of amperometric spikes to 166.6 ± 18.1% of control values (n = 8, p < 0.01, data from a total of 137 control and 139 PMA-treated cells). The effect of PMA on the time course of release is shown for one experiment in Fig. 3B. In contrast, expression of the Munc18 dGlu mutant had no effect on either the overall number of amperometric spikes per cell (21.9 ± 5.0 for control cells, n = 32 and 19.5 ± 5.2 for transfected cells, n = 35) or the time course of the cell responses (Fig. 3C).

Effect of Expression of the dGlu Munc18 Mutant on Single Vesicle Release Kinetics—From examination of the kinetics of release from single vesicles using amperometry we have previously shown that treatment with the phorbol ester PMA reduced the amount of release per vesicle (total charge) and the spike half-width and reduced both the rise and fall time of the amperometric spikes (8, 9). These effects were blocked by the PKC inhibitor bisindolylmaleimide (9) implicating PKC as the target for PMA. Identical effects were seen following expression of a Munc18 mutant (R39C) that had a reduced affinity for syntaxin (38) because Arg-39 makes an important contact with syntaxin (35). Overexpression of wild-type Munc18 had no detectable effects on either the extent or kinetics of exocytosis (38, 41). In the current series of experiments, changes in release kinetics seen as alterations in amperometric spike parameters were again observed in six of six independent experiments on different cell batches. The pooled data are shown in Fig. 4A.

The importance of Munc18 phosphorylation for the PKC effects was tested by expressing the dGlu form of Munc18. Expression of the dGlu mutant, resulted in a reduction in total charge released, in the half-width of the spikes and in rise and fall times of the spikes (Fig. 4B). These effects were essentially identical to those following PMA treatment (Fig. 4A).

In order to determine whether phosphomimetic mutations at both of the Munc18 phosphorylation sites was required for the functional effects, Munc18 with a single mutation, S306E or S313E, were also tested. Expression of either of these had no detectable effect on any spike parameters (Fig. 5, A and B). At the same time that the effect of expression of the single mutants was examined, parallel experiments on the same batch of cells demonstrated that the expected changes in spike parameters following PMA treatment did occur. To ensure that the difference between double and single mutants was not due to differences in expression levels the extent of expression was examined. All three proteins could be detected by immunofluorescence in EGF-positive transfected chromaffin cells using a concentration of antisera too low to detect the endogenous Munc18 (Fig. 6A). This and in previous studies (8, 9), we have established that close to 95% of cells coexpress proteins from both plasmids used in the transfection. Since the low efficiency of transfection of these cells (1–5%) precludes analysis by Western blotting we also used transfection of HEK cells that is efficient enough to allow Western blotting analysis. No differences were observed in expression in HEK cells (Fig. 6B). These results suggest therefore, that single mutations to Ser-306 or Ser-313 are indeed ineffective in modifying exocytosis kinetics and that both sites may need to be phosphorylated to affect exocytosis.

Comparison of the increase in the rate of rise of the amperometric spikes showed that the double glutamate Munc18 had an effect of similar magnitude to that due to PMA (Fig. 7A). We then tested whether the effect of PMA was occluded in cells expressing the double mutant as expected if the effects involved the same pathway. Expression of dGlu in this additional experiment again modified the release kinetics and increased the rate of rise of the spikes. PMA had no statistically significant effect in the dGlu mutant-expressing cells over that from the dGlu mutant alone (Fig. 7A) showing that the PMA effect was indeed occluded. We also examined the effect of PMA on cells expressing the single S306E Munc18 mutant, which did not increase the rate of rise. In this case, PMA treatment resulted in a significantly increased rate of rise of the spikes in the cells expressing this mutant.
DISCUSSION

Previous studies using phorbol esters and PKC inhibitors or following correlation of phosphorylation and secretion have failed to convincingly identify a PKC substrate linked to the regulation of a defined aspect of exocytosis. We have used, therefore, a more direct functional approach with phosphomimetic mutations to examine the role of PKC phosphorylation of Munc18 in the regulation of vesicle release kinetics. We have demonstrated that Munc18 is phosphorylated in cells on Ser-313 and that phosphorylation on Ser-313 is increased by phorbol ester treatment. The physiological significance of Munc18 phosphorylation was demonstrated by expression of Munc18 with double mutations in Ser-306 and Ser-313 to glutamate (dGlu). Recordings were made from EGFP-positive cells. EGFP-negative cells in the same dishes were recorded with the same carbon fiber to act as controls in each experiment. Typical traces are shown for control and Munc18 dGlu-expressing cells. Amperometric spikes were analyzed from 12 control (n = 466 spikes) and 15 transfected (n = 349 spikes) cells.

Fig. 4. Expression of a double glutamate mutant of Munc18 modifies the kinetics of vesicle release events in a similar manner to PMA treatment. A, typical amperometric responses from adrenal chromaffin cells, following addition of digitonin and Ca2+, before and after PMA treatment are shown. Amperometric spikes were analyzed from 93 control (n = 1636 spikes) and 84 PMA-treated cells (n = 1755 spikes) pooled from six experiments on different batches of cells. Average values for total charge, half-width, rise time to peak, and fall time of the spikes are shown. B, chromaffin cells were transfected with plasmids encoding EGFP and encoding Munc18 with both Ser-306 and Ser-313 mutated to glutamate (dGlu). Recordings were made from EGFP-positive cells. EGFP-negative cells in the same dishes were recorded with the same carbon fiber to act as controls in each experiment. Typical traces are shown for control and Munc18 dGlu-expressing cells. Amperometric spikes were analyzed from 12 control (n = 466 spikes) and 15 transfected (n = 349 spikes) cells.

Fig. 5. Amperometric recordings and analysis of spike parameters from cells expressing Munc18 with the single mutations S306E or S313E. A, amperometric recordings and analysis of spike parameters from cells expressing Munc18(S306E), (n = 8 cells, 197 spikes) and the respective control cells (n = 8 cells, 215 spikes). B, amperometric recordings and analysis of spike parameters from cells expressing Munc18(S313E) (n = 20 cells, 460 spikes) and the respective control cells (n = 24 cells, 435 spikes).

Munc18 Phosphorylation and Exocytosis
Regulation of exocytosis by protein phosphorylation has been extensively investigated (3). In particular, phosphorylation of synapsin via calmodulin-dependent kinase II has been established as a mechanism for controlling vesicle recruitment (1). It is clear, however, that protein phosphorylation has diverse effects on regulated exocytosis including direct effects of PKC activation on membrane fusion. Many of the proteins that form the machinery for exocytosis have been shown to be substrates for PKC or other kinases (49, 50) in vitro. However, few in vivo PKC substrates have been demonstrated, and the physiological regulation of exocytosis by PKC has not been functionally linked to the phosphorylation of any particular protein. Considerable work over many years has implicated protein kinase C in controlling neurotransmitter release based on the use of phorbol esters (2, 3). Very recently, however, it has been controversially suggested that all of the effects of phorbol esters are due instead to an alternative phorbol ester target, Munc13 (32). In contrast, we have now provided evidence that Munc18 is a key substrate for the regulation of one aspect of exocytosis by PKC. We have shown that it is phosphorylated on Ser-313 in response to phorbol ester in intact cells. In adrenal chromaffin cells, phorbol ester treatment has at least two effects that appear to be mediated by PKC. One is to increase the number of vesicles in the ready-releasable pool (4). The second is to modify release kinetics monitored at the level of single vesicles as described by us (8, 9) and more recently confirmed by others (51). We show here that phosphorylation of Munc18 is responsible for the regulation of exocytosis kinetics of dense-core granules seen in response to phorbol ester. It does not appear, however, to be linked to the increase in exocytotic events following phorbol ester treatment and so this must involve a distinct PKC substrate.

In using amperometry we have taken care to maximize the consistency of this assay by parallel analysis of transfected and untransfected cells in the same dishes and with the same carbon fibers. In addition, in parallel experiments we have confirmed our previously reported effects of phorbol esters on release kinetics. The robustness of our assay is demonstrated by the closeness of the control values for spike parameters from different batches of cells (Figs. 4 and 5) and by the reproducible effect of PMA treatment seen in two previous studies (8, 9) and again in the current work. In addition, the effect of the dGlu mutant of Munc18 was seen in two distinct series of experiments (Figs. 4 and 7), whereas the single Munc18 mutants had no detectable effect on any of the spike parameters. The similar effects on spike parameters of the dGlu and R39C (38) mutants of Munc18 is also notable and is consistent with both mutations reducing the affinity of Munc18 for syntaxin. Another study that examined the characteristics of amperometric spikes in chromaffin cells of the Munc18a-null mouse did not show any differences compared with control cells (52). Given the essential nature of Munc18a for neurotransmitter release (19) and Sec1 family members for other fusion steps (53), it is possible that the observed exocytotic events in the chromaffin cells from null mice were supported by low levels of other Munc18 isoforms.
Phorbol ester treatment was demonstrated to increase catecholamine release in cell population measurements on intact or permeabilized cells via activation of PKC (6, 46, 47, 54). Later work examining the rapid kinetics of exocytosis using patch clamp capacitance measurement revealed a specific increase in the size of the ready releasable pool leading to an increase in a fast component of the secretory response. This was seen as 2–3-fold increase in exocytosis in response to depolarization or flash photolysis of caged Ca2+ (4) over a subsecond time course. In other cases, the increase was more modest (around 50%) (55). We have characterized here two effects of phorbol ester, an increase in the number of exocytotic events and also changes in single vesicle release kinetics that result in a reduction in release per vesicle. Are these two effects compatible with the earlier data on cell populations? Phorbol ester treatment results in an overall increase in catecholamine release of about 40%, based on our data and that of others (6). Despite a 20% reduction in catecholamine released per spike, the overall increase in spike number by close to 70% due to PMA would be entirely compatible with an overall increase in catecholamine release due to PKC activation. We have shown, however, that the two effects of phorbol ester can be dissociated as the effect on release kinetics, but not the spike number, appears to involve PKC-mediated phosphorylation of Munc-18.

The kinetics of amperometric spikes are determined by multiple factors. During initial fusion pore formation little release of vesicle contents is detected, and the rising phase of the spike occurs as fusion pore expansion initiates (56, 57). This rising phase could be affected by the rate of fusion pore expansion but also by the rate of catecholamine dissociation from the granule core, diffusion to the carbon fiber, and consumption of the catecholamine by oxidation (44, 58). It is difficult to imagine how expression of a cytoplasmic protein such as a Munc18 mutant could modify diffusion and oxidation of catecholamine or even release from the granule core. As Munc-18 interacts directly with known components of the fusion machinery, we hypothesize that the increased rate of rise of the amperometric spikes due to expression of the dGlu Munc18 or PKC activation is likely to be a consequence of an increased rate of expansion of the fusion pore. This would be consistent with the direct demonstration that PKC activation increases the rate of fusion pore expansion in eosinophils (7). Similarly, the decrease in the amount and time of release per spike could be most easily be interpreted as being caused by faster re-closure of the expanded fusion pore, as it has been shown previously that even an expanded fusion pore can re-close abruptly (59, 60). We cannot, however, formally rule out other explanations for the data.

The importance of R39 Munc18 is predicted from the crystal structure of the Munc18-syntxin complex (35). In this structure Ser-313 and Ser-306 of Munc18 are close to a region of syntxin containing acidic residues (Asp-140 and Glu-143) (Fig. 7B), and it is likely that an acidic phosphate (or glutamate in the mutants) would disrupt the complex due to electrostatic repulsion. The increased rate of rise of the amperometric spikes could be explained by an increase in the efficiency of dissociation of Munc18 from syntxin allowing more rapid assembly of the SNARE complex and thereby driving fusion pore formation and more rapid pore expansion. Alternatively, this may be due to increased interaction of released Munc18 with other binding proteins (61). We hypothesize that the reduced half-width and decreased fall time of the spikes, suggests a second effect on accelerating vesicle retrieval by closure of the expanded fusion pore (in a kiss-and-run exocytosis, Refs. 62 and 63) and that this may have a distinct mechanistic explanation. We and others (8, 64) have obtained a variety of independent evidence that changes in amperometric spike parameters represent changes in membrane fusion dynamics and retrieval consistent with changes in the extent of kiss-and-run exocytosis. Rapid kiss-and-run exocytosis was originally proposed for sympathetic exocytosis (65) but first demonstrated for dense-core granule exocytosis (60). It was suggested to occur in hippocampal neurons (66) and its existence in a CNS synapse has recently been confirmed (67). Overall, the data point to Munc18 as a target for the modulation of neurotransmission through PKC-mediated phosphorylation that would allow both faster release kinetics and more rapid vesicle recycling. Changes in vesicle release kinetics have been suggested to cause the presynaptic changes that lead to long term depression in the hippocampus (68), and an increase in the kinetics of release have been linked to long term potentiation (69). Munc18 phosphorylation is therefore, a candidate mechanism for changes in synaptic efficacy underlying synaptic plasticity.
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