Complexin Regulates the Closure of the Fusion Pore during Regulated Vesicle Exocytosis*  

The SNAREs (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors) proteins associate to form a SNARE complex (1) that has a crucial role in membrane fusion events throughout the secretory pathway as the core of a highly conserved fusion machinery (2). The SNARE proteins syntaxin, SNAP-25, and vesicle-associated membrane protein (synaptobrevin), involved in regulated exocytosis in neurons and neuroendocrine cells, have been studied in most detail (3, 4). From in vitro studies, the neuronal SNAREs are sufficient for membrane fusion when reconstituted in liposomes (5), but this occurs in a Ca$^{2+}$-independent manner and with kinetics that are many orders of magnitude slower than exocytosis at the synapse suggesting an essential requirement for other proteins. A prime candidate for a protein that influences membrane fusion during regulated exocytosis is complexin (6, 7) as it binds specifically to the assembled SNARE complex (6, 8, 9). Regulated exocytosis differs significantly from other membrane fusion events. First, it is dependent upon an intracellular signal such as an elevated Ca$^{2+}$ concentration for its initiation (10). Second, in synapses it is specialized to be able to occur within tens of microseconds of Ca$^{2+}$ elevation (11) through the rapid formation of a transient fusion pore (12). Third, rapid retrieval of the fused membrane is essential to maintain the releasable vesicle pool (13). It is likely that proteins in addition to SNAREs are crucially important for the control and kinetics of fast, regulated exocytosis. Numerous proteins have been discovered that interact with the neuronal SNARE proteins, and some of these are not expressed in organisms such as yeast that lack regulated exocytosis. It is likely that these proteins either impose Ca$^{2+}$ sensitivity on the fusion machinery (e.g. synaptotagmin) or contribute to the fast kinetics of membrane fusion or retrieval during synaptic vesicle exocytosis. Complexin binds to the assembled SNARE complex competitively with α-SNAP (6). Knockout of either one of the two complexin isoforms, I and II, produces a mild (II) or moderate (I) phenotype (14, 15). In contrast, the double complexin I/II knockout mouse dies shortly after birth and shows a marked impairment of Ca$^{2+}$-evoked neurotransmission (15). The exact contribution of complexin to SNARE complex assembly is controversial (9, 16), but recent structural data suggest binding of a single complexin to each independent SNARE complex via association of the complexin C-terminal domain with residues from syntaxin and synaptobrevin (9, 17). Here we have investigated the function of complexin by analysis of single vesicle release events in adrenal chromaffin cells using amperometry and demonstrated a novel effect of complexin II on release kinetics that depends on its interaction with the SNARE complex. The data are consistent with a function for complexin in controlling the closure of the fusion pore to elicit rapid kiss-and-run recycling of the exocytosed vesicle.

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§ To whom correspondence should be addressed. Tel.: 44-151-794-5305; Fax: 44-151-794-5337; E-mail: burgoyne@liverpool.ac.uk.

The abbreviations used are: SNARE, soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors; SNAP, soluble N-ethylmaleimide-sensitive fusion protein; EGFP, enhanced green fluorescent protein; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid.

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bovine serum albumin, 0.5% Triton X-100 in PBS), and incubated overnight at 4°C with L668 rabbit polyclonal antibody against complexin II (provided by Dr. Harvey McMahon) at a 1:500 dilution. Cells were then washed three times, incubated for 1 h in biotinylated anti-rabbit IgG (Amersham Biosciences) at a 1:1000 dilution, washed three times, and finally incubated in streptavidin-Texas Red (Amersham Biosciences) at a 1:50 dilution for 30 min. After being mounted onto slides, cells were viewed with appropriate filters to visualize EGFP and immunofluorescence.

**Western Blotting**—HeLa cells were trypsinized, plated at a density of \(1 \times 10^5\) on 35-mm plates, and cultured in Dulbecco’s modified Eagle’s medium with 5% fetal bovine serum. After 5 h, the cells were transfected with 1 \(\mu\)g of plasmid (pCDNA3 or the plasmids encoding complexin II or complexin II(R59H)) using 3 \(\mu\)l of FuGENE transfection reagent (Roche Molecular Biochemicals). After an additional 72 h, the cells were lysed in 200 \(\mu\)l of SDS dissociation buffer. Chromaffin cells were plated onto 35-mm plates and lysed in 200 \(\mu\)l of SDS dissociation buffer after 3 days. Synaptosomes were provided by Gareth Evans (The Physiology Laboratory, University of Liverpool, UK). Samples were separated by SDS-PAGE, transferred to nitrocellulose, and probed with L668 rabbit polyclonal antibody against complexin II (1:1000), and bands were visualized using enhanced chemiluminescence (Amersham Biosciences).

**Amperometric Recording**—Cells were washed twice with PBS, incubated in bath buffer (139 mM potassium glutamate, 0.2 mM EGTA, 20 mM PIPES, 2 mM ATP, and 2 mM MglCl, pH 6.5) and viewed using a Nikon TE300 inverted microscope. Transfected cells were identified as those expressing EGFP. A 5-\(\mu\)m-diameter carbon fiber electrode was positioned in contact with a cell for stimulation. A glass micropipette filled with cell permeabilization/stimulation buffer (139 mM potassium glutamate, 20 mM PIPES, 5 mM EGTA, 2 mM ATP, 2 mM MglCl, 20 \(\mu\)M digitonin, and 10 \(\mu\)M free Ca\(^{2+}\), pH 6.5) was positioned on the opposite side of the cell from the carbon fiber. An Eppendorf Transjector was used to pressure eject the buffer onto the cell for a 20-s pulse. Amperometric responses were monitored with a VA-10 amplifier (NPI Electronic), collected at 4 kHz, and digitized with a Digidata 1322A acquisition system. Data were subsequently analyzed using an automated peak detection and analysis protocol with the technical graphics program Origin (Microcal) (19). All the data are shown as mean ± S.E., and statistical differences were assessed using the non-parametric Mann Whitney test.

**RESULTS**

Complexin I is expressed mainly in neurons, but complexin II is expressed more widely. We, therefore, examined whether complexin II is expressed in adrenal chromaffin cells by immunoblotting with anti-complexin II. A single band was detected in chromaffin cells, whereas an additional smaller band corresponding to complexin I was detected in synaptosomes. Complexin II was detected in chromaffin cells (even after prolonged exposure of the blot), whereas an additional smaller band corresponding to complexin I was detected in synaptosomes. B, overexpression of complexin II or complexin II(R59H) was observed in EGFP-expressing chromaffin cells after transfection using immunofluorescence with anti-complexin II at levels of antisera that gave only background staining of untransfected cells (arrow). C, comparison of expression levels of complexin II and complexin II(R59H) in transfected HeLa cells by immunoblotting. **CPXII**, complexin II.

The effect of overexpression of complexin II on the extent of exocytosis and the characteristics and kinetics of single vesicle release events was examined using carbon fiber amperometry (20). In each case data from transfected cells were directly compared with data from control, untransfected cells in the same dishes and using the same carbon fibers (28–38 cells for each condition). Complexin II overexpression partially inhibited exocytosis and also reduced the average charge released in each spike (Fig. 2) showing that less catecholamine was released during each exocytotic event (a reduction in quantal size). To determine whether these effects were due to interaction of complexin II with the SNARE complex or some nonspecific effect we examined the consequence of expressing the specific R59H mutant of complexin II. The specificity of the complexin II effect on exocytosis was demonstrated by the finding that complexin II(R59H) had no effect on the extent of exocytosis or on quantal size in transfected cells (Fig. 3).

The characteristics of the amperometric spikes in chromaffin cells overexpressing complexin II or the R59H mutant were examined in more detail. Overexpression of complexin II modified the shape of the amperometric spikes resulting in significant decreases in height, half-width, rise time to peak, and fall time (Fig. 4A). The effect of complexin II on the frequency distribution of events is shown for half-width, which reveals a shift to a population with narrower half-widths (Fig. 4B). In contrast, expression of complexin II(R59H) had no effect on any of the spike parameters examined. Similar reductions in half-width and rise time to those seen in complexin II-overexpressing cells have been seen in chromaffin cells expressing the Munc18(R39C) mutant that has reduced affinity for syntaxin (21) and also following phorbol ester treatment (19). In both cases these modifications were not accompanied by changes in spike height. As a consequence, spikes in cells expressing Munc18(R39C) or treated with phorbol 12-myristate 13-acetate showed a significant increase in the rate of rise of the spikes due to the decreased rise time (Fig. 4C). In contrast, this parameter was unaffected by overexpression of complexin II or the complexin II(R59H) mutant. These data suggest that complexin II does not modulate the initial rate of the release events, but the reduced half-width and charge suggest that it leads instead to premature termination of the release event. The shape of the resulting amperometric spikes demonstrates this point. The changes in amperometric spikes (Fig. 4D) were distinct from those in cells in which catecholamine was depleted as these showed both a reduction in height and in the initial rate of rise of the spikes (21).

**DISCUSSION**

Our data show that overexpression of complexin II in chromaffin cells has two effects, an inhibition of the number of
exocytic events and also changes in the kinetics of single vesicle release events consistent with kiss-and-run exocytosis (22). In the crystal structure of the complexin-SNARE complex, Arg-59 of complexin makes a crucial interaction between complexin and the SNAREs (17). The reduced length of the histidine side chain compared with arginine in the R59H mutant we tested would prevent the formation of the salt bridge observed between this residue and Asp-57 of synaptobrevin. Mutation of this residue prevented the effects of complexin overexpression on both the extent and the characteristics of the release events showing that these effects were dependent on interaction of complexin II with the SNARE complex. The interaction of complexin with Asp-57 is close to the conserved zero layer residues of the SNARE complex. Mutation of the zero layer residues does not, however, have any effect on the kinetics of exocytosis (23, 24). The inhibitory effect of complexin II on spike number is consistent with previous data showing reduced exocytosis in transfected PC12 cells overexpressing complexin I or II (25) or following acute microinjection of complexin II into Aplysia neurons (26). This inhibitory effect was ascribed to competitive inhibition by complexin of α-SNAP association with cis-SNARE complexes as it was antagonized by co-injection of α-SNAP. The effect we observed here on the kinetics of release events cannot be explained by competition with α-SNAP as we have shown that expression of a dominant negative α-SNAP mutant inhibited exocytosis in chromaffin cells but did not modify spike kinetics (27). Similarly spike kinetics were not modified by a reduction in SNARE availability in cells transfected to express clostridial neurotoxins (19).

We and others have previously demonstrated that the kinetics of catecholamine release and extent of release can be modified at the level of a single vesicle (21, 27–31). We have interpreted these data as reflecting changes in the kinetics of fusion pore expansion and fusion pore open time (32). In support of the latter aspect we have found that spike half-width and quantal size are increased by disruption of dynamin function (33) indicating that the release time course is sensitive to disruption of dynamin-dependent vesicle retrieval. The effect of complexin II overexpression on spike parameters was to some extent similar to that seen in cells expressing the Munc18(R39C) mutant or after treatment with phorbol ester (19, 21) as in all cases spike half-width and quantal size were reduced. In contrast, however, the initial rate of spike rise was increased by Munc18(R39C) and phorbol ester but not by complexin II overexpression. These data suggest that complexin II was unusual
in that it did not modify the rate of fusion pore expansion, but instead its major effect was to limit the time over which release occurred. This is consistent with complexin inducing earlier fusion pore closure to give rise to kiss-and-run exocytosis. Such a specific effect on vesicle release events has not previously been described under any other conditions.

Biological analysis of complexin has been limited to examination of its interaction with assembled cis-SNARE complexes (as would occur within the same membrane) (6, 8, 9, 17). Membrane fusion is mediated by trans-SNARE complexes that bridge two membranes. Various studies, for example using clostridial neurotoxins, have indicated the existence in cells of multiple trans-SNARE complexes assembled to differing extents prior to exocytosis (34, 35). One possible role for complexins would be to stabilize a trans-SNARE complex intermediate that would be able to allow rapid fusion pore closure after exocytosis. The rapid binding kinetics of complexins to assembled SNAREs suggests that this interaction could occur during or following membrane fusion. This suggested role for complexins in favoring kiss-and-run exocytosis could be consistent with the phenotype of complexin I/II knockout mice that show a loss of fast synchronous neurotransmission (15). This is maintained or following membrane fusion. Other functional aspects of complexin interaction with the SNARE complex cannot be ruled out, however. The ability of complexins to regulate both the extent of exocytosis and nature of vesicle recycling would make it a likely target for mechanisms regulating synaptic plasticity.

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