N-Ethylmaleimide-sensitive Factor Acts at a Prefusion ATP-dependent Step in Ca\(^{2+}\)-activated Exocytosis*

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An ATP-dependent activity of NSF (N-ethylmaleimide-sensitive factor) that rearranges soluble NSF attachment protein (SNAP) receptor (SNARE) protein complexes was proposed to be the driving force for membrane fusion. The Ca\(^{2+}\)-activated fusion of secretory vesicles with the plasma membrane in permeable PC12 cells requires ATP; however, the ATP requirement is for a priming step that precedes the Ca\(^{2+}\)-triggered fusion reaction. While phosphoinositide phosphorylation is a key reaction required for priming, additional ATP-dependent reactions are also necessary. Here we report that the NSF-catalyzed rearrangement of SNARE protein complexes occurs during ATP-dependent priming. NSF with α-SNAP (soluble NSF attachment protein) were required for ATP-dependent priming but not Ca\(^{2+}\)-triggered fusion, indicating that NSF acts at an ATP-dependent priming step rather than at fusion itself. NSF-catalyzed activation of SNARE proteins may reorganize membranes to generate a vesicle-plasma membrane fusion intermediate that is poised for full fusion by Ca\(^{2+}\)-dependent mechanisms.

The regulated fusion of vesicles with the plasma membrane in neural and endocrine cells requires a core complex of proteins (synaptobrevin, syntaxin, and SNAP-25) that are specific substrates for cloridrial neurotoxin proteases (1–4). This complex is proposed to function in vesicle targeting, docking or fusion. Identification of these neuronal synaptic proteins (termed SNAREs) as receptors for SNAP proteins that mediate the membrane association of NSF, a protein required for constitutive membrane fusion (1), suggested that NSF may be required for Ca\(^{2+}\)-regulated neurosecretion (5). Genetic studies in Drosophila have established an essential role for NSF in neural function (6). Stimulatory effects of α-SNAP on neurotransmitter secretion from chromaffin cells and squid neurons have been reported (7, 8). However, the precise stage in the regulated secretory pathway at which NSF acts has not been directly established. In vitro biochemical studies demonstrated that a 20 S complex of SNAREs, NSF, and α/β-SNAP was disassembled by the ATP-dependent activity of NSF, and it was suggested that NSF-catalyzed SNARE protein rearrangements drive membrane fusion (9). However, previous studies with permeable PC12 and adrenal cells had shown that MgATP was required for a priming step that precedes the final fusion steps triggered by Ca\(^{2+}\) (10, 11). In the present studies, the execution point of NSF and α-SNAP was established as the ATP-dependent priming step that precedes Ca\(^{2+}\)-activated fusion.

EXPERIMENTAL PROCEDURES
Preparation of Permeable PC12 Cells and Secretion Assays—PC12 cells were labeled with [\(^{3}\)H]norepinephrine (NE; Amersham Corp.) and permeabilized with a ball homogenizer (10, 12). Two stage secretion assays were in KGlubuffer (20 mM Hepes, pH 7.2, 120 mM potassium glutamate, 20 mM potassium acetate, 2 mM EGTA) with 0.1% bovine serum albumin. Thirty-min incubations at 30 °C contained 2 mM MgATP and 1.0 mg/ml rat brain cytosol, which provides PEP proteins (13, 14). Permeable cells were recovered by centrifugation, washed, and used for 1-min triggering incubations at 30 °C, which contained Ca\(^{2+}\) adjusting to 10 μM and 0.5 mg/ml rat brain cytosol, which provides the CAPS protein required for triggering (12). Recombinant His\(_5\)-NSF and His\(_5\)-αSNAP were produced in Escherichia coli using vectors kindly provided by R. H. Scheller (15), were purified >80% using Ni\(^{2+}\)-agarose beads (Qiagen) and dialyzed in KGlubuffer supplemented with 1 mg/ml MgCl\(_2\) and 0.5 mg/ml ATP.

Immunosolubilization of SNARE Protein Complexes—Permeable cells were incubated with rat brain cytosol for 30 min with or without MgATP; ATP-primed cells were further incubated in triggering reactions with Ca\(^{2+}\) where indicated. Cells recovered by centrifugation were solubilized in lysis buffer (20 mM Hepes, pH 7.2, 100 mM KC1, 2 mM EDTA, 0.5 mM ATP, 1 mM diithiothreitol, 1% Triton X-100, and 0.4 mM phenylmethylsulfonyl fluoride) and lysates were clarified at 150,000 × g for 90 min. Immunoprecipitations were conducted on supernatants with SNAP-25 (Sterneberger Monoclonals Inc.) and syntaxin (Sigma, HPC-1) monoclonal antibodies conjugated to protein G-agarose beads (Qiagen) and dialyzed in KGlubuffer supplemented with 1 mg/ml MgCl\(_2\) and 0.5 mg/ml ATP.

Electron Microscopy—2% glutaraldehyde-fixed semi-intact PC12 cells were post-fixed in 2% OsO\(_4\), embedded in Durcupan ACM (Fluka Chemical Corp.), sectioned, stained with uranyl acetate and lead citrate, and examined with a Hitachi H-600 microscope.

RESULTS AND DISCUSSION
Mechanical permeabilization of PC12 cells generates semi-intact cells that are highly permeable, allowing access to the exocytotic apparatus (12). Most of the large dense core vesicles (LDCVs) in intact PC12 cells are close to the plasma membrane in a docked state (17). LDCVs remain docked in the permeable cells at the plasma membrane (Fig. 1). Hence, permeable PC12 cells provide a means to analyze late post-docking steps in the LDCV exocytotic pathway. Ca\(^{2+}\)-regulated NE secretion via exocytosis of LDCVs is reconstructed in the permeable cells by the presence of MgATP and cytosolic proteins (12). A two-stage protocol identified an ATP-dependent priming step that precedes and is
required for Ca\textsuperscript{2+}-activated NE secretion (10). Priming preincubations with MgATP and cytosolic proteins fulfill the ATP requirement for Ca\textsuperscript{2+}-regulated exocytosis, whereas ATP\textgreek{S} fails to substitute and reversibly inhibits priming by competition with ATP (Fig. 2a, left). Incubation of extensively washed ATP-primed permeable cells with 10 μM Ca\textsuperscript{2+} plus 0.5 mg/ml dialyzed cytosol with either no MgATP, 2 mM MgATP, 2 mM MgATP, or no MgATP plus hexokinase with glucose. Panel b, NSF and α-SNAP are required for priming in NSF/SNAP-depleted cells. Permeable cells were incubated for 30 min with MgATP, washed extensively, and incubated for 30 min without MgATP. Following an additional wash, priming incubations were conducted with 2 mM MgATP, 50 μg/ml NSF, 50 μg/ml α-SNAP, and 1 mg/ml cytosol as indicated. Histograms show differences in NE release between unprimed control samples (−MgATP) and samples primed under the indicated conditions. Means of duplicate determinations with S.D. are indicated.

We sought to correlate functional stages of priming and triggering with biochemical changes in the permeable cells. The Ca\textsuperscript{2+}-dependent release of ~60% of the stored NE from permeable cells under optimal incubation conditions (12) indicated that the majority of LDCVs, probably the docked LDCVs, participate in exocytotic fusion reactions. Immunoblotting showed that a full cellular complement of the vesicle SNARE (synaptobrevin) and the plasma membrane SNAPERs (syntaxin and SNAP-25) was preserved following cell permeabilization. To assess the state of vesicle docking, the association of vesicle proteins (synaptotagmin, synaptobrevin) with plasma membrane proteins (syntaxin, SNAP-25; <1% on purified LDCVs) was determined by immunoprecipitation with syntaxin and SNAP-25 antibodies (Fig. 3a). Immune complexes isolated from detergent extracts of permeable cells prior to priming contained a significant percentage (30–40% and 10–20%, respectively) of the cellular synaptotagmin and synaptobrevin, consistent with the presence of docked vesicles prior to ATP-dependent priming. Immunoprecipitates also contained α/β-SNAP (Fig. 3a) and NSF (Fig. 3b). Hence, complexes prior to priming resembled a recently described “docking and fusion particle” (18). While SNARE-containing complexes can form readily in concentrated brain extracts following detergent extraction (16), the complexes detected in the dilute detergent extracts of PC12 cells do not represent a post-extraction artifact since they vary in composition with cell incubation conditions, are unaffected by the volume of lysis buffer used, and are observed upon rapid SDS quenching (see below).

When permeable cells were incubated under priming conditions with MgATP, significant changes in SNARE protein complexes were observed consisting of decreases in both synaptotagmin and synaptobrevin (Fig. 3, a and c). In contrast, the association of syntaxin with SNAP-25 was maintained. Extensive decreases of NSF (Fig. 3b) and α/β-SNAP (Fig. 3a) in the
SNARE complex rearrangements during priming since the reaction was inhibited by NEM and ATPγS (Fig. 4c), agents that block NSF activity (9, 5, 21), and inhibition by NEM was overcome by inclusion of recombinant NSF and α-SNAP (Fig. 4c). Neither protein alone was effective (not shown). Following ATP-dependent priming, a portion (40–70%) of the NSF and SNAP proteins present in permeable cells was released to the soluble fraction of the incubations. This release of NSF and SNAP from permeable cells depended upon MgATP and was inhibited by ATPγS and NEM (Fig. 4d), consistent with recycling of these proteins following ATP hydrolysis (5, 9). No additional release of NSF and SNAP (Fig. 4d) was detected following Ca2+-triggering of LDCV fusion in ATP-primed cells, suggesting that NSF action was restricted to priming. This was also indicated by the lack of significant further changes in SNARE protein complexes during Ca2+-triggering (Fig. 3a).

25–30% of NSF and SNAP is retained by cells following permeabilization and washing. This appears to be adequate to sustain late stages of the LDCV exocytotic pathway, since addition of recombinant NSF and α-SNAP did not stimulate ATP-dependent, Ca2+-activated NE secretion (not shown). While secretion is NEM-sensitive, inhibition by NEM was not overcome by NSF (and/or SNAP) addition presumably because other NEM-sensitive proteins are required. The further release of NSF and SNAP from permeable cells during ATP-dependent priming (see Fig. 4d), however, suggested that it may be feasible to render these proteins rate-limiting for regulated NE secretion. Priming is reversed in incubations that lack ATP and multiple cycles of priming, and its reversal can be sustained in sequential incubations (10). Hence, the permeable cells were incubated under various conditions to determine the requirements for optimal priming. Priming in these pretreated permeable cells was entirely MgATP-dependent as anticipated (Fig. 2b). However, priming was now also stimulated by a combination of NSF and α-SNAP, whereas neither protein alone was effective. Cytosol, which lacks active NSF (22), also stimulated priming as anticipated due to its content of PEP proteins (10, 13, 14). Maximal priming was observed by addition of both NSF/SNAP and cytosolic PEP proteins, indicating that both polyphosphoinositide synthesis (14) and NSF-catalyzed reactions contribute to the MgATP-dependent priming of exocytosis. The effects of NSF and α-SNAP on secretion were restricted to priming, and no stimulation by NSF and/or α-SNAP was evident in Ca2+-dependent triggering reactions (not shown).

The discovery that NSF and SNAP proteins interact with a core complex of neuronal synaptic SNARE proteins suggested a role for NSF in regulated exocytosis (5), but the precise point of action of NSF in the regulated secretory pathway has not previously been established. In vitro biochemical studies demonstrated that a 20 S complex of SNAREs, NSF, and α/β-SNAP was disassembled by the ATP-dependent activity of NSF (21), and it was suggested that NSF-catalyzed protein rearrangements drive membrane fusion (9). This model implied that the fusion step in regulated exocytosis is ATP-dependent and follows a Ca2+-dependent step (9). In contrast, our studies support an alternative model (23, 24) that the execution point of NSF action was restricted to priming, and no stimulation by NSF and/or α-SNAP was evident in Ca2+-dependent triggering reactions (not shown).

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prefusion intermediate possibly similar to that suggested for viral fusion mechanisms (25). NSF could catalyze an ATP-dependent conformational change in SNARE proteins (9, 20, 26) that alters the organization of the cytoplasmic leaflets of vesicle and plasma membrane bilayers. In constitutive fusion events, this intermediate may spontaneously resolve to full fusion. In regulated exocytosis, this intermediate would be arrested and require Ca\(^{2+}\) to allow progression to full fusion. Physical evidence for a prefusion intermediate should be sought.

REFERENCES

1. Rothman, J. E. (1994) Nature 372, 55–63
2. Jahn, R., and Sudhof, T. C. (1994) Annu. Rev. Neurosci. 17, 219–246
3. Bajjalieh, S. M., and Scheller, R. H. (1995) J. Biol. Chem. 270, 1971–1974
4. Martin, T. F. J. (1994) Curr. Opin. Neurobiol. 4, 626–632
5. Solnner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J. E. (1993) Nature 362, 318–324
6. Pallanck, L., Ordway, R. W., and Ganez, B. (1995) Nature 376, 25
7. Morgan, A., and Burgoyne, R. D. (1995) EMBO J. 14, 232–239
8. DeBello, W. M., O'Connor, V., Dresbach, T., Whiteheart, S. W., Wang, S.-H., Schweizer, F. E., Betz, H., Rothman, J. E., and Augustine, G. J. (1995) Nature 373, 626–630
9. Solin, T., Bennett, M. K., Whiteheart, S. W., Scheller, R. H., and Rothman, J. E. (1993) Cell 75, 409–418
10. Hay, J. C., and Martin, T. F. J. (1992) J. Cell Biol. 119, 139–151
11. Holz, R. W., Bittner, M. A., Peppers, S. C., Senter, R. A., and Eberhard, D. A. (1989) J. Biol. Chem. 264, 5412–5419
12. Walent, J. H., Porter, B. W., and Martin, T. F. J. (1992) Cell 70, 765–775
13. Hay, J. C., and Martin, T. F. J. (1993) Nature 366, 572–575
14. Hay, J. C., and Martin, T. F. J. (1993) Nature 366, 572–575
15. Kee, Y., Lin, R. C., Hsu, S.-C., and Scheller, R. H. (1995) Neuron 14, 991–998
16. Hayashi, T., McMahon, H., Yamazaki, S., Binz, T., Hata, Y., Sudhof, T. C., and Niemann, H. (1994) EMBO J. 13, 5051–5061
17. Schafer, T., Karl, U. O., Schweizer, F. E., and Burger, M. M. (1987) Biosci. Rep. 7, 269–279
18. Schlavo, G., Gmachi, M. J. S., Stenbeck, G., Solnner, T. H., and Rothman, J. E. (1996) Nature 378, 735–736
19. Hayashi, T., Yamazaki, S., Nauenburg, S., Binz, T., and Niemann, H. (1995) EMBO J. 14, 2317–2325
20. Hanson, P. I., Otto, H., Barton, N., and Jahn, R. (1995) J. Biol. Chem. 270, 16955–16961
21. Morgan, A., Dimatteo, R., and Burgoyne, R. D. (1994) J. Biol. Chem. 269, 29347–29350
22. Block, M. R., Glick, B. S., Wilcox, C. A., Wieland, F. T., and Rothman, J. E. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7852–7856
23. O'Connor, V., Augustine, G. J., and Betz, H. (1994) Cell 76, 785–787
24. Sudhof, T. C. (1995) Nature 375, 645–653
25. Kemble, G. W., Danielli, T., and White, J. M. (1994) Cell 76, 383–391
26. Morgan, A., and Burgoyne, R. D. (1995) Trends Cell Biol. 5, 335–339