Phosphorylation of the N-ethylmaleimide Sensitive Factor (NSF) is Associated with Depolarization-Dependent Neurotransmitter Release from Synaptosomes

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Summary:

Critical to SNARE protein function in neurotransmission are the accessory proteins, SNAP and NSF, which play a role in activation of the SNAREs for membrane fusion. In this report, we demonstrate the depolarization-induced, calcium-dependent phosphorylation of NSF in rat synaptosomes. Phosphorylation of NSF is coincident with neurotransmitter release and requires an influx of external calcium. Phospho-amino acid analysis of the radiolabeled NSF indicates a role for a serine/threonine specific kinase. Synaptosomal phosphorylation of NSF is stimulated by phorbol esters and is inhibited by staurosporine, chelerythrine, bisindolylmaleimide I, calphostin C, and Ro31-8220, but not the CaM kinase II inhibitor, Kn-93, suggesting a role for protein kinase C (PKC). Indeed, NSF is phosphorylated by PKC *in vitro* at Ser-237 of the catalytic D1 domain. Mutation of this residue to Glutamic acid or to Alanine eliminates *in vitro* phosphorylation. Molecular modeling studies suggest that Ser-237 is adjacent to an inter-subunit interface at a position where its phosphorylation could affect NSF activity. Consistently, mutation of Ser-237 to Glu, to mimic phosphorylation, results in a hexameric form of NSF that does not bind to SNAP/SNARE complexes, while the S237A mutant does form complex. These data suggest a negative regulatory role for PKC phosphorylation of NSF. (200 words)
Introduction:

The molecular mechanisms of neurotransmitter (NT)\(^1\) release have been the subject of much attention in recent years resulting in an evolving model known as the SNARE hypothesis (1-3). The SNARE hypothesis holds that membrane proteins in the vesicle (v-SNAREs, e.g. synaptobrevins) bind to a heterodimer in the target membrane (t-SNAREs, heterodimers of syntaxins and SNAP-25-like proteins). V- and t-SNAREs bind to form a 7S complex (1,4), composed of a bundle of four parallel, coiled-coil domains (5-8) which, through reconstitution studies, has been demonstrated to be minimally required for bilayer fusion (9). The N-ethylmaleimide Sensitive Factor (NSF) and the Soluble NSF Attachment Proteins (SNAPs, not to be confused with SNAP-25) affect the composition and structure of the SNARE complex. SNAPs act as adapters and are required for binding of NSF to the 7S complex. ATP hydrolysis by NSF causes the resulting 20S complex to disassemble into monomeric SNAREs (4,10), a step required for vesicle trafficking (11-13). Detailed kinetic experiments have placed one role of NSF and SNAPs at steps prior to v-/t-SNARE binding (14-16), suggesting a model in which NSF disassembles cis 7S complexes that exist in the same bilayer (17-19). The resulting monomeric SNAREs can then form trans 7S complexes that span the opposing bilayers of the vesicle and target membrane. In this manner, NSF acts as a chaperone to activate or “prime” the SNARE proteins for subsequent trans complex formation and membrane fusion.

Studies by Schweizer et al. (20), using NSF-derived peptides microinjected presynaptically, showed that NSF affects the efficiency and kinetics of neurotransmission in the squid giant axon. Studies of the comatose mutant in Drosophila indicate that active NSF is not required for initial neurotransmitter release but is instead required for sustained release upon
subsequent stimulations (21-23). These data are consistent with a role for NSF in maintaining a “ready-release” pool of synaptic vesicles, which is competent for fast response to calcium influx.

Consistently, SNAPs, when injected into crayfish axons, increase the probability of neurotransmitter release but not the rate (24). On a molecular level, these data support the model that NSF functions prior to neurotransmitter release to activate SNAREs for membrane fusion and perhaps after fusion to recycle spent SNAREs (25).

NSF is a homo-hexameric ATPase whose subunits can be divided into three domains (N, D1 and D2) (11,26,27). The N-terminal domain is required for binding to the SNAP/SNARE complex. ATP binding and hydrolysis by D1 is essential for NSF activity. Mutants in the D1 domain that fail to bind ATP also fail to interact with SNAP/SNARE complex (27). D1 mutants that fail to hydrolyze ATP are dominant negative inhibitors because they fail to disassemble 7S SNARE complexes (11,27,28). The D2 domain is required for hexamerization since its deletion yields a monomer (11). Though this domain has high affinity for ATP and low ATPase activity, neither function is essential for NSF activity (11,29). In a current model of NSF structure, the hexameric D2 domain anchors the catalytically active D1 domains and SNAP/SNARE-binding N domains.

Much work has focused on the role of phosphorylation by protein kinase C (PKC) in the control of neurotransmission (reviewed in (30)). In particular, it has clearly been shown that activation of PKC by phorbol esters can lead to an enhancement of L-glutamate release (31-34).

Several studies indicate that PKC translocation from cytosol to membrane is associated with both PKC activation and enhanced L-glutamate release (32,35,36). Moreover, phosphorylation of PKC substrates that are selectively localized in nerve terminals, such as B-50/GAP-43 and
myristoylated alanine-rich PKC substrate (MARCKS), has been related to neurotransmitter release (37-39). Among candidate protein targets for PKC that have received scrutiny are those involved in the docking/fusion process. In one such study, activation of PKC by phorbol esters induced phosphorylation of SNAP-25 and increased depolarization-dependent norepinephrine release from PC12 cells (40). That study and others (reviewed in (30)) have demonstrated a potential role for PKC in neuroendocrine secretion but have yielded little molecular insight into the precise effects of phosphorylation. Several studies have attempted to fill this gap by focusing on the components of the membrane fusion machinery (e.g. SNARE, SNAPs and NSF). Those studies have demonstrated that various machinery elements can be phosphorylated in vitro with purified kinases (e.g. PKA, PKC, CaMKII) and that the phosphorylation does affect their protein-protein interactions (30).

In the present study, we provide the first demonstration of depolarization-induced, calcium-dependent phosphorylation of NSF in rat synaptosomes. Staurosporine, chelerythrine, bisindolylmaleimide I, calphostin C, and Ro31-8220, but not Kn-93, inhibit phosphorylation of NSF. Conversely, phorbol 12-myristate 13-acetate (PMA) treatment enhances phosphorylation, thus pointing to a role for PKC. In vitro, NSF is specifically phosphorylated on Ser-237 by PKC. Mutation of Ser-237 to Glutamic acid or Alanine yields a form of NSF that cannot be phosphorylated by PKC, however only the S237E mutant fails to bind SNAP/SNARE complexes. From a regulatory standpoint, control of NSF activity via phosphorylation offers a unique mode to modulate trafficking fluxes.

**Materials and Methods:**

**Materials.** ATP and AMP-PNP (adenosine 5’-(β, γ-imido)-triphosphate) were obtained from
Boehringer Mannheim Biochemicals (Indianapolis, IN). $[^{32}\text{P}]\text{ATP}$ (4,500 Ci/m mole), and $[^{32}\text{P}]\text{PO}_4^{2+}$ (7,000 Ci/m mole) were from ICN (Costa Mesa, CA). PKC from rat brain (mix of $\alpha$, $\beta$, and $\gamma$ isoforms) was from Promega (Madison, WI) and CaM Kinase II, also from rat brain, was from Calbiochem (San Diego, CA). L-$\alpha$-phosphatidyl-L-serine, diolein, and chelerythrine were obtained from Sigma (St. Louis, MO); staurosporine, bisindolylmaleimide I, calphostin C, Ro31-8220, Kn-93 and the inactive isomer Kn-92 were from Calbiochem. All inhibitors and activators were suspended in DMSO. Nitrocellulose (0.2 $\mu$m) and PVDF membranes for immunoblotting were from Schleicher & Schuell (Keene, NH) and Waters (Milford, MA) respectively. Horseradish peroxidase conjugated, anti-immunoglobulin secondary antibodies were from Sigma. Polyethyleneimine cellulose plates for thin layer chromatography were from Selecto Scientific (Norcross, GA). Glutathione immobilized on cross-linked 4% beaded agarose was from Sigma. Calmodulin was purified from bovine testis as described previously (41). All chemicals were reagent grade.

Wild type NSF and NSF mutants were produced as recombinant proteins in \textit{E. coli} and purified as described (27). Production of His$_6$-free NSF was accomplished using the pPROExHt expression system and the TEV protease (Life Technologies, Rockville, MD). Site directed mutagenesis was accomplished using the QuickChange kit according to manufacturer’s instructions (Stratagene, La Jolla CA) and mutations were confirmed by dideoxy nucleotide sequencing. His$_6$-$\alpha$-SNAP, His$_6$-$\gamma$-SNAP, and GST-syntaxin 1 (cytosolic domain 1-265 a.a.) were produced as recombinant proteins in \textit{E. coli} and purified as described (7,27). Protein concentrations were measured with the BioRad protein assay reagent (BioRad, Hercules CA) using ovalbumin as a standard. The anti-NSF monoclonal (2E5) and polyclonal antibodies were
described previously (11,27). The anti-α-SNAP antibody was from Gamma One Laboratories (Lexington, KY).

**Bath Experiments with Synaptosomes.** An *in situ* preparation of Percoll-gradient purified rat cerebral cortical synaptosomes (42) was used to measure both K⁺-evoked, Ca²⁺-dependent NSF phosphorylation and NT release. The composition of all buffers has been described (43). Synaptosomes were resuspended in Krebs/sucrose buffer without phosphate and incubated with ³²Pi (7,000 Ci/m mole) for 30 min at 37° under 95%O₂, 5%CO₂. ³²Pi-loaded synaptosomes were then placed in Krebs/Ringer bicarbonate buffer with either 1.5 mM Ca²⁺ or 10 mM Mg²⁺. Samples were depolarized by addition of K⁺ (25 mM final concentration) and the process was stopped at 30 sec by the addition of SDS/ortho-vanadate (8). L-glutamate release was measured in parallel samples using an enzyme coupled fluorometric assay (44,45). Synaptosomes were depolarized on addition of 25 mM KCl to an incubation mixture containing NADP (1 mM), glutamate dehydrogenase (50 U/ml), and either CaCl₂ (1.5 mM) or MgCl₂ (10 mM). NADPH fluorescence was monitored using excitation and emission wavelengths of 340 and 460 nm respectively. Data were accumulated at 1 sec intervals using a Perkin-Elmer LS5B spectrofluorometer fitted with stirred, thermostatted cuvettes at 37°.

**Immunoprecipitation of NSF.** Prior to immunoprecipitation (IP), Triton X-100 was added to the solubilized samples to neutralize the SDS. Samples were then diluted in IP buffer (10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.2 mM sodium o-vanadate, 0.2 mM phenylmethyl-sulfonyl fluoride) and Protein G Sepharose beads with covalently coupled anti-NSF antibody were added (46). NSF was immunoprecipitated by incubation for 1 h at 4°C. The beads were harvested and washed 5 times in 0.5 mL of IP buffer; the protein-
antibody complexes were eluted with SDS-PAGE sample buffer and analyzed by SDS-PAGE and immuno-blotting.

**NSF Phosphorylation by PKC or CaM Kinase II in vitro.** Recombinant NSF or NSF mutants (6 µg) were treated for 1 h at 25°C with PKC (0.5 mU) or CaM Kinase II (0.05 mU) and 1 mM ATP plus 1 µl [γ-32P] ATP (4,500 Ci/m mole) in PKC buffer (20 mM HEPES, pH 7.4, 100 mM KCl, 10 mM MgCl2 1 mM CaCl2, 1 mM DTT, 200 µg/ml L-α-phosphatidyl-L-serine and 10 µg/ml diolein) or CaM Kinase II buffer (50 mM Tris/HCl, pH 7.6, 1 mM DTT, 10 mM MgCl2, 1 mM CaCl2, 100 mM KCl, 1 μM calmodulin) in a total reaction volume of 40 µL. Phosphorylation reactions were terminated by addition of 1% SDS, 1% 2-mercaptoethanol and boiling for 5 min. The radiolabeled proteins were resolved by SDS-PAGE and the gels were stained with 0.02% Coomassie Brilliant Blue R-250, 40% methanol and 10% acetic acid, washed overnight, and dried. The levels of phosphorylation were assessed by analysis with a Storm Phosphorimager (Molecular Dynamics, Sunnyvale, CA) with ImagQuant software.

**Characterization of Phosphorylated NSF.** IP samples or in vitro phosphorylated NSF were resolved by SDS-PAGE and transferred to PVDF (47). The position of NSF was detected by enhanced chemifluorescence with the anti-NSF antibodies noted above using the Attophos detection system (Amersham Pharmacia Biotech, Piscataway, NJ). 32P-labeling and chemifluorescence immunodetection were quantified using phosphorimager analysis as noted above.

Radiolabeled NSF samples from in situ and in vitro labeling experiments were recovered from the PVDF membranes after western blotting. Phosphoamino acid analysis was performed by 2D electrophoresis after acid hydrolysis of the PVDF-blotted material as described by Duclos
et al. (48). Phosphopeptide analysis was done on NSF that had been phosphorylated with PKC in vitro (see above). After incubation, the radiolabeled NSF was recovered by precipitation with TCA and the pellets were washed extensively to remove unincorporated label. The protein was denatured in 4 M urea, 4 M guanidine HCl, 7 mM DTT and 100 mM Tris-HCl, pH 8.5 then cysteine-thiols derivatized by addition of 2 M acrylamide for 2 h at 37°C. The protein was desalted by acetone precipitation and digested overnight at 37°C in 80 mM Tris-HCl, pH 8.5, 1% (v/v) hydrogenated Triton X-100 (Calbiochem, La Jolla, CA) and 10% (v/v) acetonitrile with either 1 µg of trypsin (Promega, Madison, WI) or 0.1 µg of endoproteinase Lys C (Roche Molecular Biochemicals, Indianapolis, IN). The digestion reactions were acidified with TFA and the peptides were fractionated by C18 reverse phase HPLC and monitored by both UV absorbance and liquid scintillation counting. Radiolabeled peptides, thus purified, were covalently attached to acrylamide-derivatized discs (Sequelon-AA discs, Millipore Corp., Bedford, MA) and subjected to chemical sequencing in an Applied Biosystems 494 Peptide/Protein sequencer (Foster City, CA). A portion (40%) from each cycle was analyzed directly by online HPLC to identify and quantify released PTH derivatized amino acids. The remainder was collected and monitored for $^{32}$P by Cherenkov counting using the preset $^3$H windows.

SNAP/SNARE Complex Binding Assay. The complex formation procedure was modified from our previously described method (29). GST-syntaxin 1 (cytosolic domain) was incubated with pre-swollen, glutathione-agarose beads (100 µg protein per 100 µl beads) at 4°C in phosphate-buffered saline with 0.01% (v/v) Tween 20, 0.1% (v/v) β-mercaptoethanol and 2 mM EDTA. After 1 h of incubation, the beads were washed four times in the same buffer, and then equal
volumes of the beads were aliquotted into the reaction tubes. Particle formation reactions were performed in a final volume of 500 µl containing 15 µl of beads with GST-syntaxin 1 in binding buffer [20 mM HEPES/KOH, pH 7.4, 250 mM imidazole, 150 mM potassium acetate, 5 mM EGTA, 1 mM AMP-PNP, 5 mM MgCl2, 1% (w/v) glycerol, 1% (w/v) Triton X-100 and 10% (w/v) ovalbumin] and saturating amounts of α-SNAP and wild-type or mutant NSF. After 3 h of incubation at 4°C with rotation, the beads were washed five times in binding buffer without ovalbumin. The bound proteins were eluted with SDS-PAGE sample buffer, subjected to SDS-PAGE and analyzed by western blotting using the 2E5 monoclonal antibody, which equally detects both mutant and wild-type NSF. In all cases NSF binding was SNAP dependent.

Results:

Depolarization-Dependent Phosphorylation of NSF in Isolated Synaptosomes. The use of purified synaptosomes, metabolically labeled with high specific activity 32P-inorganic phosphate, permits direct correlation of Ca2+- and depolarization-dependent neurotransmitter release with specific protein phosphorylation events. Figure 1A shows phosphorimager and ECF-immunoblot analyses of SDS-PAGE resolved and blotted NSF isolated by immunoprecipitation with immobilized antibody from pre-labeled synaptosomes after various treatments. Incubation for 30 seconds following depolarization with 25 mM KCl in the presence of 1.5 mM Ca2+ (Ca Rel) led to substantial 32P labeling of NSF. Little 32P incorporation was observed either on depolarization in the presence of 10 mM Mg2+ (Mg Rel) to block calcium entry through SPM calcium channels or without KCl addition to induce depolarization (Ca Pre and Mg Pre). Indeed, 32P incorporation for all controls was less than 10% of that obtained with
Ca$^{2+}$ + K$^+$-depolarization as judged by quantifying the relative extents of labeling under these conditions as shown in Figure 1B (hashed bars). It should be noted that equal amounts of NSF were recovered from the lysed rat brain synaptosomes by immunoprecipitation with either the rabbit polyclonal anti-NSF antibody used for the samples shown in Figure 1A or with a mouse monoclonal antibody (2E5, data not shown). In addition, no NSF protein or radiolabel was recovered with Protein G-Sepharose alone. In a related experiment, no radiolabeled $\alpha$-SNAP from either resting or stimulated synaptosomes was detected in immunoprecipitates using anti-$\alpha$-SNAP antibodies (data not shown).

The relative extents of NSF $^{32}$P-labeling correlated exactly with the extents of glutamate release from synaptosomes measured under the same conditions at 30 seconds of incubation in the release solution (Figure 1B, solid bars). Depolarization in the presence of Ca$^{2+}$ gave 6 fold greater glutamate release than depolarization in the presence of Mg$^{2+}$ while release without depolarization was negligible. These data are derived from continuous release assays using the coupled glutamate dehydrogenase spectrophotometric assay described in Methods. Release reached a plateau value after ~ 2 minutes in every case. The results for both phosphorylation index and glutamate release assays were highly reproducible as shown in the figure.

**Characterization of NSF Phosphorylation in Synaptosomes.** To further characterize the nature of the Ca$^{2+}$- and depolarization-dependent phosphorylation of NSF and the possible identity of the responsible protein kinase(s), $^{32}$P-labeled NSF was prepared from synaptosomes treated exactly as shown in Figure 1A (Ca Rel) and subjected to 2D-TLE phosphoamino acid analysis following acid hydrolysis (48). Both phosphoserine and phosphothreonine, but not phosphotyrosine, were
detected from this *in situ* labeled NSF (Figure 2A). This suggests the involvement of one of the many neuronal Ser/Thr protein kinases (*i.e.* PKC, PKA, CaMKII). NSF contains potential kinase recognition sites for PKC, CaMKII, and casein kinase II but does not have consensus PKA sites. When recombinant NSF was labeled *in vitro* with purified PKC, phosphoamino acid analyses showed the presence of only phosphoserine (Figure 2B). Experiments have shown that CaMKII, but not PKA or casein kinase II can modify NSF *in vitro* ((49) and see Figure 3). Protein kinase inhibitors were used to further examine the kinase(s) responsible for NSF phosphorylation in synaptosomes. Figure 2C shows that the depolarization-dependent phosphorylation of NSF was inhibited by the inclusion 5 µM staurosporine, but not by the addition of 4 µM Kn-93, which is 10 times the reported K\(_i\) for CaM kinase II (50). Other inhibitors, more specific for protein kinase C also affected phosphorylation. Bisindolmaleimide I (100 nM), calphostin C (500 nM), chelerythrine (6.6 µM) and Ro31-8220 (100 nM) inhibited NSF phosphorylation by between 55% for chelerythrine and 80% for calphostin C (Figure 2D). The appearance of phospho-NSF was not enhanced significantly by 1 µM okadaic acid suggesting that protein phosphatases 1 and 2A may not be involved in NSF dephosphorylation. Treatment of synaptosomes with the PKC activator PMA (0.16 µM) increased NSF phosphorylation by 77% in the presence of EGTA and by 61% in the presence of calcium (Figure 2C). Under these same conditions, PMA did not stimulate glutamate release, however it did enhance both the extent of release (199.7±32.9 versus 126.3±12.0 pmole/mg synaptosome protein (p= 0.05)) and the initial rate of release (253.8±43.6 versus 126.8±18.6 pmole/mg synaptosome protein/min (p=0.03)).

**Characterization of NSF and SNAP Phosphorylation *in vitro***. Previous studies (49) reported that CaMKII can phosphorylate NSF *in vitro*, however, PKC was not tested. To reconcile those studies with present data, the ability of purified rat brain-derived CaMKII and PKC to
phosphorylate recombinant NSF was compared (Figure 3). Both kinases phosphorylated NSF in vitro, however the level of NSF phosphorylation with PKC (0.1-1 mole of PO₄/mole of NSF subunit; n=3) was 100 fold greater than that obtained with CaMKII under similar conditions. Consistent with Hirling and Scheller (49), both CaMKII and PKC phosphorylated α- and γ-SNAP under these conditions (Figure 3) indicating that the low extent of phosphorylation of NSF observed with CaMKII is an inherent property of NSF as a substrate. Further characterization of in vitro phosphorylation of NSF by PKC showed that the reaction was time dependent, saturable (data not shown), and required the PKC activators phosphatidylserine, diolein, and calcium (Figure 3). As noted above, phosphoamino acid analysis of the in vitro PKC-phosphorylated NSF showed the presence of only phosphoserine (Figure 2B). These data suggest that PKC may be one of the enzymes responsible for in situ phosphorylation of NSF. [It should be noted that the presence of phosphothreonine in the in situ but not in vitro phosphorylated NSF could be explained by contaminating phosphoproteins in the material isolated from synaptosomes. Alternatively, the commercial mixture of PKC isoforms (predominantly α, β, and γ) used in vitro may not contain all of the kinases involved.]

There are twelve canonical PKC recognition sites on the NSF subunit and several potential non-canonical recognition sites. To determine the exact sites at which the recombinant NSF was phosphorylated in vitro, labeled NSF was subjected to peptide mapping coupled with radiochemical sequencing. Initial 2D tryptic peptide maps gave a single major radioactive spot, which yielded phosphoserine after hydrolysis (data not shown). Subsequently, PKC-phosphorylated NSF was digested with either trypsin or Lys C and the released 32P-labeled phosphopeptides were fractionated by reverse-phase HPLC as shown in Figure 4A. Only one
major radiolabeled peak (other than the unabsorbed fraction containing inorganic phosphate) was observed in each case. However, the tryptic fragment eluted early in the acetonitrile gradient, commensurate with a small peptide, while the Lys C product eluted much later, as expected for a longer peptide. Radiochemical sequencing of the trypsin fragment isolated from the separation shown in Figure 4A gave release of the bulk of the $^{32}$P at cycle 4 (data not shown) whereas release of radiolabel on degradation of the Lys C phosphopeptide was observed after cycle 4 and cycle 12 as shown in Figure 4B. These data suggest that the phosphorylated serine in the recombinant NSF was 4 residues from an Arginine or Lysine (trypsin digest) and 12 residues from a Lysine (Lys C digest). [It should be noted that the primary sequence detected on PTH-amino acid analysis of these fractions was that for the amino terminal portion of the recombinant molecule containing the His$_6$-tag preceded by the sequence MRGS. Removal of this tag by TEV protease digestion however did not lead to a significant reduction in in vitro phosphorylation (data not shown)]. The only residue that completely fulfills these criteria, Ser-237, is part of the sequence RRAFASRVF that is a non-canonical PKC recognition site but is conserved in 17 of the 18 NSF sequences in the database. To demonstrate that this serine was phosphorylated in vitro, site-directed mutagenesis was used to change the residue to either an Alanine or a Glutamate. The resulting S237A and S237E mutant forms of NSF retain native oligomeric structure (see below), but are not phosphorylated significantly by PKC in vitro as shown in Figure 4C. Thus, Ser-237 is the only modification site consistent with the radiochemical sequencing data and its mutation results in a non-phosphorylatable form of NSF.

**Potential Role of Ser-237 Phosphorylation.** Initial experiments did not detect any significant difference between in vitro phosphorylated and unmodified NSF in SNAP/SNARE binding
assays (data not shown). The stoichiometry of phosphorylation was variable in those experiments. Therefore, we were not certain if all of the subunits of the NSF hexamer were modified. Previous reports have shown that NSF can bind to SNAP/SNARE complexes when monomeric or when the hexamer lacks a full complement of intact subunits (27) suggesting that NSF binding might not be affected unless all of the subunits are modified. The Ser-237 to Glu point mutation described above should mimic the negative charge that would be present in wild-type NSF after phosphorylation. Therefore, all six subunits would have the identical negative charge at the Ser-237 position mimicking complete phosphorylation of an NSF hexamer. The resulting mutant protein, S237E, was hexameric and had the same chromatographic properties on Superose 6 as wild-type NSF (data not shown). However, when tested for SNAP/SNARE complex binding, the S237E mutant showed reduced binding under standard conditions (Figure 4D). The lack of binding is most likely due to the introduction of a negative charge at S237 since another mutant, S237A showed wild-type levels of SNAP/SNARE binding. This defect in SNAP/SNARE binding is consistent with an inability of the NSF molecule to attain a binding competent conformation. Such binding incompetence occurs when NSF is in the ADP-bound form (29) and has been seen in mutants that lack or have mutations in the N-domain ((27) and Matveeva et al. in preparation) or that are unable to bind nucleotide in the D1 domain (27).

Discussion:

In this study, we demonstrate a depolarization-induced phosphorylation of NSF in rat synaptosomes that is dependent on influx of Ca\(^{2+}\) into the synaptosome. Phosphoamino acid analysis of radiolabeled NSF shows the presence of phosphoserine and phosphothreonine but no phosphotyrosine suggesting a role for a neuronal Ser/Thr kinase. Previous work has shown that
NSF is not a substrate, \emph{in vitro}, for casein kinase II or PKA (49). Here, we show that the depolarization-induced phosphorylation of NSF \emph{in situ} is not sensitive to the CaM kinase II inhibitor Kn-93 but is sensitive to staurosporine as well as, bisindolmaleimide I, calphostin C, chelerythrine, and Ro31-8220, suggesting a role for PKC. We further demonstrate that PKC phosphorylates NSF \emph{in vitro}, specifically at Ser-237, which lies in the catalytically important D1 domain. Mutation of Ser-237 to either Ala or Glu eliminates \emph{in vitro} phosphorylation by PKC confirming the phosphopeptide analysis. Both mutants are hexameric, based on sizing chromatography. However, only the S237E mutant looses its ability bind to SNAP/SNARE complexes. This inactivation of NSF can be partially understood using the known structure for the homologous D2 domain. A negative charge at Ser-237 (such as a phosphate or glutamate) is in a position to greatly affect the flexibility of the catalytic D1 domain (see below). Our observations are the first of which we are aware that connect control of a physiologic process to a phosphorylation event involving a protein of the docking/fusion apparatus. They are significant because they indicate that in response to depolarization, synaptosomal PKC may down-regulate NSF activity by effecting its ability to interact with its substrates, SNAPs and SNAREs. We were able to document this phosphorylation because the synaptosomal preparation offers an isolated region of the cell where the docking/fusion components involved in neurotransmitter release are concentrated; 0.5 to 1% of the total synaptosomal protein is NSF.

**Role of PKC in Neurotransmission.** Numerous studies have shown a presynaptic role for PKC in neurotransmission (for a recent review see (30)). It is generally accepted that activation of PKC, by either phorbol esters or agonist, leads to an increase in the stimulus-induced release of neurotransmitters. Several studies, all \emph{in vitro}, have attempted to identify the relevant PKC substrates responsible for this enhancement of secretion. Others have attempted to associate
changes in the phosphorylation profile of PKC substrates or the compartmentalization of PKC isozymes with physiologic processes (e.g., LTP) and pathologic processes (e.g., kindled epilepsy). Thus LTP of synaptic transmission in the hippocampus, a model of learning and memory (for review see (51)), has been associated with PKC translocation to the membrane (35,52) and to phosphorylation by PKC of F1/GAP-43 (53). Similarly, kindled epilepsy evoked by stimulation at multiple brain sites has been associated with both transient (54,55) and long-term (56,57) increases in membrane-associated PKC enzyme activity but not protein levels. However, no direct association has been made between enhanced glutamate release associated with LTP or kindling and phosphorylation of the secretory machinery (43,58). Our studies provide the first evidence for the linkage between phosphorylation of secretory machinery, namely NSF, and neurotransmitter release and also provide a potential site for alteration in LTP and kindling.

To date, SNAP-25 (40,59,60), syntaxins (60-62), α- and γ-SNAPs (49), Munc18 (63,64), synaptotagmins (65,66), and now NSF are substrates for PKC at least in vitro. In most of these reports the PKC-mediated phosphorylation prevents the association of proteins (i.e. phospho-Munc18a and syntaxin-1 (63) or phospho-α-SNAP and syntaxin-1 (49)). In this report, this also appears to be true based on the behavior of the S237E mutant NSF, which cannot bind to the SNAP/SNARE complex. At this point, it is unclear how these PKC-mediated phosphorylation events can regulate the secretory cycle in the synaptosome. Here, we report depolarization-dependent phosphorylation of NSF that requires calcium influx, suggesting that NSF phosphorylation occurs after exocytosis. The physiological role of this modification in controlling NSF function is yet to be determined.

Molecular analysis of the effects of NSF phosphorylation. Two models of NSF function in
neurons suggest two distinct interpretations of the phosphorylation data presented here. Initially studies of NSF function in *Drosophila* and chromaffin cells suggested that NSF is important for the maintenance of the “ready-release” pool of vesicles by disassembling *cis*-SNARE complexes to promote the formation of functional *trans*-SNARE complexes (22,23,67). However, in a more recent study, inactivation of NSF through the addition of N-ethylmaleimide was shown to increase the pool of hyperosmotically-sensitive synaptic vesicles (68). This pool is thought to be equivalent to the kinetically defined “ready-release” pool. In this second model, inhibition of NSF is thought to block disassembly of *trans*-SNARE complexes thereby maintaining the functional complexes for membrane fusion. In these two models, NSF inactivation by phosphorylation could lead to distinct outcomes. Based on the first model, phosphorylation of NSF would down regulate a nerve terminal by lessening the activation of SNAREs and therefore lowering the pool of “ready-release” vesicles. For the second, inactivation of NSF would lead to an increase in fusion competent vesicles because it would lengthen the lifetime of the active *trans*-SNARE complexes. At this stage, it is difficult to be more specific since both up and down regulation of fusion-competent synaptic vesicle pools has been observed.

Structurally, phosphorylation of NSF at Ser-237 could have rather drastic effects. Since no crystal structure is available for the D1 domain of NSF, we must use the structure of the homologous, but not identical, D2 domain as the basis for discussion. By structure-based sequence alignments (69), Ser-237 would be in the middle of the α1 helix, which is solvent-accessible and is adjacent to the inter-subunit interface that is important for hexamerization. This residue (Ser-237) is well within 15 Å of the adjacent subunit particularly the loop between the α8 and α9 helices. Such a position could be important since the α8 helix has several key
residues that make contacts with the ATP nucleotide (red, Figure 5B) and with the adjacent subunit (yellow, Figure 5B). Phosphorylation at Ser-237 could affect the positioning or flexibility of the α8 and therefore the conformational changes associated with ATP hydrolysis or with nucleotide exchange. When one models in the residues that are present in the predicted α8-α9 loop of D1, the potential for an effect becomes more striking (Figure 5B). This region of D1 has a net higher positive charge than the corresponding region of D2 (Figure 5A) with the Arg-463 and Lys-466 being within 9 Å of the phospho-amino acid (or the modeled Glu in Figure 5B). This is well within the reach of charge influences from a negatively charged phosphate on the adjacent subunit. Such charge-charge interactions could restrict the movement of the α8 helix and thereby could affect the catalytically important conformational change(s) in the D1 domain. At this stage, the proposed effects of the negative charge at the Ser-237 position remain speculative until a structure is available for the D1 domain.
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46. Sollner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S.,


Abbreviations:

NSF, N-ethylmaleimide Sensitive Factor; SNAP, Soluble NSF Attachment Protein; SNAP-25, synaptosomal associated protein of 25 kDa; PKA, protein kinase A; PKC, protein kinase C; CaMKII, Ca\(^{2+}\)/calmodulin dependent protein kinase II; PMA, phorbol 12-myristate 13-acetate; KRB, Krebs - Ringer bicarbonate buffer; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; TLC-TLE, thin layer chromatography-thin layer electrophoresis; NT, neurotransmitter; ECF, enhanced chemifluorescence; SPM, synaptic plasma membrane; LTP, long term potentiation.
Figure Legends:

Figure 1. Depolarization-Induced, Calcium-Dependent Phosphorylation of Synaptosomal NSF.

Panel A shows phosphorimager (\(^{32}\text{P}\)) and immunoblot (Ab) analysis of NSF immunoprecipitated from solubilized synaptosomes pre-loaded with \(^{32}\text{P}\) then treated for 30 sec under conditions that trigger NT release (\(Ca\ \text{Rel} - 1.5\ \text{mM} \ \text{Ca}^{2+}, 25\ \text{mM} \ \text{K}^+\)) or various controls (\(Ca\ \text{Pre} - 1.5\ \text{mM} \ \text{Ca}^{2+}, 1\ \text{mM} \ \text{K}^+; Mg\ \text{Rel} - 10\ \text{mM} \ \text{Mg}^{2+}, 25\ \text{mM} \ \text{K}^+; Mg\ \text{Pre} - 10\ \text{mM} \ \text{Mg}^{2+}, 1\ \text{mM} \ \text{K}^+\)). The position of NSF was detected by enhanced chemifluorescence and the \(^{32}\text{P}\)-label was detected by phosphorimager analysis following a 5 day exposure. Quantification of relative labeling (Panel B) was obtained by dividing \(^{32}\text{P}\) phosphorimager densities by ECF intensities for these samples. The values shown (hashed bars) are averaged values for two experiments normalized to Ca Rel as 100%. Error bars represent deviation from the average. Glutamate release (solid bars) was measured at 30 sec from a parallel set of samples using the continuous spectrophotometric assay outlined in Methods. The data shown are averages of six separate experiments with error bars indicating the range of values.

Figure 2. \textit{In Situ} and \textit{In Vitro} Phosphorylation of NSF by Protein Kinase C. Panel A shows the results of 2D TLE analysis of phosphoamino acids recovered from the \(^{32}\text{P}\) labeled NSF from one of the \(Ca\ \text{Rel}\) samples shown in Fig. 1. Panel B is a similar analysis of \textit{in vitro}, PKC-phosphorylated, recombinant NSF shown in the right panel of Figure 3. Regions outlined by dotted lines denote the positions of the indicated phospho-amino acid standards (1 \(\mu\text{g}\) each), which were added to the sample prior to analysis and detected by ninhydrin. Panel C. The
indicated inhibitors (staurosporine, Kn-93) or activator (PMA) were added, from DMSO dissolved stocks, to synaptosomes prior to depolarization with potassium in the presence of calcium or EGTA where noted. The incubations were stopped by the addition of SDS and boiling. NSF was recovered by immunoprecipitation and detected by phosphoimager analysis. Kn-93 (and its inactive isomer Kn-92), were added at 10 times the K_i for CaMKII. Okadaic acid (OA) was added at levels that would block protein phosphatases 1 and 2A. The numbers above each lane of the right hand panel of C represent the ratio of phosphoimager signal to Coomassie stained NSF protein. Panel D. Synaptosomes were incubated with calphostin C (Calph, 500 nM), chelerythrine (Chel, 6.6 µM) and Ro31-8220 (Ro31, 100 nM) or bisindolmaleimide I (Bis, 100 nM) 15 min prior to depolarization. Reactions were stopped and NSF was recovered by immunoprecipitation. Phosphorylation was evaluated as the ratio of phosphoimager signal to Coomassie stained NSF protein. The data represents two separate experiments, which were compared by setting the depolarization-induced phosphorylation (Cal Rel) as 100%. Error bars represent deviation from the average. Calphostin C was activated, after addition to synaptosomes, by exposure to fluorescence lighting for 30 min at room temperature (70).

**Figure 3. Comparison of NSF and SNAP Phosphorylation by Ca^{2+}-Dependent Protein Kinases**

In Vitro. Samples of recombinant NSF (6 µg), α-SNAP (10 µg) or γ-SNAP (10 µg) were treated for 1 h at 25°C in a total volume of 40 µl with CaM Kinase II (0.05 mU) or PKC (0.5 mU) + 1 mM γ-32P-ATP [S.A. = 4,500 Ci/mmol]. Phosphorylated samples were immediately disrupted by boiling for 5 min in 1 % SDS - 1 % 2-mercaptoethanol, resolved by SDS-PAGE, and then
electroblotted onto PVDF yielding the phosphorimager analyses shown following 48 h exposure.

Samples contained various combinations of Ca$^{2+}$ (1 mM), calmodulin (1 µM), phosphatidylserine (200 µg/ml) or diolein (10 µg/ml) as indicated. Auto-phosphorylation of the CaMKII α-chain is the major labeled band in the left-hand panel.

**Figure 4. Identification and Functional Significance of Ser-237 as the Protein Kinase C Phosphorylation Site in NSF.** Panel A shows the HPLC profile of the phosphopeptides recovered from trypsin (dashed line) and Lys C (solid line) digestion of *in vitro* phosphorylated NSF. Panel B shows the $[^{32}P]$ radiolabel recovered from the Lys C generated phosphopeptides after each cycle of the chemical sequencing reactions corrected for repetitive yield (92.5%). Panel C shows the results of *in vitro* phosphorylation of NSF by purified PKC. His$_6$-tagged NSF mutants S237A and S237E were phosphorylated by PKC, *in vitro*, and separated by SDS-PAGE. The left side shows the Coomassie stained gel used to generate the phosphoimage at the right. Panel D shows the results from a SNAP/SNARE binding experiment. Mutants S237E and S237A mutants or wild type NSF were incubated with GST-syntaxin and α-SNAP in the presence of AMP-PNP. Complexes were recovered on GSH-agarose beads and the amount of wt or mutant NSF bound was determined by western blotting (*Bound*). The band at the left side is pure NSF standard.

**Figure 5. Proposed Molecular Mechanism for the Regulation of NSF Activity by Ser-237 Phosphorylation.** Panel A shows the structure-based sequence alignment of the α8-loop-α9 region of the D2 domain compared to D1. Panel B. The position of Ser-237 in the D1 domain
was modeled onto the crystal structure of the D2 domain (69). This representation shows a S237E mutation made in one subunit (red) and the potentially interacting residues on the adjacent subunit (blue). The residues in the inter-helix loop have been modeled based on the D1 sequence. The \( \alpha_8 \) helix is green and the \( \alpha_9 \) is purple. The yellow residues in \( \alpha_8 \) make up part of the inter-subunit interface that is critical to hexamer formation those in red make up part of the nucleotide-binding pocket of the blue subunit. Panel B was constructed with Swiss PDB Viewer and rendered with Pov-Ray.
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**Matveeva et al. Figure 3**
D2: IKKLLMLIEMLQMDPEYRVRKFLALLRE

D1: LEGLVRAAQSTAMNRHIKASTKVEVDMEK
Phosphorylation of the N-ethylmaleimide sensitive factor (NSF) is associated with depolarization-dependent neurotransmitter release from synaptosomes
Elena A. Matveeva, Sidney W. Whiteheart, Thomas C. Vanaman and John T. Slevin

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