MgATP-Dependent and MgATP-Independent [^{3}H]Noradrenaline Release from Perforated Synaptosomes Both Use *N*-Ethylmaleimide-Sensitive Fusion Protein†

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ABSTRACT: In streptolysin-O (SLO)-perforated rat brain cortical synaptosomes, Ca^{2+}-induced [^{3}H]-noradrenaline (^{3}H-NA) release began with a phase lasting about 1 min that did not depend on MgATP. Subsequent release became increasingly MgATP-dependent. The first phase involved release from previously "primed" synaptic vesicles. MgATP-dependent release, on the other hand, was release from unprimed vesicles that needed to be primed by ATP hydrolysis before they could be fused with the presynaptic membrane. Vesicle depriming was detected by observing that the initial release decreased when the synaptosomes were perforated and incubated for 2 min in the absence of MgATP before increasing Ca^{2+} to promote release. One millimolar *N*-ethylmaleimide (NEM) inhibited both MgATP-dependent and MgATP-independent release at all times of incubation (0.5−5 min), and inhibition by NEM was partially reversed at short (0.5 min) and longer (5 min) times by adding intact *N*-ethylmaleimide sensitive fusion protein (NSF) to the perforated synaptosomes. Polyclonal antibodies against the N-terminal domain of NSF produced dose-dependent inhibition of Ca^{2+}-induced ^{3}H-NA release. This inhibition occurred in both early and late release phases and was highly significant at early times if the perforated synaptosomes were preincubated for 2 min with anti-NSF. These results indicate participation of NSF both after vesicular fusion, probably for separation of SNARE proteins in v/t-SNARE complexes before endocytosis, and, surprisingly, after docking, possibly to maintain vesicles in a primed state and reverse depriming during regulated secretion.

Neurotransmitters are released from nerve terminals via Ca^{2+}-dependent exocytosis, probably by molecular mechanisms similar to those of intracellular vesicle transport (*1*−*3*). The cytosolic protein *N*-ethylmaleimide-sensitive fusion protein (NSF),[1] soluble NSF attachment proteins (SNAPs), and membrane SNAP receptors (SNAREs) have been identified as proteins essential for in vivo vesicle transport, docking, and/or membrane fusion (*1*−*4*). However, only unassembled t- and v-SNAREs in opposite membranes are required for complex formation and membrane fusion in vitro (*5*).

Functional studies of large dense core vesicle (LDCV) exocytosis from permeabilized chromaffin cells (*6*, *7*) and PC12 cells (*8*) showed that the process of readying a LDCV for Ca^{2+}-induced catecholamine release could be separated into a MgATP-dependent priming step followed by a MgATP-independent, Ca^{2+}-triggered step. MgATP-dependent priming allowed secretory LDCVs to proceed to the Ca^{2+}-triggered step, and only primed LDCVs were able to undergo exocytotic fusion in response to Ca^{2+} in the absence of MgATP, suggesting that NSF functions before Ca^{2+}-triggering. Currently, a role for NSF prior to docking is being actively investigated in PC12 cells and yeast (*9*−*12*).

The emerging picture of constitutive membrane fusion (*13*) is that membrane and vesicle SNARE proteins interact to produce a parallel coiled-coil structure that brings membranes close together and produces membrane fusion. After fusion, these SNARE protein complexes are dissolved by NSF, so vesicular membrane materials can be recycled for reuse. We investigated ^{3}H-NA release from perforated rat cortex synaptosomes by applying *N*-ethylmaleimide (NEM), NSF, and anti-NSF antibodies to better understand if this model was applicable to regulated neurotransmitter release from nerve terminals.

EXPERIMENTAL PROCEDURES

Materials. One-month-old (100−120 g) male Wistar rats were used according to NIH guidelines in all experiments. Streptolysin-O (SLO) was from Murex (Norcross, GA). Percoll and E-Z-SEP antibody purification kits were from Pharmacia (Uppsala, Sweden). Levo[7-^{3}H]noradrenaline (^{3}H-NA) (specific activity 12.4 Ci/mmol) was from DuPont (Boston, MA). *Staphylococcus aureus* α-toxin was from Gibco (Gaithersburg, MD). *N*-Ethylmaleimide (NEM), glutathione, goat IgG, and all nucleotides were from Sigma (St. Louis, MO). Monoclonal and polyclonal anti-NSF antibodies and the large amount of NSF needed for the restoration

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[1] Abbreviations: SLO, streptolysin-O; NA, noradrenaline; NSF, *N*-ethylmaleimide-sensitive fusion protein; NEM, *N*-ethylmaleimide; SNAP, soluble NSF attachment protein; SNARE, soluble NSF attachment protein receptor; LDCV, large dense core vesicle; BSA, bovine serum albumin.

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experiments were generously provided by Dr. S. W. Whiteheart.

Calculation of Free Ca^{2+} Concentrations. Ca/EGTA buffers were calculated and prepared as described by Föhr et al. (*14*), using a computer program kindly supplied by Dr. M. Gratzl.

Ca^{2+}-Induced ^{3}H-NA Release from Perforated Synaptosomes. Synaptosomes were prepared from rat cerebrocortex, purified by Percoll-Sucrose density gradient centrifugation, labeled with ^{3}H-NA, washed, and resuspended as described previously (*15*, *16*). Aliquots (120 μL, ~120 μg of protein) of synaptosomes were perforated with 0.5 unit/mL SLO in 600 μL of release buffer [123 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1.15 mM NaH_2PO_4, 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 5.6 mM D-(+)-glucose, pH 6.8, 10^{-8} or 10^{-5} M free Ca^{2+}, supplemented with 2−5 mM ATP] at 37 °C. Thirty seconds before the indicated times, 120 μL of the incubation was withdrawn and centrifuged for 30 s at 15800*g*, 4 °C. Released ^{3}H-NA was determined in 90 μL of supernatant by liquid scintillation counting and was expressed as a percentage of the total ^{3}H-NA incorporated into prelabeled synaptosomes after volume correction. For studies of MgATP-independent release, $MgCl_2$, ATP, and glucose were omitted from the incubations, 3 mM EDTA was included to chelate endogenous Mg^{2+}, and the free Ca^{2+} concentration was maintained by adjusting the [$CaCl_2$] added. For studies of priming and depriming, synaptosomes were first perforated in 10^{-8} M Ca^{2+} buffer with 0.5 unit/mL SLO, with or without 2 mM MgATP for 2 min at 37 °C. Then the free Ca^{2+} concentration was either maintained at 10^{-8} M or elevated to 10^{-5} M. Aliquots were removed 30 s before the indicated times for ^{3}H-NA determination as described above. Small corrections (usually about 5%) were made for ^{3}H-NA leakage from the synaptosomes while they were maintained on ice before being used in the experiments. Ca^{2+}-induced release was calculated by subtracting ^{3}H-NA release in the presence of 10^{-8} M Ca^{2+} from release in the presence of 10^{-5} M Ca^{2+} for each individual measurement because there is a continuous Ca^{2+}-independent efflux of ^{3}H-NA from the synaptic vesicles and synaptosomes that is elevated by chelating Mg^{2+} with EDTA.

Effects of Anti-NSF Antibodies on Ca^{2+}-Induced Release. Monoclonal and polyclonal anti-NSF as well as goat IgG were further purified (>90%) with E-Z-SEP antibody purification kits (Pharmacia) and dialyzed against 10 mM Tris-HCl buffer, pH 7.0. Twenty microliters of labeled synaptosomes (~20 μg of protein) was permeabilized with 0.5 unit/mL SLO in 100 μL of release buffer (with 2 mM ATP) in the presence of either goat IgG or anti-NSF at the indicated concentrations; 30 s before the indicated times, incubations were stopped by 30 s centrifugation at 4 °C, and Ca^{2+}-induced ^{3}H-NA release was determined as described above.

Effects of NEM on Ca^{2+}-Induced Release. ^{3}H-NA-labeled synaptosomes were incubated with 1 mM NEM (from 50 mM stock in H_2O) for 5 min on ice; then 2 mM glutathione was added to quench the unreacted NEM, and the incubations were allowed to stand on ice for another 5 min. In control experiments, 1 mM NEM was first-mixed with 2 mM glutathione before adding the synaptosomes. Synaptosomes were washed 3 times by centrifugation (10 min once, 5 min twice at 15000*g*, 4 °C) and resuspended in wash buffer (release buffer minus Ca^{2+} and ATP) at 1 mg of protein/mL. The time course of Ca^{2+}-induced ^{3}H-NA release from both control and NEM-treated synaptosomes was studied as described above, sometimes in the presence of added NSF for NSF restoration experiments. Inhibited NSF was prepared by incubating 2 mg/mL NSF with 1 mM NEM at 4 °C for 5 min. Then 2 mM glutathione was added, and the resulting inactivated preparation was used directly.

Protein Determinations. Protein concentrations were determined by the Bradford method (*17*) using bovine serum albumin as standards.

Presentation of Data. For each study described here, three independent experiments were carried out unless otherwise indicated. Data were presented as the mean of 6−12 determinations on different synaptosomal preparations ± SEM.

RESULTS

Ca^{2+}-Induced ^{3}H-NA Release in the Presence and Absence of MgATP. Ca^{2+}-induced exocytotic release, defined as ^{3}H-NA release from the perforated synaptosomes in the presence of 10^{-5} M free Ca^{2+} minus release in the presence of 10^{-8} M free Ca^{2+}, was measured over time from synaptosomes incubated in the presence of 5 mM ATP and 0.5 mM free Mg^{2+} (Figure 1A). Ca^{2+}-induced release increased in the first 2 min of incubation before leveling off at about 20% of total ^{3}H-NA in the synaptosomes at the start of the incubations. If, however, 3 mM EDTA was substituted for the added Mg^{2+} to reduce endogenous Mg^{2+} to low levels (calculated to be <1 μM), then the Ca^{2+}-induced release was comparable to the previous incubations with added MgATP only for the first minute. Release after 1 min in the absence of free Mg^{2+} leveled off and then rapidly declined. Also indicated is the MgATP dependency calculated as the Ca^{2+}-induced release in the presence of Mg^{2+} minus the Ca^{2+}-induced release in the absence of Mg^{2+} (presence of EDTA). Clearly, Ca^{2+}-induced release was independent of MgATP for the first minute, but became increasingly Mg^{2+}-dependent after the first minute. Release during the first minute probably was from vesicles that were already primed with MgATP before release was stimulated. The falloff of Ca^{2+}-induced release occurred because unprimed synaptic vesicles could not be primed in the absence of Mg^{2+}. Chelating Mg^{2+} with EDTA undoubtedly eliminated the ability of ATP to act as an energy source for neurotransmitter release because MgATP is generally acknowledged to be the fuel for release in most secretory systems. Also, MgATP is the substrate for NSF, which is proposed to play a critical role in exocytosis (*18*).

This interpretation is supported by the results of incubations performed without exogenous ATP (Figure 1B). Again, Ca^{2+}-induced release increased rapidly in the first minute, then leveled off, and declined afterward whether or not EDTA was added, so this effect was not due to the presence of EDTA. Here, the increasing dependence of release on MgATP noted previously was absent. Apparently, endogenous MgATP was consumed rapidly, so the results are similar to release in the absence of Mg^{2+} (Figure 1A). Taken together, these experiments show that ^{3}H-NA release from SLO-permeabilized synaptosomes can be resolved into an initial (1 min) ATP-independent phase (because Ca^{2+}-

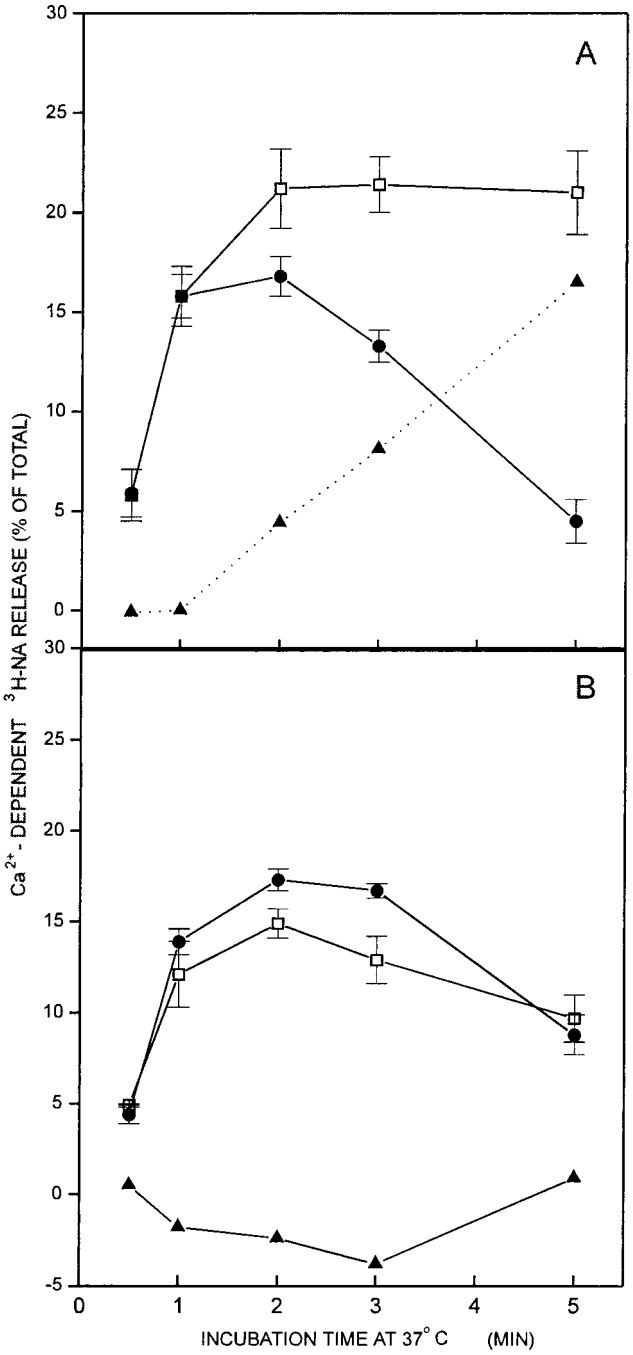

FIGURE 1: Time course of Ca^{2+}-induced release in the presence and absence of MgATP. ^{3}H-NA-labeled synaptosomes were permeabilized with 0.5 unit/mL SLO, in the presence of 5 mM ATP, 0.5 mM free Mg^{2+} or in the absence of added ATP and Mg^{2+} plus 3 mM EDTA, in the presence of either 10^{-8} M or 10^{-5} M free Ca^{2+}. At the indicated times, aliquots were taken, and Ca^{2+}-induced release was determined as described under Experimental Procedures. MgATP dependency was calculated by subtracting Ca^{2+}-induced release in the absence of MgATP from Ca^{2+}-induced release in the presence of MgATP. (A) Incubations in the presence of 5 mM ATP with (●—●) or without (□—□) mM EDTA. (▲···▲) indicates Mg^{2+} dependency. (B) Incubations in the absence of added MgATP with (●—●) or without (□—□) added EDTA. (▲—▲) indicates Mg^{2+} dependency. Data are from 3−4 independent experiments (n = 9−12).

dependent release had no MgATP dependency) that occurs from synaptic vesicles primed before release was initiated, and a later phase that becomes increasingly dependent on MgATP. This is because, as primed vesicles are gradually

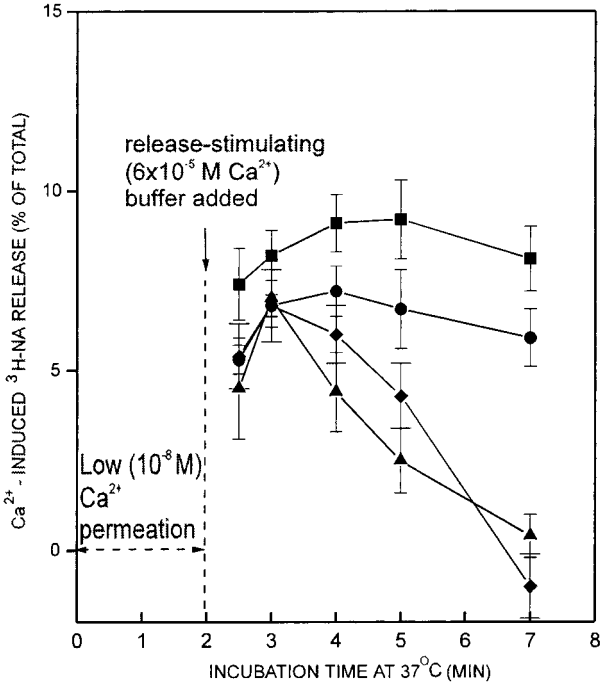

FIGURE 2: Effects of MgATP on vesicle priming and depriming. ^{3}H-NA-labeled synaptosomes were first permeabilized with 0.5 unit/mL SLO in 10^{-8} M Ca^{2+} buffer for 2 min (broken horizontal arrow), with or without 2 mM MgATP. Then a correction buffer was added (vertical arrow) to increase Ca^{2+} to 6 × 10^{-5} M or to maintain Ca^{2+} at 10^{-8} M; 2 mM MgATP also was added to some incubations during the Ca^{2+}-triggered step as indicated. ^{3}H-NA release was determined at the indicated times as described and Ca^{2+}-induced release was plotted. (▲) Absence of Mg^{2+} (presence of high EDTA) all 7 min of incubation. (◆) Incubation with MgATP for the first 2 min; Mg^{2+} chelation thereafter. (●) Mg^{2+} chelation for the first 2 min; MgATP present thereafter. (■) Continuous presence of MgATP all 7 min of incubation. Data were from four independent experiments (n = 9−12).

used during minutes 1−5 of incubation, a growing fraction of the synaptic vesicles must be primed with MgATP before they can fuse with the presynaptic membrane and release their contents through Ca^{2+}-induced exocytosis. This demonstrates that MgATP-dependent priming occurs before Ca^{2+} triggering for synaptic vesicles in nerve endings, similar to adrenal chromaffin and PC12 cell LDCVs.

Priming and Depriming of Synaptic Vesicles. Using a different type of experiment, we further examined whether synaptic vesicles could be primed and deprimed, as has been shown for LDCVs (*6*, *7*). Here, synaptosomes were perforated with SLO in 10^{-8} M free Ca^{2+} buffer in the presence or absence of 2 mM ATP. After 2 min of incubation, Ca^{2+} levels either were increased to 10^{-5} M to initiate Ca^{2+}-induced release or were maintained in 10^{-8} M free Ca^{2+} buffer, sometimes with the addition of 2 mM MgATP (Figure 2).

When MgATP was absent (EDTA was present) during both parts of the experiment (▲), there was an initial 1 min surge of Ca^{2+}-induced release that subsequently declined rapidly. This falloff was the reverse of the rise in MgATP dependency observed before (Figure 1A). This surge of release probably corresponds to release from primed vesicles that already have passed the ATP hydrolysis step and can fuse in the absence of MgATP.

When MgATP was added after the 2 min perforation incubation (●), Ca^{2+}-induced release was similar for the first

minute after Ca^{2+} triggering, but did not decrease significantly thereafter. Apparently, release after the first minute of stimulation was sustained by the added MgATP.

When MgATP was present during the 2 min low Ca^{2+} perforation incubation and Mg^{2+} was chelated after 2 min as high Ca^{2+} was added, release began like the second condition, but fell off after the first minute of stimulated release (◆). However, for technical reasons, these incubations could not be made identical to the other conditions. When MgATP and free Mg^{2+} are maintained at 2 mM and 0.5 mM, respectively, for the first 2 min, the free Mg^{2+} can only be lowered to about 17 μM subsequently by adding EDTA to 10 mM. The technical limitation is that increasing EDTA above 10 mM produces nonspecific effects. Whether or not this relatively high level of free Mg^{2+} caused the low initial value of Ca^{2+}-induced release in these incubations is unclear at present.

The highest levels of release were obtained when MgATP was included in both parts of the incubation (■). Including MgATP during perforation elevates the initial release significantly, suggesting that depriming reactions occurred during the 2 minute perforation incubation in the absence of MgATP (see Discussion). For the first time, these data show that in nerve endings, synaptic vesicles undergo priming and depriming reactions similar to LDCVs.

N-Ethylmaleimide (NEM) Inhibits both ATP-Dependent and ATP-Independent Release. To examine NSF involvement in synaptosomal release, we studied the effect of NEM treatment on ATP-dependent and ATP-independent release. NEM could modify several steps in vesicle transport in addition to NSF-dependent vesicle priming. However, the synaptosome is the isolated terminal transport compartment, so the effects of NEM are restricted to late stage(s) of exocytosis and vesicle recycling. In the presence of 2 mM MgATP (Figure 3A), release from NEM-treated synaptosomes was significantly lower than control release, even at the earliest times examined (0.5 min). In the presence of NEM, little Ca^{2+}-induced release was observed (<5%) during 5 min incubations. Inhibition of release by NEM during the first minute suggested that ATP-independent release, presumably from primed vesicles, was impaired. To test this, release from NEM-treated and control synaptosomes incubated in the absence of MgATP (in the presence of 3 mM EDTA) was examined. Figure 3B shows that as before (Figure 1B), release rises rapidly, levels off after 1 min, and then declines for control synaptosomes. However, in the NEM-treated synaptosomes, MgATP-independent release was essentially eliminated.

Exogenous NSF Partially Restores ^{3}H-NA Release from NEM-Inhibited, Perforated Synaptosomes. We added NSF back to NEM-inhibited, perforated synaptosomes to determine whether NSF played a part in the reduction of release by NEM. Figure 4 shows that, in both 1 and 5 min incubations, exogenous NSF significantly increased ^{3}H-NA release compared to equivalent amounts of bovine serum albumin. Similar results were obtained for 3 min incubations.[2] An additional control for the 5 min incubations showed no release stimulation when NEM-inhibited NSF was substituted for NSF (Figure 4). These results show that

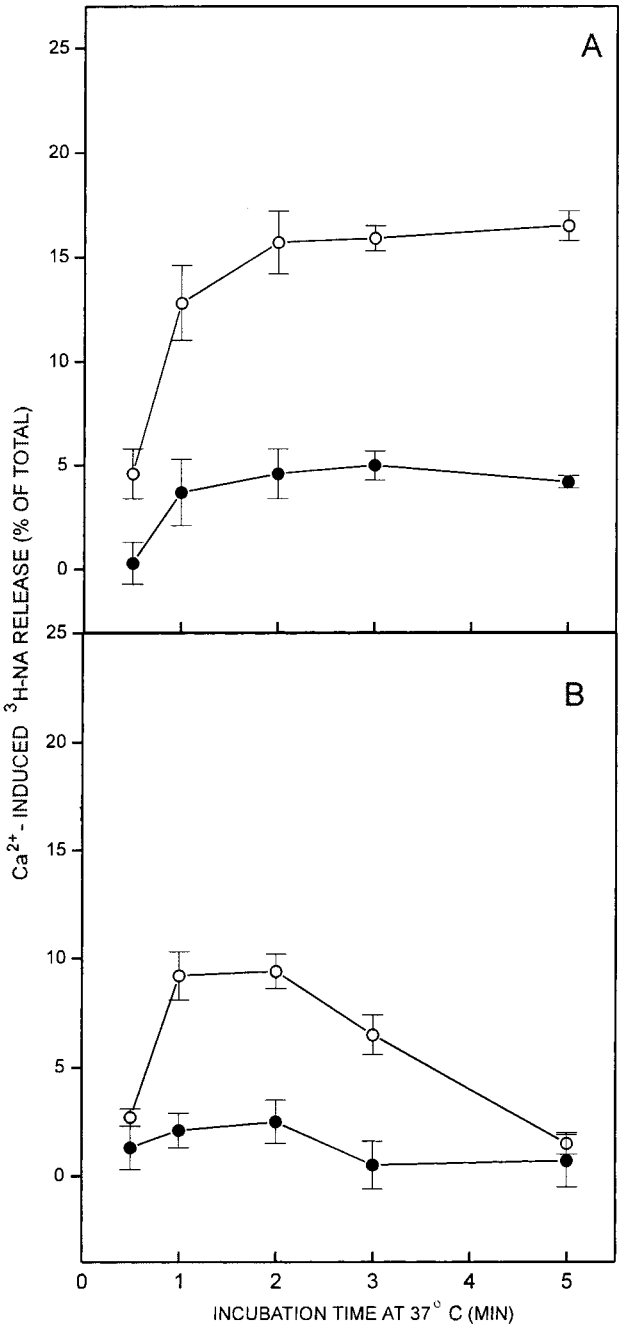

FIGURE 3: Effect of NEM on Ca^{2+}-induced release. ^{3}H-NA-labeled synaptosomes were incubated with 1 mM NEM for 5 min on ice. Then 2 mM glutathione was added to quench the unreacted NEM (●). For control experiments, 1 mM NEM was first mixed with 2 mM glutathione before addition to the synaptosomes (○). Treated synaptosomes were washed and resuspended as described. The time course of Ca^{2+}-induced release from both control and NEM-treated synaptosomes was examined. Release experiments were conducted either in the presence of 2 mM MgATP **(A)** or in the absence of MgATP (in the presence of 3 mM EDTA) **(B)**. Data were from two independent experiments ($n = 6$).

inactivation of synaptosomal NSF was, at least in part, responsible for the inhibition of ^{3}H-NA release by NEM at both early and late stages. Of course, these results do not exclude the possibility that NEM inhibition of other proteins, e.g., lipid kinases or Hrs-2, may be involved in this inhibition.

Anti-NSF Antibodies Inhibit Ca^{2+}-Induced Release. The role of NSF as a fusogen in LDCV exocytosis was challenged when NSF was shown to leave the fusion complex before

[2] Zheng and Bobich, unpublished observations.

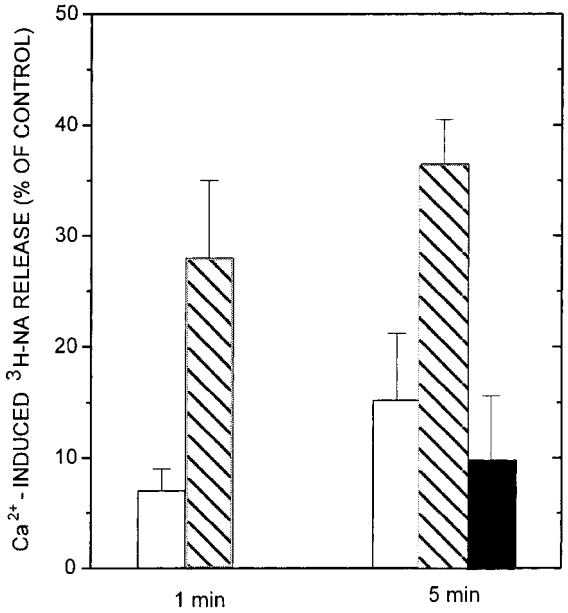

FIGURE 4: Effects of NSF on NEM-treated synaptosomes. Synaptosomes were treated with NEM as described in the legend to Figure 3. NEM-treated synaptosomes were perforated in the presence of 0.2 mg/mL BSA (white bars), NSF (hatched bars), or NEM-inhibited NSF (black bar) for indicated times. Ca^{2+}-induced release was measured and plotted as a percentage of controls where NEM was mixed with glutathione before addition to the synaptosomes. The presence of NSF elevated the release significantly above BSA ($p < 0.05$, $n = 6-12$) or NEM-inhibited NSF ($p < 0.01$, $n = 3$).

Ca^{2+} triggering in semi-intact chromaffin cells (*11*). To further understand the role of NSF in neurotransmitter release in nerve endings, we studied the effects of several anti-NSF antibodies on Ca^{2+}-induced release from perforated synaptosomes. These antibodies were further purified to remove other contaminating proteins present in serum or ascites fluid and dialyzed against 10 mM Tris-HCl, pH 7.0. Each anti-NSF—but not anti-goat—IgG recognized a single band of $M_r \approx 76$K on Western blots (for example, Figure 5A, inset). Figure 5A demonstrates that all the anti-NSF antibodies inhibited Ca^{2+}-induced release when applied to perforated synaptosomes, while identically treated control IgG did not change release significantly. The polyclonal anti-NSF antibody (R3230), examined in more detail, displayed concentration-dependent inhibition, with $IC_{50} \approx 75$ μg of IgG/mL at 200 μg/mL synaptosomal protein (Figure 5B).

An additional series of experiments examined the effects of polyclonal anti-NSF on the time course of release. When the antibody was applied at the start of synaptosomal perforation (Figure 6A), inhibition of release was highly significant by 1 minute, and the antibody continued to inhibit release throughout the 5 min incubation. These effects are not due to a loss of ATP because incubations were supplemented with 5 mM ATP and 0.5 mM free Mg^{2+}.

The lack of significant inhibition by anti-NSF at the first (30 s) timepoint could be due to the absence of an antibody effect on primed vesicles or to slow entry of the antibody into the synaptosomes. We tested these possibilities by incubating perforated synaptosomes with the same antibody in low-Ca^{2+} (10^{-8} M) buffer with 5 mM ATP for 2 min before stimulating release (Figure 6B). We observed much greater inhibition of release by the antibody after 30 s of release stimulation (Figure 6B) than if the antibody was

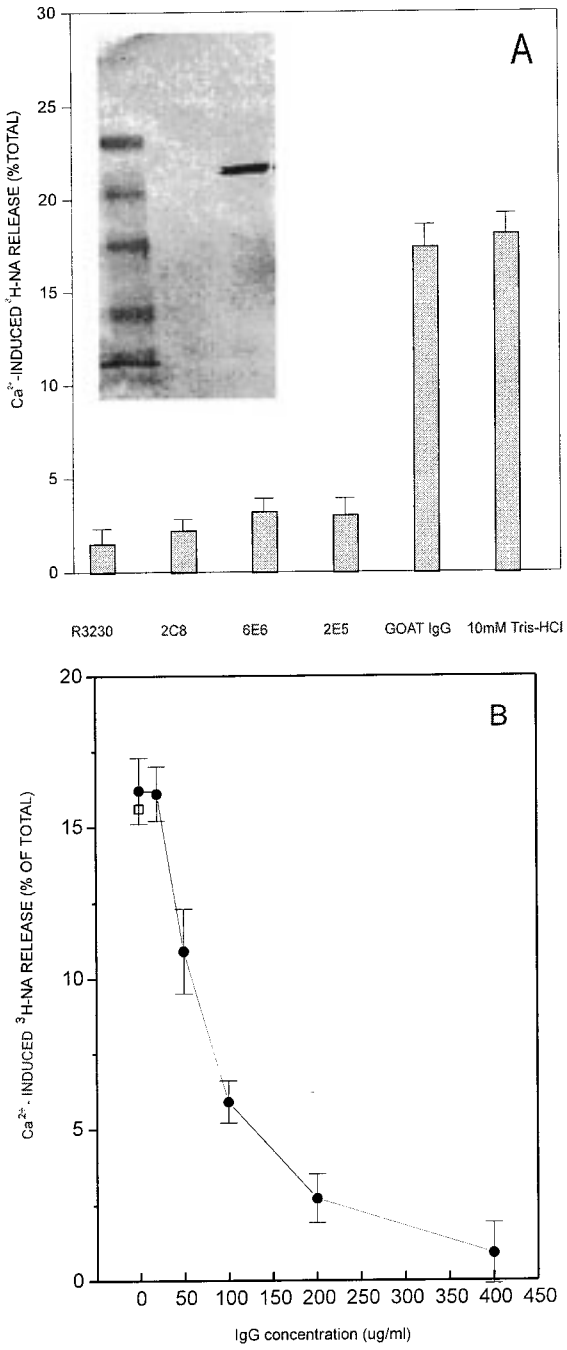

FIGURE 5: Effects of anti-NSF antibodies on Ca^{2+}-induced release. (A) Polyclonal (R3230) and monoclonal (2C8, 6E6, and 2E5) anti-NSF antibodies as well as goat IgG were further purified (>90%) by the E-Z-SEP antibody purification kit (Pharmacia). A 0.4 mg/mL aliquot of each antibody was added to permeabilized synaptosomes and incubated for 5 min at 37 °C, and the Ca^{2+}-induced release was determined as described. Inset: molecular weight markers (left lane) on a western blot of whole synaptosomal proteins separated on a 10% polyacrylamide gel and treated with the polyclonal antibody (right lane). (B) Concentration dependence of polyclonal anti-NSF (R3230) inhibition of Ca^{2+}-induced release. 400 μg/mL goat IgG (indicated as 0 μg/mL anti-NSF) and 25, 50, 100, 200, and 400 μg/mL anti-NSF were incubated with perforated synaptosomes in the presence of 10^{-8} M or 10^{-5} M Ca^{2+} for 5 min (□: Tris buffer control). Ca^{2+}-induced release under each condition was determined and plotted. Data were from two independent experiments ($n = 6$).

added simultaneously with perforation and release stimulation (Figure 6A). This indicates that the antibody is slowly entering the synaptosomes, but is inhibiting release from

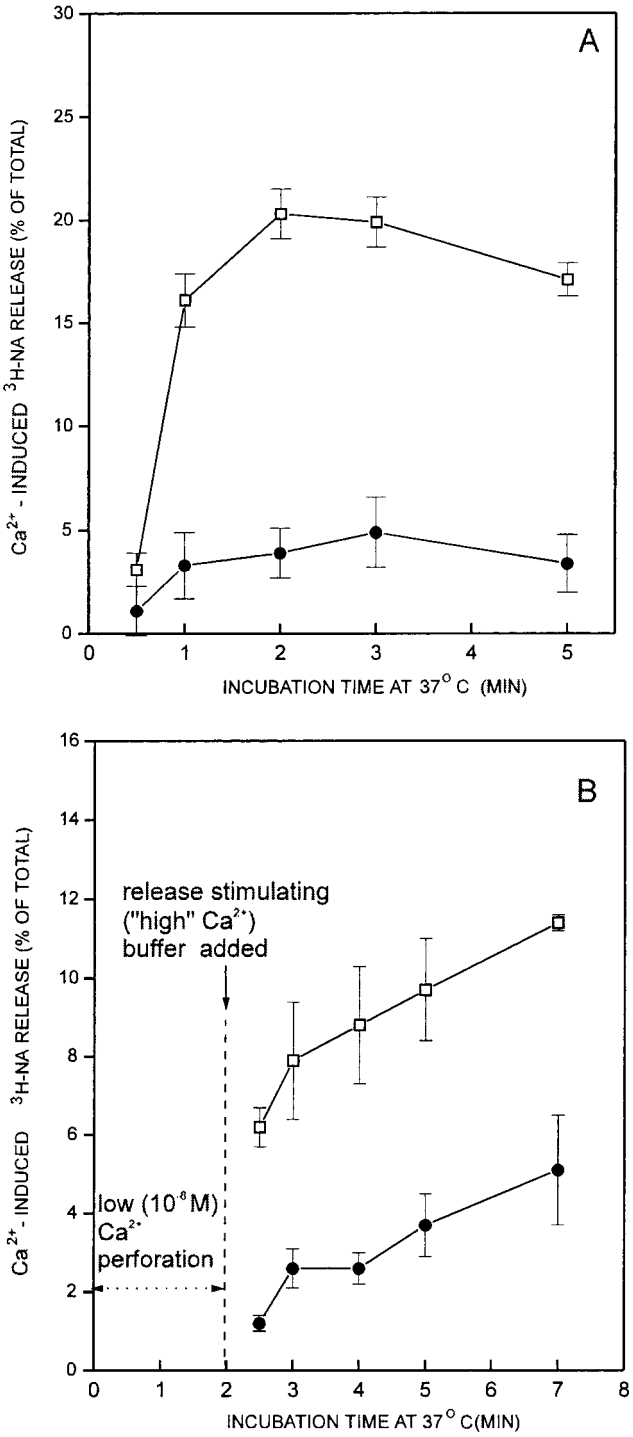

FIGURE 6: Effects of polyclonal anti-NSF on the time course of Ca^{2+}-induced release. ^{3}H-NA-labeled synaptosomes were perforated with 0.5 unit/mL SLO in the presence of 5 mM ATP, 0.5 mM free Mg^{2+}, and either 0.2 mg/mL goat IgG (□) or 0.2 mg/mL polyclonal anti-NSF (R3230) (●). Free Ca^{2+} was increased from 10^{-8} M to 10^{-5} M either simultaneous with perforation (A) or 2 min after perforation (B) to induce release, and the time course of Ca^{2+}-induced release was measured for an additional 5 min.

primed synaptic vesicles (cf. Figure 2). These effects are due to the anti-NSF IgG because adding this polyclonal antibody to synaptosomes perforated with *Staphylococcus aureus* α-toxin, which produces holes in the synaptosomal membrane too small to admit antibody molecules (*19*), had no significant effect on Ca^{2+}-induced release *(20)*. In addition, anti-NSF Fab fragments inhibited release less effectively than equivalent concentrations of whole antibody binding sites.[2] This suggests that the effects are on NSF because cooperative conformational rearrangements associated with its function might be inhibited to a greater degree by a bivalent binding molecule rather than two separately binding Fab fragments.

DISCUSSION

The work of several groups (summarized in *13*) has revealed that NSF separates v/t-SNARE complexes, and then individual SNARE proteins in opposed membranes combine to produce membrane fusion. Such a cycle of constitutive membrane fusion and re-formation contains a large free energy decrease when separated v- and t-SNARE proteins form coiled-coils immediately before membrane fusion. That decrease in free energy must be compensated by the ATPase activity of NSF "priming" and separating the coiled-coils, so the cycle can continue. A population of vesicles moving through this cycle would display uniform dependence on MgATP hydrolysis at all times, unless a metastable processing step halted cycling for a demonstrable length of time.

In regulated secretion, on the other hand, a metastable docking step clearly does occur, apparently thanks to synaptotagmin (*1, 2*). Here NSF action after membrane fusion corresponds to the steadily intensifying requirement for MgATP hydrolysis after the first minute of synaptosomal incubation (Figure 1). Therefore, it is not surprising that NEM (Figure 3) and anti-NSF (Figure 5) would have inhibitory effects on Ca^{2+}-dependent (exocytotic) release during this time. However, it is surprising that these two inhibitory probes also reduce exocytosis (Figures 3 and 6) during the MgATP-independent phase (first minute) of incubation, which undoubtedly corresponds to the fusion of previously primed, "docked but blocked" (by synaptotagmin) vesicles.

Does this indicate that NSF plays (an) additional roles(s) in regulated secretion that do(es) not involve MgATP hydrolysis? Several recent reports have shown that NSF can bind to membranes in an ATP-independent manner. Hong et al. (*21*) showed that synaptic vesicle-associated NSF was not dissociated by incubations with MgATP, and similar observations were made by Steel et al. (*22*) and Tagaya et al. (*23*) on coated vesicles and neuronal growth cones of PC12 cells, respectively. In yeast, Mayer et al. (*24*) observed that Sec18p (the yeast NSF homologue) was retained on vacuole membranes where it may bind to Pep12p (a probable yeast SNARE protein) (*25*). More recently, Colombo et al. (*26*) showed that NSF binds to a protein in endosomal membranes in the absence of α-SNAP, and β- and γ-SNAP are retained on the membranes after NSF action releases α-SNAP. Also, NSF, β-SNAP, and synaptotagmin form a complex that is not dissociated by MgATP (*27*). Taken together, these results suggest that in nerve endings binding sites for NSF could exist to permit NSF and β- and γ-SNAP retention for participation in events after MgATP hydrolysis. NSF staying at the membrane throughout fusion could provide interactions that would help to explain docked vesicles in the presence of botulinum toxins. An alternative interpretation is that, even if NSF does not participate after priming, it still binds to sites that are close enough to the primed vesicles that when inactivated by NEM or anti-NSF,

it will physically interfere with vesicle fusion. Though NSF's ATPase activity is inhibited by NEM, it does not interfere with some binding properties (*28*). The incomplete restoration of NEM inhibition by added NSF (Figure 4) may be due to the synaptosomes retaining the bulk of their NEM−inhibited NSF in a form that cannot be displaced by added NSF. Alternatively, incomplete restoration also may indicate that other NEM-inhibitable components are involved in exocytosis.

The existence of depriming (Figure 2) shows that the metastable, docked but blocked state is maintained by the continued provision of MgATP, and priming will reverse if MgATP is removed. The existence of a deprimed, off-pathway vesicle condition that must be reprimed by MgATP hydrolysis may be another reason NSF is kept close to the docked vesicle, which would enhance neuronal efficiency. Application of anti-NSF antibodies to deprimed vesicles may shed more light on this possibility.

We agree with Bannerjee et al. (*11)* that NEM inhibits priming and SNARE complex rearrangements. Nevertheless, the idea that NSF stays in the fusion complex after vesicle priming and possibly plays a role before Ca^{2+}-triggered membrane fusion differs from the finding that in mechanically permeabilized, semi-intact PC12 cells, NSF was released from fusion complexes after priming. Binding of NSF to the fusion complex could become weaker after ATP hydrolysis, or the lengthy incubations of semi-intact PC12 cells (30 min at 30 °C) might dissociate bound NSF from the complex as suggested by the selective losses of some proteins from the complexes (*11*). Alternatively, NSF may function differently in different cell types (possibly due to the presence of different accessory proteins) and/or NSF may have (an) additional role(s) in synaptic vesicle fusion.

Two major concerns when using perforated synaptosomes to study exocytosis as compared to perforated cells are the following: (a) the higher nonspecific efflux of transmitter molecules due to dynamic storage in synaptic vesicles compared with large dense core vesicles, which is enhanced by increasing temperature and the removal of ATP (*27*); and (b) the apparently rapid run-down of exocytosis (Figure 1). Plotting the data as we do provides a conservative, but reasonably accurate, reflection of real Ca^{2+}-dependent exocytosis; conservative because, as exocytosis decreases with incubation time, the amounts of measurable (tritiated) NA in each synaptic vesicle decrease due to continuing nonspecific neurotransmitter efflux. However, we recently have found (Bobich et al., in preparation) that this problem may be improved by the addition of GTP, which is not used by NSF (*29*), but is used by vesicular H^+-ATPase (*30*) to better keep neurotransmitter molecules in the synaptic vesicles. This increased efflux of neurotransmitter molecules in the absence of MgATP is nevertheless small enough up to 2 min of incubation to not alter the conclusion that the time course of release can be separated into MgATP-independent and MgATP-dependent stages, reflecting the docked and recycling stages of regulated synaptic vesicle exocytosis.

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