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Early requirement for α-SNAP and NSF in the secretory cascade in chromaffin cells

Tao Xu[1], Uri Ashery[2], Robert D.Burgoyne[3] and Erwin Neher

Department of Membrane Biophysics, Max-Planck-Institute for Biophysical Chemistry, Am Fassberg 11, D-37077, Göttingen, Germany and [3]The Physiological Laboratory, University of Liverpool, Liverpool L69 3BX, UK

[1]Present address: Department of Physiology and Biophysics, University of Washington, Seattle, WA 98195, USA

[2]Corresponding author
e-mail: uashery@gwdg.de

NSF and α-SNAP have been shown to be required for SNARE complex disassembly and exocytosis. However, the exact requirement for NSF and α-SNAP in vesicular traffic through the secretory pathway remains controversial. We performed a study on the kinetics of exocytosis from bovine chromaffin cells using high time resolution capacitance measurement and electrochemical amperometry, combined with flash photolysis of caged Ca^{2+} as a fast stimulus. α-SNAP, a C-terminal mutant of α-SNAP, and NEM were assayed for their effects on secretion kinetics. Two kinetically distinct components of catecholamine release can be observed upon fast step-like elevation of $[Ca^{2+}]_i$. One is the exocytotic burst, thought to represent the readily releasable pool of vesicles. Following the exocytotic burst, secretion proceeds slowly at maintained high $[Ca^{2+}]_i$, which may represent vesicle maturation/recruitment, i.e. some priming steps after docking. α-SNAP increased the amplitude of both the exocytotic burst and the slow component but did not change their kinetics, which we examined with millisecond time resolution. In addition, NEM only partially inhibited the slow component without altering the exocytotic burst, fusion kinetics and the rate of endocytosis. These results suggest a role for α-SNAP/NSF in priming granules for release at an early step, but not modifying the fusion of readily releasable granules.

Keywords: α-SNAP/caged calcium/exocytosis/membrane capacitance measurements/NSF

Introduction

Soluble NSF attachment proteins (α-SNAP) and *N*-ethylmaleimide-sensitive factor (NSF) have been proven to be essential for traffic through the Golgi complex (Wilson *et al*., 1989; Clary *et al*., 1990; Whiteheart *et al*., 1993) and for exocytosis (Morgan and Burgoyne, 1995a; Littleton *et al*., 1998). A set of three synaptic membrane proteins, including the synaptic vesicle protein synaptobrevin (VAMP), the plasma membrane proteins syntaxin and SNAP-25 (synaptosome-associated protein of 25 kDa), were identified as the receptors for SNAPs and NSF (Söllner *et al*., 1993). These three proteins were designated SNAREs (SNAP receptors) (Söllner *et al*., 1993). The SNAREs can form a tight 7S complex, which forms a high affinity binding site for α-SNAP and NSF. Upon binding of α-SNAP and NSF, the so-called 20S complex is formed and is disassembled due to NSF's ATPase activity, which is stimulated by α-SNAP (Morgan *et al*., 1994). Since the first proposal of the SNARE hypothesis (Rothman and Warren, 1994), many experimental data have been obtained in attempts to understand the physiological meaning of the disassembly of the SNARE complex by α-SNAP and NSF. However, the precise stages in which disassembly takes place along the sequence of vesicle traffic remain controversial.

In Rothman's original SNARE hypothesis, it was proposed that the disassembly of 20S complex by NSF provides the energy for fusion and should be the last step in secretion. On the other hand, there is potentially conflicting evidence against this version of the SNARE hypothesis, pointing instead to an early role for SNAPs and NSF (Morgan and Burgoyne, 1995b; Burgoyne and Morgan, 1998). First, it has been shown that most of the components of the 20S complex including α-SNAP and NSF are found on synaptic vesicles and chromaffin granules (Hong *et al*., 1994; Tagaya *et al*., 1995; Walch-Solimena *et al*., 1995; Burgoyne and Williams, 1997). This raises the question of why these so-called vesicle SNARE (v-SNARE) and target SNAREs (t-SNAREs) should be present on the same membranes. What is the physiological function of the t-SNAREs on the vesicles? More surprisingly, in a recent experiment addressing the assembly and disassembly of the ternary complex in the same membrane of synaptic vesicles, it has been shown that synaptobrevin, syntaxin and SNAP-25 readily associate with each other as soon as NSF is removed, and all of the ternary complexes are reversibly disassembled by treating synaptic vesicles with ATP-NSF and SNAPs (Otto *et al*., 1997). Similarly, SDS-resistant complexes recently have been observed also in purified fractions of chromaffin granules (Hohne-Zell and Gratzl, 1996). Furthermore, using a combination of genetics and biochemistry, Nichols *et al*. (1997) tested yeast vacuolar fusion after manipulation of the SNARE composition of vacuoles. Their results indicated the requirement of SNARE proteins for fusion, although a more recent study in the same system (Ungermann *et al*., 1998) postulated that the action of SNAREs is transient and not directly linked to the final fusion step. The studies in yeast showed that α-SNAP and NSF were only required before the vacuoles were mixed, which suggested that ATP hydrolysis is not necessary for the final fusion step but rather is required to prime individual SNAREs, probably to change their conformation to an activated state. This view is supported also by *in vitro* experiments from mammalian endosomes (Colombo *et al*., 1996).

Contrary to the *in vitro* studies, recent experiments in squid giant synapses suggested a requirement for NSF and α-SNAP at a late step in regulated exocytosis. By injecting peptides that inhibit the ATPase activity of NSF into the giant presynaptic terminal, it was shown that the amount of neurotransmitter release was reduced in a use-dependent manner. Surprisingly, the kinetics of exocytosis following a single action potential was slowed down. This was interpreted as a result of NSF actions that required vesicle turnover and occurred at a step subsequent to vesicle docking (Schweizer *et al*., 1998). Desynchronization of vesicle fusion events and slowing of the dilation of a fusion pore were discussed as possible consequences of NSF action in this context. Other physiological studies, however, indicated that ATP hydrolysis is not required for the final fusion step (Parsons *et al*., 1995; Banerjee *et al*., 1996). However, the possibility of early NSF action with late consequences in retarding fusion pore kinetics was not tested.

Given the apparent controversy, it is necessary to check the role of α-SNAP and NSF in the final fusion steps with high time resolution. Furthermore, a model for regulated exocytosis other than the yeast system seems to be important to address the dispute because the reactions in regulated exocytosis occur at a time scale which differs by that of the yeast system by three to six orders of magnitude. Adrenal chromaffin cells serve as an ideal system for this purpose, not only because it is possible to perform millisecond exocytosis assays using capacitance measurement and amperometry to monitor the time course of release from individual secretory granules, but also due to the ability to separate distinct kinetic components of secretion (Heinemann *et al*., 1994; Xu *et al*., 1998). In addition, by monitoring cell membrane capacitance and amperometric signals simultaneously, it is possible to estimate the rate of endocytosis, which can be used to address the question of whether NSF acts directly after fusion in the endocytotic pathway. Hence, α-SNAP, a C-terminally truncated α-SNAP, a mutant of α-SNAP, and NEM were dialyzed into adrenal chromaffin cells in whole-cell patch–clamp mode to test their effects on the kinetics of secretion. Our results suggest that α-SNAP and NSF action and thus the disassembly of the ternary complex occurs at an early stage of vesicle recruitment rather than at the late step for fusion itself. No changes in the kinetics of catecholamine release following individual fusion events are apparent in the amperometric recordings after NEM treatment. Moreover, the rate of endocytosis is not influenced in the presence of NEM.

Results

Chromaffin cells were studied in the whole-cell patch–clamp configuration in order to dialyze into the cytosol the reagents of interest and to measure the whole-cell membrane capacitance as an indicator of exocytosis. As we have described, there are three different components of Cm increases in chromaffin cells when $[Ca^{2+}]_i$ is elevated to certain levels, only two of which are related to catecholamine release (Xu *et al*., 1998). We call these two components the exocytic burst and slow component, according to Parsons *et al*. (1995). The exocytic burst happens within a few hundred milliseconds, and may represent the vesicles that are already primed and ready to release. We called these vesicles the readily releasable pool (RRP) (Neher and Zuker, 1993; Gillis *et al*., 1996). The slow component has a time constant on the order of 10 s. It thus may represent those vesicles that must undergo multiple steps of priming and maturation before they are ready for fusion, as we suggested in our recent paper (Xu *et al*., 1998). An example of these two components is shown in Figure 1A.

It has been suggested that there are multiple ATP-dependent steps in the exocytotic pathway (Holz *et al*., 1989; Chamberlain *et al*., 1995; Martin, 1997). In our previous paper (Xu *et al*., 1998), we have shown that the non-hydrolyzable ATP analogues, adenosine 5′-[β,γ-methylene]triphosphate (AMP-PCP) and β,γ-imido-adenosine 5′-triphosphate (AMP-PNP), completely block secretion after 5 min of pre-incubation. These reagents could not be hydrolyzed either by ATPases or kinases (Yount, 1975). In this study, we further tested another non-hydrolyzable ATP analogue adenosine 5′-o-(3-thiotriphosphate) (ATP-γS) since there is evidence that ATP-γS can be used by some protein kinases but not by ATPases (Yount, 1975). We included 2 mM ATP-γS instead of ATP in our internal solution and after 5–6 min for diffusion and exchange for endogenous ATP, a step-like $[Ca^{2+}]_i$ increase was elicited by flash photorelease of Ca^{2+}. In contrast to robust Cm increases in control cells, ATP-γS reduced both the exocytotic burst and the slow component, as shown in Figure 1B and C. The result complements our previously finding that ATP is required for maintaining both the exocytotic burst and the slow component and provides more information that this kind of ATP requirement does not involve certain protein kinases, such as PKC and PKA. We noticed that after 5–6 min of ATP-γS dialysis, at a time when all the endogenous ATP should have diffused out of the cell and the cytosol ATP-γS will be equilibrated with the patch pipette (Pusch and Neher, 1988; Parsons *et al*., 1995), there still remained a very small portion of Cm response to the first flash. We then asked the question whether the kinetics of the remaining exocytotic burst would be affected by ATP-γS. By comparing the kinetics of the exocytotic burst in control cells and cells treated with ATP-γS (data not shown), we concluded that ATP-γS itself did not change the kinetics of the exocytotic burst, which suggests that ATP hydrolysis is not involved in the final fusion event.

To test the effects of α-SNAP on secretion, 42 μg/ml α-SNAP protein was included in the patch pipette and 8–10 min were allowed for diffusion and action of the protein. Then step-like $[Ca^{2+}]_i$ jumps were elicited by photolysis of caged-Ca^{2+}. The Cm responses to these $[Ca^{2+}]_i$ increases were compared with those of control cells. The averaged Cm responses are shown in Figure 2A. Clearly, α-SNAP increased the amplitudes of both the exocytotic burst and the slow component. On average, α-SNAP increased the exocytotic burst by 69% while the slow component was increased by 129% (Figure 2C; see also Table I). The fact that α-SNAP increases the slow component more than the exocytotic burst is in favor of the idea that α-SNAP acts mainly on the slow process of vesicle recruitment. Two minutes after the first flash, we triggered a second flash. In cells dialyzed with α-SNAP, the Cm responses to the second flash are also larger, as

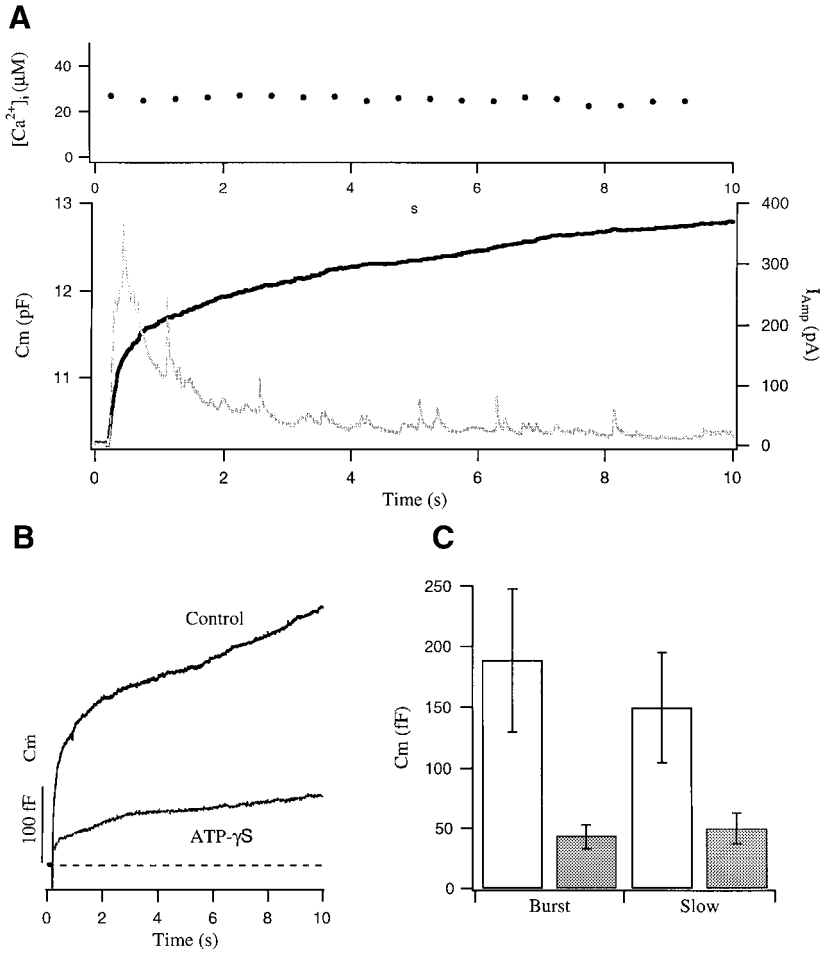

Fig. 1. ATP-γS reduces the exocytotic burst and the slow component. (**A**) An example of a typical exocytosis response triggered by a step-like $[Ca^{2+}]_i$ elevation. The Cm increases with a fast exocytotic burst followed by a slow component (lower panel, solid line). The corresponding amperometric signal confirms the two components (lower panel, gray line). (**B**) Both the exocytotic burst and the slow component are reduced in the presence of 2 mM ATP-γS. Flash photolysis was triggered after 5–6 min of dialysis in whole-cell configuration. (**C**) Comparison of the amplitude of the exocytotic burst and the slow component between control cells (open, $n = 10$) and cells dialyzed with ATP-γS (shaded, $n = 11$). The amplitude of the exocytotic burst is measured as the Cm increase within 0.8 s after the flash, while the slow component is measured as the Cm increase between 0.8 and 10 s after the flash.

Table I. Comparison of the effects of different treatments on Cm increase (in fF)

	Exocytotic burst		Slow component	
	Control	Application	Control	Application
α-SNAP	317 ± 60 ($n = 13$)	537 ± 80 ($n = 22$)[a]	179 ± 46 ($n = 13$)	410 ± 80 ($n = 22$)[b]
α-SNAP(L294A)	432 ± 63 ($n = 6$)	489 ± 106 ($n = 11$)	133 ± 39 ($n = 6$)	174 ± 90 ($n = 11$)
α-SNAP(1–285)	370 ± 59 ($n = 10$)	382 ± 49 ($n = 12$)	239 ± 68 ($n = 10$)	298 ± 73 ($n = 12$)
NEM	582 ± 67 ($n = 21$)	490 ± 62 ($n = 33$)	428 ± 70 ($n = 21$)	171 ± 47 ($n = 33$)[b]
NEM (2nd flash)	594 ± 136 ($n = 21$)	167 ± 40 ($n = 33$)[b]	231 ± 55 ($n = 21$)	59 ± 28 ($n = 33$)[b]
ATP-γS	189 ± 59 ($n = 10$)	44 ± 9 ($n = 11$)[a]	150 ± 50 ($n = 10$)	49 ± 13 ($n = 11$)[a]

[a]$p < 0.05$; [b]$p < 0.01$.

shown in Figure 2B. This provides further evidence that α-SNAP accelerates the recruitment of RRP. We then checked the influence of α-SNAP on the kinetics of the exocytotic burst. Figure 2D shows the normalized Cm increase of the exocytotic burst. No major change in the kinetics of the exocytotic burst could be detected. Since the kinetics of the exocytotic burst is steeply dependent on $[Ca^{2+}]_i$, we further compared the $[Ca^{2+}]_i$ dependence of the exocytotic burst in control and α-SNAP-treated cells. As pointed out by Heinemann *et al*. (1994), the exocytotic burst itself can be fitted with two exponentials. This is also the case in our Cm responses, and so we fitted the individual exocytotic burst with two exponentials and plotted the faster rate constant versus $[Ca^{2+}]_i$ (Figure 2E). Obviously, no major change can be observed between control and α-SNAP-treated cells. Furthermore, the slower rate constant was also not changed (data not shown). Thus, α-SNAP seems to increase the size of RRP by

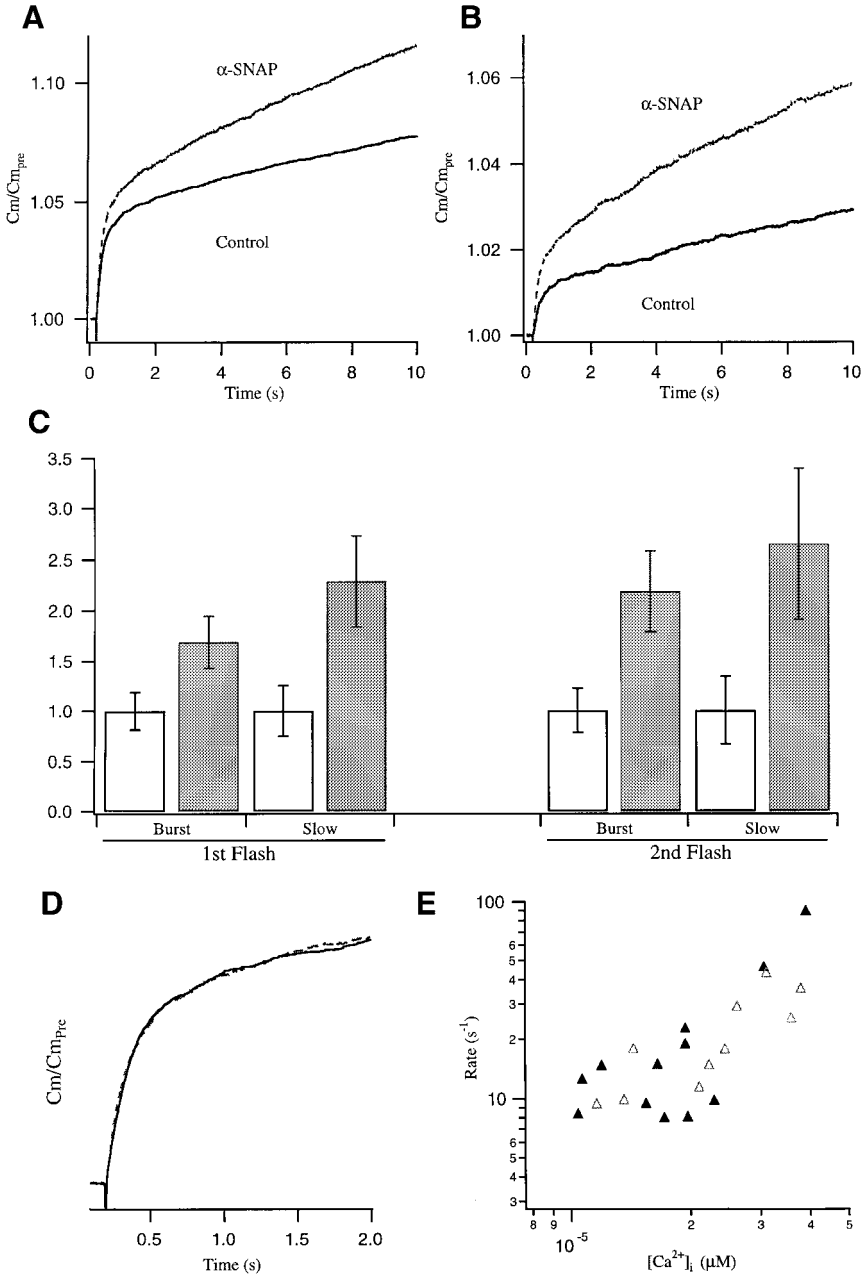

Fig. 2. α-SNAP increases the exocytotic burst and the slow component without affecting the kinetics of the exocytotic burst. (**A**) Amplitudes of the exocytotic burst and the slow component in response to the first flash were increased by α-SNAP. Averaged Cm responses from 13 control cells and 22 α-SNAP-containing cells were normalized to their pre-flash values. (**B**) Similar to (A), but for the subsequent second flashes. The larger Cm increase in the case of α-SNAP demonstrates that α-SNAP facilitates refilling of the vesicles. (**C**) Comparison of the amplitude of the exocytotic burst and the slow component between control cells (open, $n = 13$) and cells dialyzed with α-SNAP (shaded, $n = 22$) for the first and the second flash. (**D**) Normalized Cm traces from control cells (solid line) and α-SNAP-treated cells (dashed line) show no major change in the time course of the exocytotic burst in the presence of α-SNAP. (**E**) The dependence of the rate constant of the exocytotic burst on $[Ca^{2+}]_i$ is not altered by α-SNAP (filled triangles). Control, open triangles. The exocytotic burst was fitted by two exponentials and the faster rate constant is presented here.

accelerating the replenishment of vesicles to the RRP and does not change the kinetics of the final fusion step.

We further examined the effect of a C-terminal truncated mutant of α-SNAP [α-SNAP(1–285)] and a mutation of α-SNAP from leucine 294 to alanine [α-SNAP(L294A)]. These two proteins had no significant effect either on the size of Cm increase or on the kinetics. The effect of 40 μg/ml α-SNAP(1–285) is shown in Figure 3. Both α-SNAP(1–285) and α-SNAP(L294A) have decreased ability to stimulate NSF ATPase activity (Barnard *et al*., 1997) compared with wild-type α-SNAP (Morgan *et al*., 1994), but have similar affinities to bind NSF. It is interesting that α-SNAP(1–285) and α-SNAP(L294A) were not able to inhibit the slow component, which is consistent with the effects of these proteins in permeabilized chromaffin cells (Barnard *et al*., 1997). The reason for this is unclear, but it is likely that endogenous α-SNAP that is already associated with the SNAREs complex is not replaced by exogenous one within the 10 min of incubation, although addition of intact α-SNAP increases the number of functional complexes. Indeed, lack of inhibitory effect of these α-SNAP mutants was seen

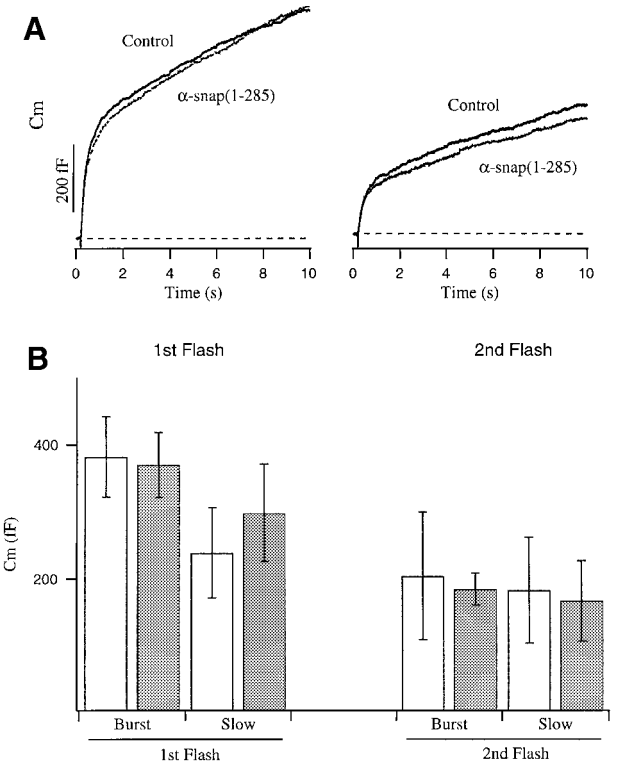

Fig. 3. α-SNAP(1–285) has no effect on exocytosis. (**A**) Averaged Cm traces in response to the first flashes (left panel) and second flashes (right panel) from control cells (solid line) and α-SNAP(1–285) containing cells (dashed line). (**B**) Summary of the amplitudes of the exocytotic burst and of the slow component between control cells (open, $n = 10$) and α-SNAP(1–285) containing cells (shaded, $n = 12$). Cells were dialyzed with α-SNAP(1–285) at 40 μg/ml for 10 min.

in permeabilized cells even after use of much higher concentrations (Barnard *et al*., 1997) and prolonged incubations with the proteins for up to 45 min (R.J.O. Barnard, A.Morgan and R.D.Burgoyne, unpublished observations). Over this time, a proportion endogenous α-SNAP still remained membrane associated (Morgan and Burgoyne, 1995a). Nevertheless, the α-SNAP mutants provide an important control for the stimulatory effect of wild-type α-SNAP.

We then investigated the effect of α-SNAP on the size of the RRP and on the kinetics of recruitment to the RRP following electrical stimulation. For that purpose, we took advantage of the double dual-pulse protocol described previously (Gillis *et al*., 1996; Smith *et al*., 1998). Briefly, the size of the RRP in chromaffin cells was estimated by two depolorazing pulses given in rapid succession. Then an upper bound to the RRP, B_{max}, can be calculated by

$$B_{max} = S/(1–R^2) \quad (1)$$

where S represents the sum of the capacitance response to the first (ΔCm1) and the second (ΔCm2) depolarizations, and R is defined as the ratio of ΔCm2/ΔCm1. We restricted the analysis to cells with *R*-values <0.6, because an accurate estimate of the RRP is only possible when substantial depletion of RRP occurs during the first pulse (see Gillis *et al*., 1996). By using this dual-pulse protocol at varying time intervals, one can estimate not only the pool size but also the dynamics of RRP recovery from

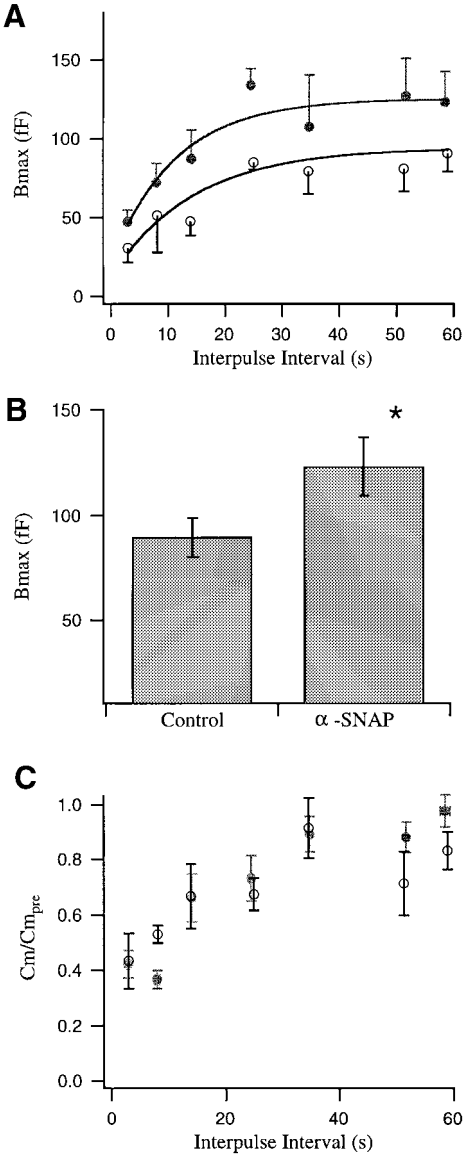

Fig. 4. α-SNAP causes an increase in the size of the RRP but does not change the refilling kinetics. (**A**) Plot of averaged B_{max} (a measure of RRP size) in control cells (open circles) and α-SNAP treated cells (filled circles) versus the time interval between stimuli. Superimposed traces are exponential fits with similar time constants. (**B**) B_{max} values for time intervals >35 s were pooled for control and α-SNAP-containing cells in order to estimate the steady-state size of RRP. The steady-state RRP size was 123 ± 14 fF for cells containing α-SNAP and only 89 ± 9 fF for control cells. The difference is significant (*t*-test, $p < 0.05$). (**C**) RRP size was normalized to its preceding size and presented as a percentage of the recovery. The kinetics of the refilling process is similar for control (open circles) and α-SNAP-treated cells (filled circles).

depletion (Moser and Neher, 1997; Smith *et al*., 1998). Here, we investigated the size of the RRP as well as the recovery of the RRP in the absence and presence of 42 μg/ml α-SNAP (Figure 4). Figure 4A shows that α-SNAP consistently increased the size of the RRP (filled circle). In this series of experiments, the steady-state RRP size was increased from 89 ± 9 fF ($n = 24$) in control cells to 123 ± 14 fF ($n = 37$; $p < 0.05$) in cells dialyzed with α-SNAP. The percentage increase (38%) is comparable with that of the increase which we saw in the exocytotic bursts in flash experiments. To compare the

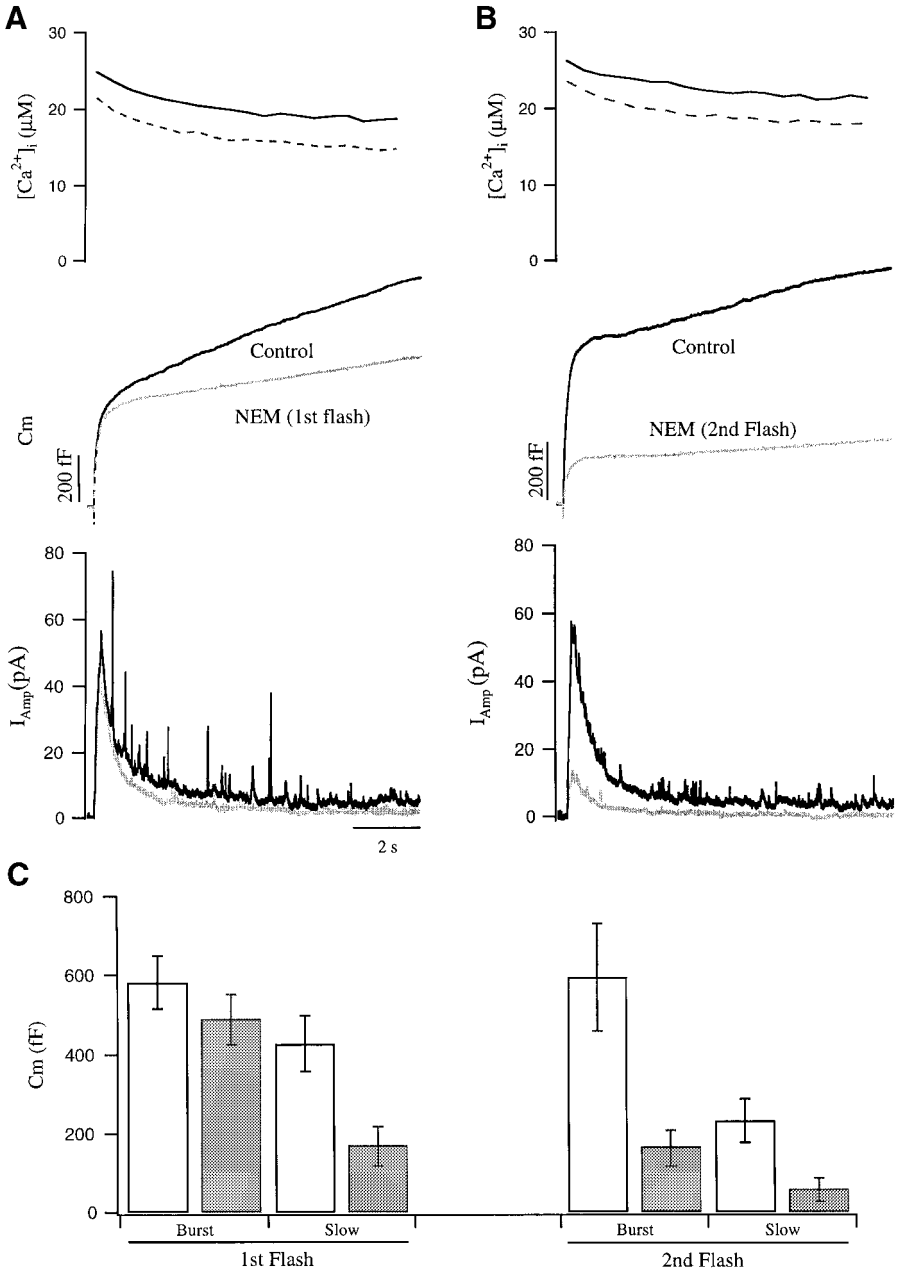

Fig. 5. NEM reduces the size of the slow component but leaves the exocytotic burst unaltered. The figure displays the averaged $[Ca^{2+}]_i$ levels, capacitance traces and amperometric currents in response to the first (**A**) and the second flash (**B**) in control condition (solid line, $n = 21$) and in the presence of 0.5–l mM NEM (dashed or grey line, $n = 33$). In the first flash applied to a given cell, NEM does not reduce the amplitude of the exocytotic burst significantly, but causes a significant decrease in the slow component which is confirmed by the amperometric signals. For the second flash (B), both the exocytotic burst and the slow component were substantially reduced. (**C**) The amplitude of the exocytotic burst following the first flash in control cells (open, $n = 21$) is almost unchanged by NEM (shaded, $n = 33$). However, the slow component of the first flash is attenuated by >50% in the presence of NEM. As a result of a block of the refilling process, the amplitude of the exocytotic burst and the slow component in the successive second flash are greatly reduced by NEM.

time course of recovery of RRP, the RRP size was normalized to its preceding steady-state RRP size and presented as a percentage of the recovery (Figure 4C). The resulting two curves overlap each other, demonstrating that the kinetics of RRP refilling after pool depletion was not changed by α-SNAP. According to the flash experiments (Figure 2), we suggested that α-SNAP increases the rate of replenishment of vesicles to the RRP. Thus, on a first look Figure 4C seems to contradict Figure 2, but actually the two results are well compatible when examined within the framework of a simple two-step model of secretion control, as suggested by Heinemann *et al*. (1993). In this model, vesicles are assumed to adopt any one of three separate states:

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} B \xrightarrow{k_2} C$$

Pool A is considered to be a large reserve pool of vesicles which are primed, by some way, into the release-ready vesicles of pool B (RRP). Vesicles in pool B either return to pool A, or undergo evoked Ca^{2+}-dependent

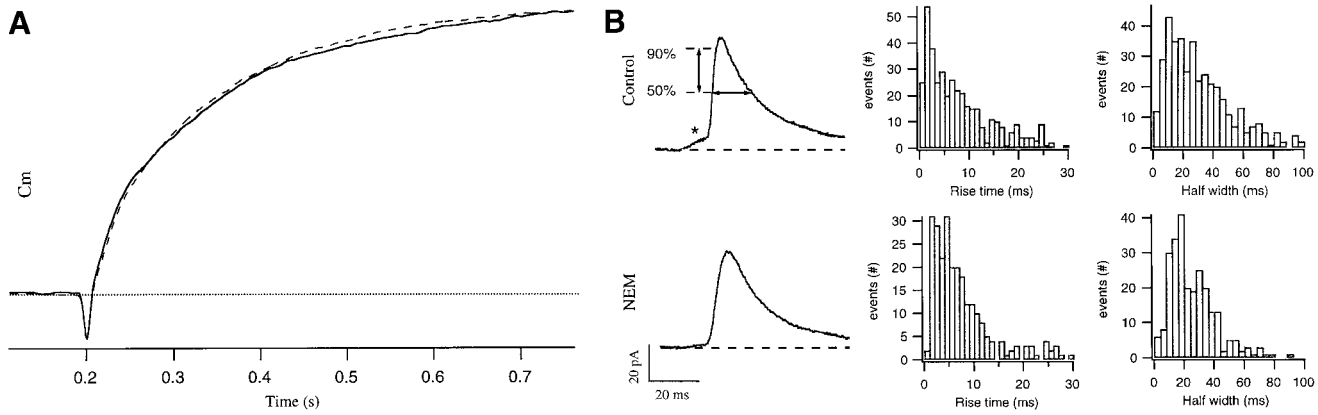

Fig. 6. NEM does not change the kinetics of fusion. (**A**) Normalized averaged Cm traces demonstrate that the kinetics of the exocytotic burst is unchanged by NEM (dashed line). (**B**) Left panels show representative amperometric spikes recorded from control (upper) and NEM-treated cells (lower). The vertical arrow marks the 50–90% of the spike peak amplitude. The horizontal arrow represents the half width. The foot is marked with an asterisk. The corresponding rise time and half-width distributions are shown in the middle and right panels, respectively. The average rise time is 7.8 ± 6.6 ms (mean ± SD) in control cells (389 spikes from 18 cells) and 7.39 ± 6.6 ms (mean ± SD) in NEM-treated cells (253 spikes from 26 cells). The average half-width was 30.1 ± 21.0 ms and 25.4 ± 15.2 ms (mean ± SD) for control and NEM-treated cells, respectively.

secretion and transition to pool C. In such a model the time constant of changes of pool B during period of rest (when k_2 is small) is given by the inverse of k_{-1}, as long as pool A is much larger than pool B (Heinemann *et al*., 1993), while the amplitude of pool B is given by Ak_1/k_{-1}. Although this model definitely is an oversimplification, it does describe many of the features of chromaffin cell secretion (Klingauf and Neher, 1997; Smith *et al*., 1998), and we therefore expect no major changes in the recovery time constant of pool B but only an increase in the amplitude of the recovery process, when either the forward rate k_1 or pool A is increased.

We have described above that α-SNAP does not change the kinetics of the exocytotic burst. However, this evidence may not be sufficient to conclude that α-SNAP is not involved in the final fusion event. If most of the endogenous α-SNAP were already part of the 20S complexes, the exogenous α-SNAP which might facilitate the exocytic burst, would not affect the kinetics too much. Hence we tested further the effect of NEM treatment, which blocks the NSF/ATPase activity. We tried to examine the effect of NEM on RRP size using the double-pulse protocol. However, NEM severely inhibited Ca current and thereby blocked secretion (data not shown). Therefore, we used only the Ca-uncaging experiments in the case of NEM. The idea is that we would expect to see some interference with the exocytotic burst if NSF and α-SNAP acted at the final step of exocytosis. We included 0.5–1 mM NEM in the pipette and waited for 5–10 min after establishing the whole-cell configuration. Figure 5 displays the comparison of the Cm responses to first flashes of individual cells. It can be seen that NEM largely (but not completely) blocked the slow component but left the exocytotic burst unaltered (Figure 5A). NEM inhibits the slow sustained amperometric signal verifying that the reduction of the slow Cm component by NEM is not due to an increased rate of endocytosis. In response to the second flash from the same batch of cells, there is a clear reduction in the exocytotic burst as well, suggesting the refilling of RRP is reduced by NEM treatment (Figure 5B and C).

Figure 6A documents in more detail that the kinetics of the exocytotic burst was unchanged by NEM. Since it has been reported that the kinetics of neurotransmitter release was slowed down as a result of blockade of NSF action (Schweizer *et al*., 1998), it is interesting to check whether the expansion of the fusion pore is retarded by NEM treatment. Thus, we analyzed the kinetic properties of individual amperometric spikes. If the NSF/ATPase activity plays a role at the last step of fusion or in dilation of the fusion pore, its inhibition might cause a decrease in the speed of granule fusion. This will be reflected as an increase in the rise time and the half-width of the amperometric spikes (Schroeder *et al*., 1996). However, the distributions of spike rise time and half-width from NEM-treated cells were similar to those from control cells (Figure 6B). The average rise time for control cells is not significantly different from that of NEM-treated cells (see Figure 6 legend). These results provide direct evidence that inhibition of NSF does not change the kinetics of fusion and support an involvement for NSF at early stages in the secretory cascade rather than direct involvement in the fusion process.

If NSF acts at early stages, when is the stable SNARE complex disassembled for another round of vesicle docking and fusion? It is possible that α-SNAP and NSF act after fusion to separate the SNARE proteins, which are then segregated to vesicle and plasma membrane for another round of secretion. Here, we explore the possible influence of NEM on the rate of endocytosis. Previous studies (Heinemann *et al*., 1994; Smith and Neher, 1997; Engisch and Nowycky, 1998) have revealed the co-existence of rapid excess endocytosis and slow compensatory endocytosis in chromaffin cells. In the case where rapid endocytosis is avoided by restricting intracellular $[Ca^{2+}]_i$ to values <50 μM (Haller *et al*., 1998; Xu *et al*., 1998; this study), the rapid phase of exocytosis measured by capacitance seems to be little contaminated by endocytosis and agrees well with the amperometric signals (Heinemann *et al*., 1994; Haller *et al*., 1998). Thus, by scaling the capacitance response and the integral of simultaneously recorded amperometric current according to their fast phases, one can tell the rate of endocytosis from the difference between the later sections of these two measurements. To quantify the level of endocytosis during the

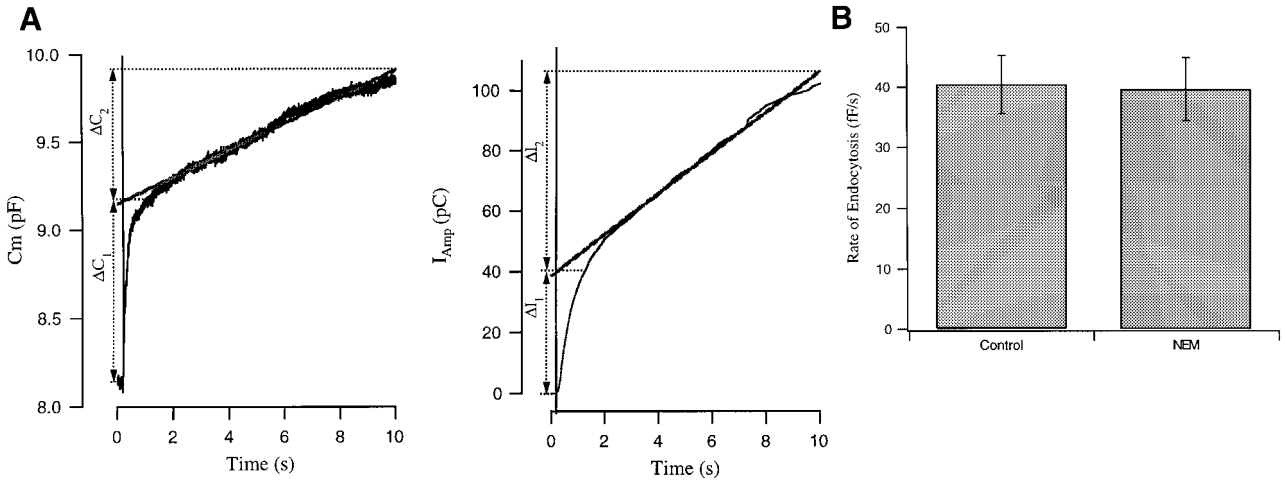

Fig. 7. NEM does not change the rate of endocytosis. (**A**) An example of membrane capacitance response (left) and integral of the amperometric signal (right) from a control cell. A line was fitted to the slow component and extrapolated back to 0.2 s to estimate the amplitude of the exocytotic burst (ΔC_1, and ΔI_1) and the slow component (ΔC_2 and ΔI_2). (**B**) The rate of endocytosis was calculated as described in the Materials and methods section. It was 40.4 ± 4.9 fF/s (n = 41) in control cells, not significantly different from 39.6 ± 5.3 fF/s (n = 32) in NEM-treated cells.

10 s stimulus, we calculated the ratio of the amplitude of the slow component over the exocytotic burst in the Cm measurement (R_{Cm}; see Materials and methods). R_{Cm} was then compared with the ratio of the amplitude of the slow component over the exocytotic burst as measured in the integral of amperometric signal (R_{Amp}). The difference between R_{Cm} and R_{Amp} will reflect the degree of endocytosis that occurred during capacitance measurements. It was found that R_{Cm} was consistently smaller than R_{Amp}, indicating that endocytosis activity occurred simultaneously (see Figure 7A). The rate of endocytosis was then calculated according to the formulas described in the Materials and methods section. The rate of endocytosis was found to be 40.4 ± 4.9 fF/s in control cells, which is not significantly different from 39.6 ± 5.3 fF/s in NEM-treated cells (Figure 7B). Thus, NEM inhibits specifically the slow component of secretion without any effect on endocytosis. In conclusion, α-SNAP and NSF seem to act at an early step in the exocytotic pathway rather than at the last step of vesicle fusion.

Discussion

The role of α-SNAP and NSF in vesicle recruitment

The exact site of the NSF/ATPase requirement in the secretory pathway remains to be established. Our data provides evidence that α-SNAP and NSF act at an early step in the exocytotic pathway rather than at a late step, and that exogenous α-SNAP increased recruitment of secretory vesicles into a RRP. Based on the presence of ternary SNARE complexes in the membrane of synaptic vesicles as well as on chromaffin granules, and the finding that SNARE complexes assemble in parallel with respect to their transmembrane anchors, Hanson *et al*. (1997) proposed a new model to explain the significance of the disassembly of ternary complexes by NSF. They postulate that the requirement of NSF/ATPase is to disassemble SNARE complexes in a given membrane in order to liberate the proteins for forming new complexes between membranes. The subsequent formation of the 'parallel' complexes by v- and t-SNAREs, which Hanson *et al*. called 'productive reassembly', would force the two membranes into close apposition and thereby enable the vesicle to become fusion competent (Hanson *et al*., 1997). This view is consistent with our findings that α-SNAP increases the amplitude of the slow component and NEM only partially blocks the slow component. It seems that the so-called productive reassembly is a rate-limiting step of our slow component in flash experiments. The stimulating effect of α-SNAP, then, is a consequence of its effect on disassembly of more SNARE complexes, which makes more vesicles available for intermembrane SNAREs reassembly. NEM, on the other hand, prevents disassembly of SNARE complexes in the single membrane of vesicles and reduces the number of vesicles available for subsequent formation of new SNARE complexes, as indicated by the inhibition of the slow component in the first flash and the blockade of the exocytosis in the successive flash. It is interesting that our NEM treatment does not eliminate the fusion of the exocytotic burst in the first flash. If the SNARE complexes are turning over in chromaffin cells (Xu *et al*., 1998), then it is possible that a longer period of pre-treatment or higher concentrations of NEM might become inhibitory to the initial burst of exocytosis, provided that the de-priming of these vesicles does not require NSF. However if de-priming of these vesicles depends on the activity of NSF to separate SNARE complexes, NEM may also prevent the loss of vesicles from the RRP under resting conditions and thus, may leave the RRP unaffected. With both possibilities, however, longer incubation periods or higher concentrations would be very problematic due to non-specific effects of NEM. Nevertheless, the point of these experiments is to test the effect of NEM (and thus the requirement for NSF) on the initial burst of exocytosis (due to docked and already primed granules) and on the slow recruiting process. It is clear that, already at the concentration and time used, NEM substantially inhibited the slow component and the second burst (presumably requiring some SNARE to prime new granules), but not the initial burst.

Although the conclusion reached from this study is not very different from that of recent studies on vacuolar

fusion in yeast (Ungermann *et al*., 1998), we do think that our findings add to the understanding of regulated fusion. We consider it important to demonstrate that the same sequence of steps is suggested in a secretion assay that is complete within seconds, as is found with an assay that measures secretion over a period of 3 h. Surprisingly, the result from the slow vacuolar system seem to indicate that SNARE proteins are not involved in the final step of membrane fusion (Ungermann *et al*., 1998), whereas our data on Botulinum toxin type A action (Xu *et al*., 1998) indicate that SNAP-25, the target of Botulinum toxin type A, is required in its intact form in order for the fastest (probably last) step of the exocytotic burst to reach its full speed. This indicates that further experiments are needed to identify the correspondence between steps in the two systems.

In this study, we further checked the possible role of NSF in the endocytotic pathway and in the process of fusion pore dilation. Two forms of endocytosis have been reported in chromaffin cells. One rapid form, termed excess retrieval, sequesters membrane very rapidly within seconds and requires very strong stimuli. The other, termed compensatory retrieval, happens at a much slower rate and seems to be responsible for most of physiological membrane recycling (Smith and Neher, 1997; Engisch and Nowycky, 1998). It is found that fast excess retrieval can be avoided by restricting the stimulation strength. Thus, we focused on the slow endocytosis in this study by applying $[Ca^{2+}]_i$ jumps to 10–40 μM in flash experiments. Interestingly, NEM treatment did not change the rate of slow endocytosis, indicating that the mechanism for retrieval of membrane does not need the involvement of α-SNAP and NSF, and therefore does not require prior disassembly of the SNARE complexes.

The high time resolution assay of secretion in our study does not reveal a slow down of the final fusion kinetics by NEM treatment. This disagrees somewhat with the experiment in squid giant presynaptic terminal where NSF peptides slowed the release kinetics (Schweizer *et al*., 1998). The reason for such a discrepancy remains to be clarified. At least one difference between the two systems should be considered, that is, that we mostly checked one round of vesicle fusion in our experiment, while in squid synapse the fusion of locally recycled vesicles might be prominent during repetitive stimulation. Thus, if the fusion of single vesicle involves multiple SNARE complexes (Vogel *et al*., 1996), one may get less SNARE complexes for each recycled vesicle when NSF is prevented from dissociating SNARE complexes after fusion. This may result in a retarding of the overall fusion reaction. Nevertheless, our data provide direct evidence that NSF and α-SNAP per se are not required for the final fusion event.

Multiple ATP-dependent 'priming' steps

It has been shown that the release capability of permeabilized cells can be primed by pre-incubation with ATP and deprimed by pre-incubation in the absence of ATP (Holz *et al*., 1989). The term 'priming', however, is very ambiguous since there seem to be multiple ATP-dependent steps along the secretory cascade involving several proteins (Hay and Martin, 1993; Chamberlain *et al*., 1995), all of which this term has been used for. At present, at least four possible roles for ATP in the secretory pathway must be considered. First, the requirement of ATP by the ATPase NSF, as discussed above. Secondly, an additional ATP-requiring step in exocytosis has been suggested to be the formation of phosphatidylinositol-4,5-phosphate-2 (PIP2) by phosphoinositide-4-kinases (PI4K) and phosphatidylinositol-4-phosphate-5-kinase (PIP5K). Both proteins were shown to be required for the ATP-dependent priming of exocytosis (Hay and Martin, 1993; Hay *et al*., 1995). PIP2 has been suggested to be important for the interaction between secretory granules and the cytoskeleton as well as with other secretory proteins such as CAPS (Martin *et al*., 1995; Martin, 1997). Thirdly, one should also consider the utilization of ATP by various kinases which have been shown to be relevant to increase the size of the RRP (Gillis *et al*., 1996) and even have been postulated to trigger exocytosis directly. Protein kinases A (PKA) and C (PKC) have both been shown to increase the frequency of spontaneous neurotransmitter release events (Singer and Goldberg, 1969; Llano and Gerschenfeld, 1993; Capogna *et al*., 1995). In addition, in pituitary gonadotrophes, PKC has been postulated to trigger exocytosis directly (Billiard *et al*., 1997). Finally, the ATP requirement for recruitment of vesicles by ATP-dependent motors such as myosin II and protein kinases like the Ca^{2+}-regulated myosin light chain kinase have also been suggested. Inhibition of ATP-dependent secretion by inhibitors of myosin light chain kinase and an activity-dependent inhibition of neurosecretion by myosin II antibody suggest a role for myosin-actin system in vesicle recruitment (Kumakura *et al*., 1994; Mochida, 1995). Disassembly of cortical F-actin has been shown to increase vesicle recruitment into the RRP (Roth and Burgoyne, 1995; Vitale *et al*., 1995).

Sequence of MgATP requirement in the secretory cascade

It may be tempting to rank the different MgATP requirements in the multistep processes of vesicle exocytosis. MgATP appears to be essential for a vesicle-recruitment step prior to docking as well as ATP-dependent priming reactions after vesicle docking. The term 'docking' is ambiguous due to its different meaning when different definitions are used: morphological (vesicle close to the plasma membrane revealed by electron microscopy), functional (docked vesicles readily undergo fusion upon stimulation) and biochemical (formation of SNARE complexes between vesicle and plasma membrane). In chromaffin cells, during typical ATP priming experiments, the release during test episodes is found to be dependent on ATP in preceding priming periods (Holz *et al*., 1989; Parsons *et al*., 1995), implying a role for MgATP in the recruitment of vesicles prior to docking. A recent experiment in goldfish bipolar terminals also provides evidence that MgATP is required for the recruitment of vesicles (Heidelberger, 1998). As for the functional and biochemical definition of docking, this ATP requirement may involve the NSF/ATPase activity to disassemble the SNARE complexes on the same vesicle membrane for subsequent intermembrane interaction. Another possibility could be ATP-dependent motors and protein kinases that are involved in releasing vesicles from the actin cytoskeleton for docking.

After vesicle docking, there may be additional ATP-dependent reactions. No evidence has been obtained that

the lack of ATP or the replacement of ATP by non-hydrolyzable ATP analogues would change the kinetics of the final Ca^{2+}-triggered fusion event, either in chromaffin cells (Parsons *et al*., 1995; this paper) or in bipolar terminals (Heidelberger, 1998). Thus, it appears that this ATP-dependent priming step happens before the fusion reaction. In a cell-free membrane fragment system containing docked (or tethered) granules, it has been shown that a post-docking ATP-dependent priming step is essential for exocytosis (Martin and Kowalchyk, 1997). In our previous study, we have shown that non-hydrolyzable ATP analogues AMP-PCP and AMP-PNP completely block secretion in chromaffin cells (Xu *et al*., 1998). In the present study, we found that ATP-γS, although it can be used by some protein kinases, blocked both the exocytotic burst and the slow component. Our results suggest an ATP requirement essential for the maintenance of fusion competence of the RRP of vesicles. NSF-catalyzed ATP hydrolysis cannot be the only requirement for preserving the exocytotic burst, because NEM treatment has little effect on the exocytotic burst while ATP-γS treatment does. A likely candidate for this ATP requirement might be PIP2 synthesis, which is ATP dependent. The synthesis of PIP2 requires the lipid kinases PI4K and PIP5K. Both enzymes need ATP to donate phosphate to phosphoinositide (Hay and Martin, 1993; Hay *et al*., 1995; Martin *et al*., 1995). So far, there is no direct evidence as to whether PI4K and PIP5K can use ATP-γS, but it is likely that ATP-γS is not a substrate of these enzymes (T.F.J.Martin, personal communication). If it is the case that these enzymes cannot use ATP-γS, the breakdown of PIP2 by PLC would lead to the depletion of PIP2 in the case of our ATP-γS dialysis experiments. According to our experiments, this could happen within <5 min. Thus, it is likely that the maintenance of a certain level of PIP2 plays an essential role after docking in somehow directing vesicles towards fusion. Synaptotagmin, the putative Ca^{2+} sensor, has been shown to exhibit Ca^{2+}-dependent PIP2 binding (Schiavo *et al*., 1996).

It remains to be established whether the ATP requirement by various protein kinases occurs before or after docking. PKA has been suggested to increase the Ca^{2+} sensitivity of the secretory machinery rather than increase the number of morphologically docked vesicles in hippocampal neurons (Trudeau *et al*., 1998). An earlier role for PKC in enhancing the docking of secretory granules through disruption of a cortical actin 'barrier' has also been suggested (Vitale *et al*., 1995). However, the fact that the RRP is only part of the morphologically defined 'docked' vesicles (Parsons *et al*., 1995; Plattner *et al*., 1997) does not allow us to rule out the possibility that PKC may act after docking to speed the maturation of docked granules to a fully fusion-competent state (Gillis *et al*., 1996).

Materials and methods

Cell preparation and solutions

Chromaffin cells from bovine adrenal glands were prepared and cultured as described previously (Smith *et al*., 1998). Cells were used 1–3 days after preparation. The external bathing solutions for experiments contained 150 mM NaCl, 2.8 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES and 2 mg/ml glucose (pH 7.2, 320 mosm). For preparing pipette solutions, we generally used 2× concentrated buffers, which contained 250 mM Cs-glutamate, 80 mM HEPES (pH 7.2). We added to the 2× buffer NP-EGTA (Molecular Probes, Eugene, OR), fura-2 (Texas Fluorescence Labs, Austin, TX), furaptra (Molecular Probes, Eugene, OR), $CaCl_2$, ATP, ATP-γS (Calbiochem) etc. for different purposes, as indicated in the text. The resulting mixtures were diluted with double distilled water for the appropriate osmolarity (310 mosm). The NP-EGTA containing internal solutions for control, prepared this way, consisted of 110 mM Cs-glutamate, 5 mM NP-EGTA, 4 mM $CaCl_2$, 2 mM MgATP, 0.3 mM GTP, 0.5 mM furaptra, 35 mM HEPES. The basal $[Ca^{2+}]_i$ was measured to be 100–300 nM by fura-2. The pipette solution was adjusted to pH 7.2 by either HCl or CsOH. All experiments were performed at 32–33°C.

Recombinant fusion proteins

α-SNAP, α-SNAP(1–285) and α-SNAP(L294A) were expressed in *Escherichia coli* M15 [pREP4] (Qiagen) induced by treatment with IPTG and purified on Ni-NTA–agarose as described previously (Whiteheart *et al*., 1993; Barnard *et al*., 1997).

Photolysis of caged Ca^{2+} and $[Ca^{2+}]_i$ measurement

Flashes of UV light and fluorescence excitation light were generated as described by Xu *et al*. (1997). To avoid any influence resulting from a 'loading transient' (Neher and Zuker, 1993), we used the more Ca^{2+}-selective caged compound, Nitrophenyl-EGTA (Ellis-Davies and Kaplan, 1994). The method for measuring the flash photolysis efficiency has been described previously (Xu *et al*., 1998). Since the $[Ca^{2+}]_i$ should decay significantly during 10 s of Cm measurement after flashes (Xu *et al*., 1997), we used the fluorescence excitation light to measure $[Ca^{2+}]_i$ and to simultaneously photorelease Ca^{2+} after the flashes in order to keep $[Ca^{2+}]_i$ more or less constant. $[Ca^{2+}]_i$ was calculated from the fluorescence ratio R according to Grynkiewicz *et al*. (1985).

Whole-cell patch–clamp and capacitance measurement

Conventional whole-cell recordings were performed with sylgard-coated 2–3 MΩ pipettes. Series resistance ranged from 4 to 12 MΩ. An EPC-9 patch–clamp amplifier was used together with Pulse software (HEKA Electronics, Lambrecht, Germany). Capacitance measurements were performed using the Lindau-Neher technique implemented as the 'sine+dc' mode (Gillis, 1995) of the software lock-in extension of pulse, which allowed long duration Cm measurement in single sweeps. An 800 Hz, 50 mV peak-to-peak sinusoid voltage stimulus was superimposed onto a DC holding potential of –70 mV. Currents were filtered at 2 kHz and sampled at 12 kHz. The capacitance traces were imported to IGOR Pro (WaveMetrics, Inc., Lake Oswego, OR). For the analysis of dual-pulse experiments, we used an IGOR macro adapted from Dr Corey Smith. The analyses were conducted on a PC computer using IGOR Pro. Unless otherwise stated, the data were given as mean ± SE.

Amperometry

Carbon fiber electrodes were prepared from 10 μm diameter carbon fibers (Amoco performance products, Greenville, SC) and were cannulated through glass capillaries. A constant voltage of 780 mV versus Ag/AgCl reference was applied to the electrode. The tip of the carbon fiber electrode was gently pressed against the cell surface. The amperometric current was filtered at 3 kHz, sampled at 10 kHz and further digitally filtered at 1 kHz. Artifacts of amperometry due to flash irradiation were subtracted using the averaged trace for the same fiber at the end of the experiment when there was no secretion. Single amperometric spikes were analyzed with IGOR macro adapted from Dr R.H.Chow. A spike was accepted if the amplitude was >3 times the value of the r.m.s. noise. Overlapping spikes were eliminated from analysis. Some spikes associate with a 'foot' that precede the spike and represent a transient release of catecholamine through the fusion pore (Chow *et al*., 1992). In some cases, the amplitudes of the foot reached up to 40% of the spike peak amplitude. In order to eliminate possible influence by the foot durations and amplitudes on the rise time measurements we calculated the rise-time from 50 to 90% of the spike amplitude.

Endocytosis measurement

The rate of endocytosis that occurs during capacitance measurement was estimated as follows. The slow Cm component was fitted by a line and was extrapolated to the *y*-axis at the beginning of the flash. The difference between the Cm baseline and the extrapolation represents the amount of secretion during the exocytotic burst (ΔC_1). The difference between the maximum secretion and ΔC_1 was designated as ΔC_2 (see Figure 7A).

The ratio between these two values is:

$$R_{Cm} = \Delta C_2/\Delta C_1 \quad (2)$$

The amperometric signal was integrated over the 10 s measurement to get a cumulative measure of release of catecholamine. In analogy to Cm analysis, the ratio between the slow component (ΔI_2) and the exocytotic burst (ΔI_1) (see also Figure 7A) of the integral of the amperometric signal was defined as R_{Amp}:

$$R_{Amp} = \Delta I_2/\Delta I_1 \quad (3)$$

If no endocytosis takes place, $R_{Cm} = R_{Amp}$. On the other hand, if the slow component of Cm increase is contaminated by endocytosis, then

$$\Delta C_2 = \Delta C_{2'} - \Delta Endo \quad (4)$$

where $\Delta C_{2'}$ represents the amount of exocytosis. Since $\Delta C_{2'}/\Delta C_1 = \Delta I_2/\Delta I_1$, we obtain

$$R_{Amp} - R_{Cm} = \Delta Endo/\Delta C_1 \quad (5)$$

Hence, the amount of endocytosis ΔEndo can be estimated by

$$\Delta Endo = (R_{Amp} - R_{Cm})^* \Delta C_1 \quad (6)$$

The rate of endocytosis then can be calculated by dividing ΔEndo by the duration of our Cm measurement after flashes, which is 9.8 s in our experiments.

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