Multiple kinetic components of exocytosis distinguished by neurotoxin sensitivity

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The secretion of synaptic and other vesicles is a complex process involving multiple steps. Many molecular components of the secretory apparatus have been identified, but how they relate to the different stages of vesicle release is not clear. We examined this issue in adrenal chromaffin cells, where capacitance measurements and amperometry allow us to measure vesicle fusion and hormone release simultaneously. Using flash photolysis of caged intracellular calcium to induce exocytosis, we observed three distinct kinetic components to vesicle fusion, of which only two are related to catecholamine release. Intracellular dialysis with botulinum neurotoxin E, D or C1 or tetanus-toxin light chains abolishes the catecholamine-related components, but leaves the third component untouched. Botulinum neurotoxin A, which removes nine amino acids from the carboxy(C)-terminal end of SNAP-25, does not eliminate catecholamine release completely, but slows down both catecholamine-related components. Thus we assign a dual role to SNAP-25 and suggest that its nine C-terminal amino acids are directly involved in coupling the calcium sensor to the final step in exocytosis.

Functional studies on secretory cells have supplied ample evidence that synaptic and other secretory vesicles can exist in distinct functional states[1–4]. Typically, only a fraction of all vesicles of a secretory cell can be released in a certain time window, independent of the strength of a given stimulus. These vesicles in a specific release-ready state are termed a 'pool'. The question arises whether pools defined in this or other ways can be associated with well defined macromolecular complexes of synaptic proteins, which have recently been characterized biochemically[5–7]. Some of the proteins of these complexes, such as SNAP-25, synaptobrevin and syntaxin, are the targets of very specific proteolytic neurotoxins, which cleave the proteins at unique sites[8–10]. These proteins are highly susceptible to toxin attack in monomeric form, whereas they are protected to various degrees in macromolecular complexes[11]. This property and differences between the toxins' actions may allow us to link certain functional pools to some of the biochemically defined complexes by their toxin susceptibility. Indeed, different kinetic components are impaired differentially by toxins[12,13]. Also, botulinum neurotoxin type A may be special among the clostridial neurotoxins, as its action in some preparations can be overcome by strong stimuli[14,15].

For a precise dissociation of functional steps and their correlation with molecular data, it would be desirable to study exocytosis at fast resolution, as the final steps in the neurosecretory pathway are known to be fast, and most likely neurotoxins act at later steps. We therefore studied catecholamine secretion from bovine chromaffin cells using capacitance measurement as a fast assay of exocytosis combined with flash photolysis of caged calcium as a fast and strong stimulus. We characterized two kinetic components that were differentially affected by clostridial neurotoxins. We compared the capacitance signal with simultaneously measured catecholamine release, and thereby identified a third very prominent kinetic component, which apparently was not related to catecholamine release, and which was not affected by toxins. Together, these data suggest possible relationships between late steps in the release process and their biochemically defined molecular counterparts.

Results

Multiple components of exocytosis in chromaffin cells

To study the kinetic components of secretion, we employed fast-resolution capacitance measurements to estimate the secretory response after spatially homogeneous elevation of internal calcium concentration ($[Ca^{2+}]_i$) by photorelease of caged calcium, nitrophenyl-EGTA. Membrane capacitance (Cm) is proportional to the surface area of the cell, and it increases when secretory vesicles fuse with the plasma membrane[16,17]. In most of our measurements, we used the following protocol (Fig. 1a). After ten minutes of whole-cell dialysis with either toxin-free or toxin-containing pipette solutions, we first gave low-intensity flashes (usually two) to generate small $[Ca^{2+}]_i$ jumps to about 20 μM. This readily elicited secretory responses (Fig. 1b). When there was a clear indication that secretion was depressed, we then gave strong flashes to elevate $[Ca^{2+}]_i$ to higher values (over 100 μM). Intervals between the flashes were 120 seconds. In response to the first flash, there was always a robust Cm increase with two clearly distinct phases, which we call the exocytic burst[18] and the slow component. Our

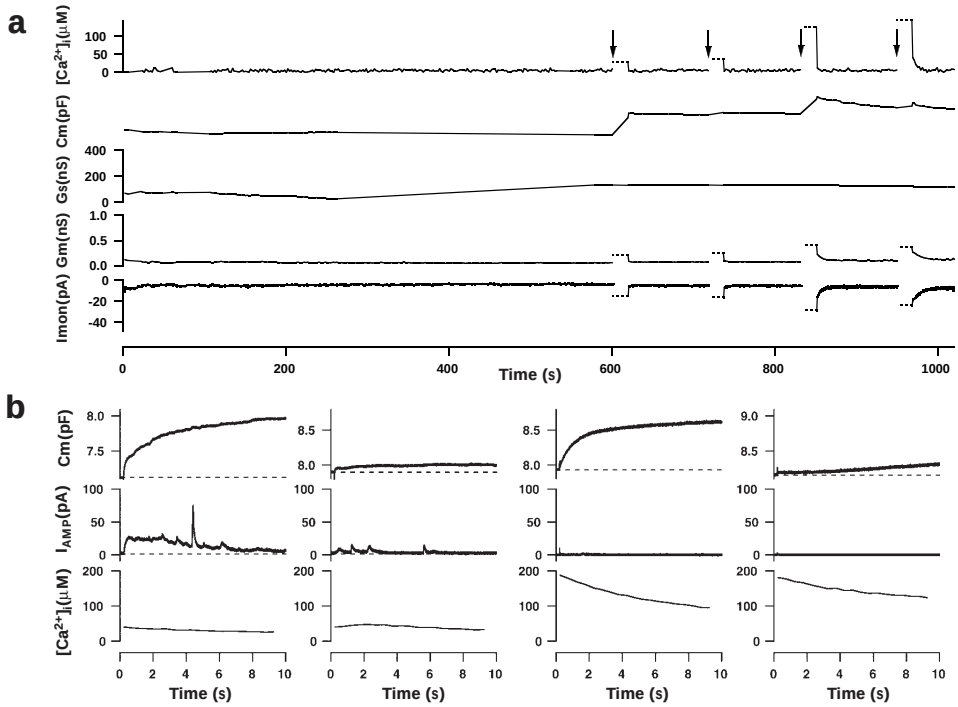

Fig. 1. Multiple secretory components in response to different $[Ca^{2+}]_i$ levels. **(a)** Overview of the experiment. Sample responses from one cell are illustrated. The individual traces are intracellular free calcium concentration ($[Ca^{2+}]_i$) as measured by furaptra, membrane capacitance (Cm), series conductance of the equivalent circuit (Gs), membrane conductance (Gm) and membrane current (Imon). Arrows indicate time points, where flashes are given. They are followed by gaps in the record, during which data sampling switched to a high-resolution mode. During ten minutes of whole-cell dialysis, no loading transient was seen in NP-EGTA-containing internal solution. The Cm traces remained flat until the onset of flashes (arrows). The flashes were given every 120 s. **(b)** Fast-resolution recordings of Cm, amperometric current and $[Ca^{2+}]_i$ in response to individual flashes in (a) are displayed. The flashes were triggered at 200 ms in each trace.

working hypothesis is that the exocytic burst represents those granules that are in a release-ready state and require only elevation of $[Ca^{2+}]_i$ for exocytosis. Subsequently, granules have to undergo slower steps of recruitment to the release-ready pool before they can exocytose. Therefore, further capacitance increase occurs at a much slower rate (see Discussion for details and a model). In 84 experiments, exocytic bursts were evoked at $[Ca^{2+}]_i$ averaging 29.3 ± 18.9 μM (mean ± SD). The time course could be fitted by double exponentials with average time constants of 62.6 ± 5.9 ms (n = 44 of 84) and 295.6 ± 23.1 ms (n = 76 of 84), and average amplitudes of 368 ± 40 fF and 302 ± 27 fF, respectively. This is similar to previously reported values[19] except for somewhat slower kinetics. The slow component showed a time constant in the range of ten seconds, but this is probably an underestimate, as capacitance at late times may be reduced by endocytosis and may be curtailed by a slow decline in $[Ca^{2+}]_i$. The amperometric current, which remained on a plateau level for up to ten seconds in some cases, also suggests that the time constant of this slow component may be over ten seconds. The increase in Cm always was accompanied by two phases of catecholamine release as monitored by carbon fibers (Fig. 2a). It should be noted that the faster component of the exocytic burst was also accompanied by catecholamine release (Fig. 2a, inset).

Following flashes, $[Ca^{2+}]_i$ dropped back to basal values at times longer than ten seconds. Second flashes in a given experiment usually elicited very small Cm increases and only rarely were accompanied by amperometric spikes, which might suggest the depletion of a vesicle pool during first flashes. However, when $[Ca^{2+}]_i$ was raised above 100 μM in third flashes, again an intense Cm increase was observed (Fig. 2b). This low-calcium-affinity Cm component increased with two exponentials. One had an amplitude of 404 ± 76 fF and a time constant of 1.1 ± 0.2 s. The second exponential had a time constant of 6.4 ± 0.9 s and 361 ± 62 fF in amplitude (n = 12). This large membrane increase, which constituted roughly 10% of the total cell membrane, was not associated with catecholamine release (see Figs. 1b and 6). Also, the time constant did not change with $[Ca^{2+}]_i$ (Fig. 2b). It rather seemed that Cm increases of this kind were elicited whenever $[Ca^{2+}]_i$ exceeded a certain threshold.

ATP DEPENDENCE OF CALCIUM-TRIGGERED EXOCYTOSIS

To test the ATP dependence of different exocytic components, we dialyzed the cytosol of chromaffin cells with 80% calcium-loaded NP-EGTA internal solution containing nonhydrolyzable ATP analogs, either adenosine 5'-[β,γ-methylene]triphosphate (AMP-PCP) or β,γ-Imidoadenosine 5'-triphosphate (AMP-PNP), via the patch pipette. While the basal $[Ca^{2+}]_i$ was kept at 100–300 nM, too low to support exocytosis, ATP was washed out of the cell and replaced by the analogs during the first five minutes of perfusion. Subsequently no responses could be elicited by flash photolysis, neither when secretion was measured by Cm nor by amperometry (Fig. 3a; n = 28 cells in paired experiments). These data complement the finding that the release capability of permeabilized cells can be increased ('primed') by preincubation with ATP and diminished ('deprimed') by preincubation in the absence of ATP[1]. Our results differ from a report that MgATP-independent exocytosis remained undiminished for even six minutes[2], although that experiment was done at a lower temperature than ours. The authors suggested that this form of 'depriming' may require magnesium, which was absent in their experiments. We therefore performed additional experiments

using an internal solution without magnesium and DM-nitrophen to chelate magnesium. The free concentration of magnesium in this solution was estimated to be less than 10 nM. Again no secretion was observed in the presence of 2 mM AMP-PCP (data not shown). Thus we conclude that magnesium does not matter for this effect.

We also used AMP-PNP to replace ATP. The effect of AMP-PNP was weaker than that of AMP-PCP (Fig. 3b). In some cells, AMP-PNP only partially blocked exocytosis, whereas in other cells AMP-PNP showed strong inhibition of exocytosis. However, AMP-PNP completely blocked the secretion elicited by a second flash, suggesting that ATP is necessary for the refilling of vesicles (Fig. 3c).

Our results confirm the importance of ATP in secretion. Although replacement of ATP by the nonhydrolyzable analogs AMP-PCP and AMP-PNP completely blocked both the exocytic burst and the slow exocytosis within five minutes, it is unlikely that ATP hydrolysis is involved in the exocytic burst for the following reasons. Experiments using the caged calcium DM-Nitrophen have shown that exocytosis can readily be induced earlier in a whole-cell recording in the complete absence of free magnesium and in a nominally ATP-free solution[2,4,18]. This conclusion is in line with studies on permeabilized cells[20–21] and with recent investigations showing that the action of N-ethylmaleimide fusion protein (NSF) is required before contact of vesicle and target membrane[22,23].

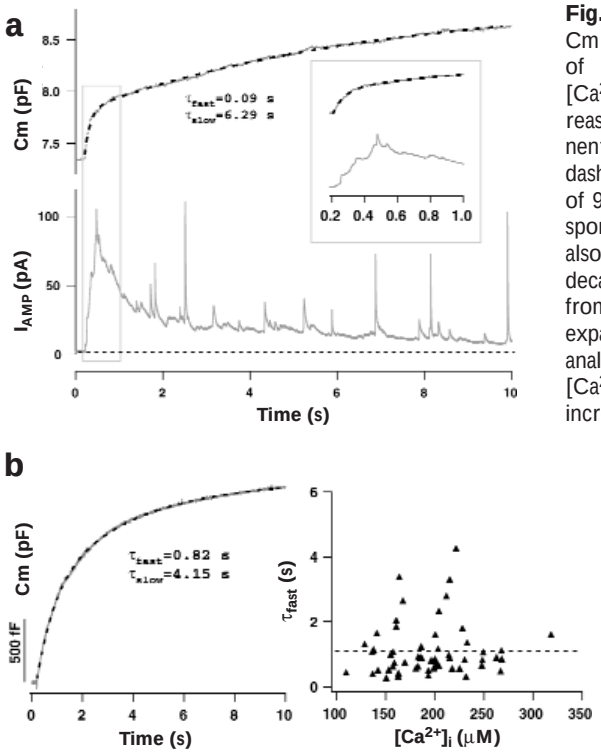

Fig. 2. Kinetic analysis of multiple Cm components. **(a)** An example of secretory kinetics at low $[Ca^{2+}]_i$ (27 μM). The Cm trace is reasonably fitted by two exponentials (superimposed thick dashed line) with time constants of 90 ms and 6.29 s. The corresponding amperometric current also shows a double exponential decay. The onset of secretion from 0.2 to 1 s (boxed area) is expanded in the inset. **(b)** Kinetic analysis of Cm response to high $[Ca^{2+}]_i$. One example of Cm increase at $[Ca^{2+}]_i$ of 287 μM is shown in the left panel. The Cm trace is reasonably fitted by two exponentials (superimposed thick dashed line) with time constants of 0.82 s and 4.15 s. The right panel displays a summary of the faster time constants of the Cm increases versus the $[Ca^{2+}]_i$ levels from 58 experiments. The dashed line is the mean value.

Complexes of synaptobrevin, syntaxin and SNAP-25 (termed SNARE complexes) are found in synaptic vesicle membrane *in vitro*[24,25], and such ternary complexes are reversibly disassembled by treating synaptic vesicles with ATP-NSF and SNAPs[25]. It is therefore possible that, in living cells, most of the SNARE complexes are continuously formed on the vesicle and continuously disassembled because of the activity of ATP-NSF, which might be the last step of ATP hydroly-

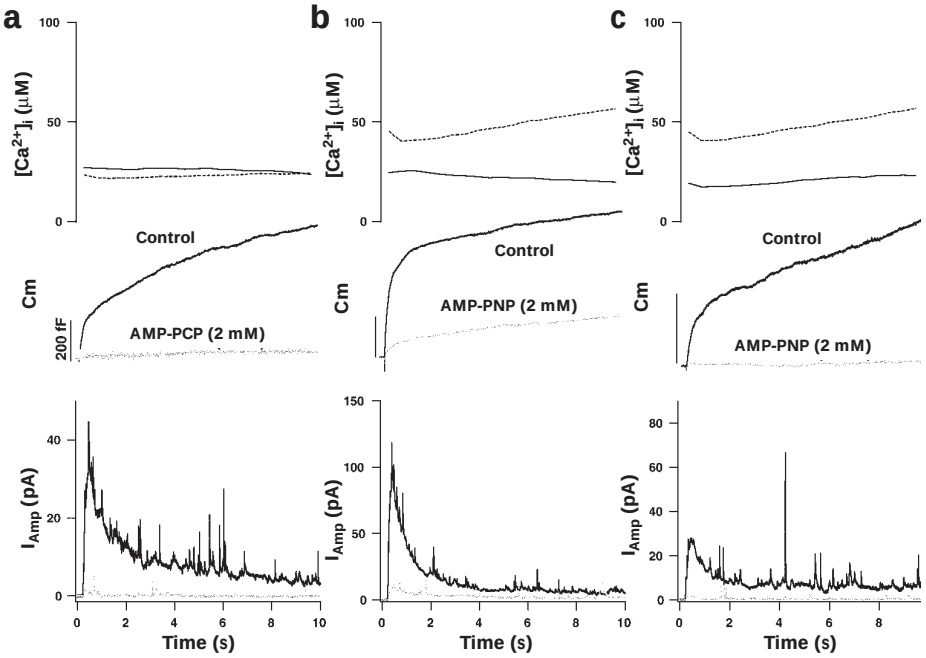

Fig. 3. ATP dependence of secretion. **(a)** The effect of 5–6-minute dialysis of AMP-PCP on secretion. Averaged $[Ca^{2+}]_i$ levels, capacitance traces and amperometric currents in response to the first flashes from paired experiments in the presence of MgATP as control (solid lines; *n* = 11) and in the presence of 2 mM AMP-PCP substituting for MgATP (dashed lines; *n* = 17) are displayed. Flashes were triggered at 200 ms in the graph. **(b)** The effect of 5–6 minute dialysis of AMP-PNP on secretion. Similar to (a), but 2 mM AMP-PNP was used to substitute for MgATP (*n* = 12). Control responses from paired experiments are from 11 cells. **(c)** Similar to (b) but for the successive second flashes. Cells are the same as those in (b).

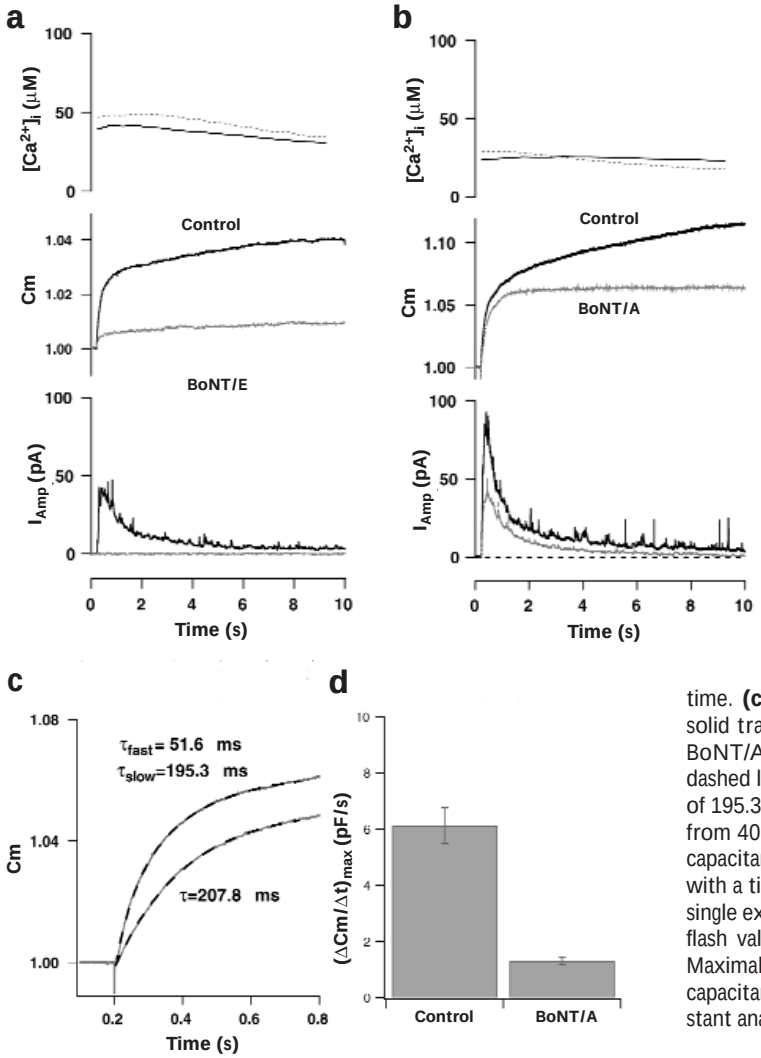

Fig. 4. The effect of BoNT/E and BoNT/A on secretion. **(a)** BoNT/E blocks both components of secretion at low $[Ca^{2+}]_i$. Averaged $[Ca^{2+}]_i$ levels, capacitance traces and amperometric currents in response to the first flashes from paired experiments in control (solid lines; n = 18) and in the presence of 400 nM BoNT/E-LC (dashed lines; n = 15) are displayed. Flashes were triggered after 10 min of whole cell dialysis, corresponding to 200 ms in the graph. The capacitance was normalized to its pre-flash values. **(b)** BoNT/A partially blocks secretion at low $[Ca^{2+}]_i$. Averaged $[Ca^{2+}]_i$ levels, capacitance traces and amperometric currents in response to the first flashes from paired experiments in control (solid lines; n = 24) and in the presence of 800 nM BoNT/A-LC (dashed lines; n = 37) are displayed. The capacitance was normalized to its pre-flash values. Flashes were triggered after 10 min of whole cell dialysis, corresponding to 200 ms in the graph. The capacitance trace gives the impression that the slow phase of secretion is blocked completely, whereas the amperometry trace reports some continuing release two seconds following the flash. This discrepancy may be due to slow endocytosis, which conceals a slow-release process in the capacitance trace at later time. **(c)** BoNT/A slows down the exocytic burst. The upper solid trace is the averaged Cm response from 55 cells without BoNT/A. It can be fitted by two exponentials (superimposed dashed line) with a fast time constant of 51.6 ms and a slow one of 195.3 ms. The lower solid trace is the averaged Cm response from 40 cells poisoned with 800 nM BoNT/A-LC. The onset of capacitance increase only shows a slow exponential component with a time constant of 207.8 ms. Superimposed dashed line is a single exponential fit. The capacitance was normalized to its pre-flash values. **(d)** BoNT/A reduced maximal rate of secretion. Maximal rates of secretion ΔCm/Δt were measured from the capacitance response. Δt was set to half the fastest time constant analyzed in the exponential fit of the cell.

sis. We have shown that replacement of ATP by AMP-PCP after a period of five minutes completely blocked both components of secretion. The *in vitro* results mentioned above offer an explanation for these findings: when ATP is replaced by a nonhydrolyzable analogue, the continuous disassembly of SNARE complex stops. Therefore the SNAREs on a given vesicle tend to form ternary complexes and are no longer available for complexes linking vesicle and plasma membrane. However, our data do not exclude additional requirements for ATP, such as in maintaining PtdIns-4,5P2 levels[26–28]. In fact, our finding that the exocytic burst is lost within five minutes in the absence of MgATP implies either that one of the last steps before fusion is affected by the prolonged absence of MgATP, or else that transmembrane SNARE complexes can be disintegrated without the action of NSF.

Clostridial neurotoxins and catecholamine secretion

Clostridial neurotoxins are potent inhibitors of synaptic-vesicle exocytosis in nerve terminals. It is now well established that the light chains (LC) of clostridial neurotoxins act as zinc-dependent metallopreoteases, which specifically cleave SNARE proteins. In particular, tetanus neurotoxin (TeNT) and botulinum neurotoxin (BoNT) serotypes B, D, F and G specifically cleave synaptobrevin at unique peptide bonds[8–10]. BoNT serotypes A and E cleave SNAP-25 at two different sites located close to the carboxyl terminals[29,30], whereas the targets of BoNT serotype C are syntaxin and SNAP-25[31,32]. To explore the role of SNARE proteins in the secretory pathway, we asked whether the kinetic components described above are differentially influenced by different clostridial neurotoxins.

If we assume there are 22,000 vesicles per chromaffin cell[33], and each vesicle contains 100 copies of synaptobrevin, the final concentration of synaptobrevin is calculated to be 3 μM in a single chromaffin cell. To test the time course of the cleavage of our recombinant light chains of clostridial neurotoxins, we first performed *in vitro* cleavage assays. The results show that, in our internal solution at 32°C, 330 nM TeNT-LC are capable of cleaving more than 95% of 5 μM recombinant GST-synaptobrevin2 within three minutes, and 40 nM BoNT/E-LC can cleave more than 95% of 5 μM recombinant SNAP-25His6 (data not shown). For a 10 MΩ access-resistance pipette, a protein with molecular weight of 50 kD, which is the size of the light chains of clostridial neurotoxins, can diffuse into the cytosol with a time constant of five minutes[34]. Thus within

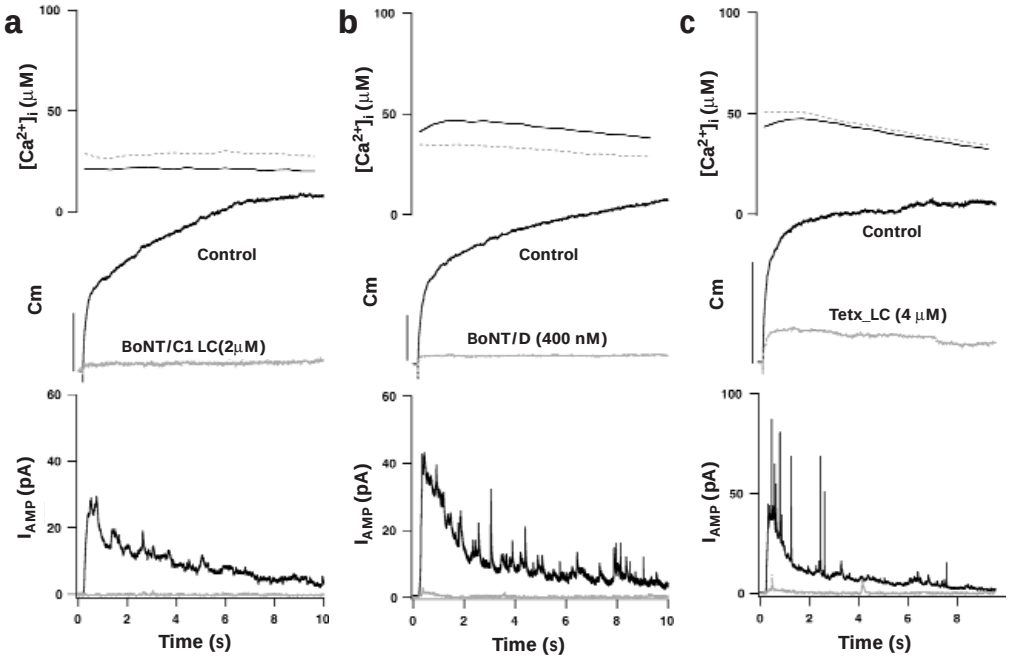

Fig. 5. The effect of BoNT/C1, BoNT/D and TeNT on secretion. BoNT/C1 **(a)**, BoNT/D **(b)** and TeNT **(c)** block both components of secretion at low $[Ca^{2+}]_i$. The figure displays the averaged $[Ca^{2+}]_i$ levels, capacitance traces and amperometric currents in response to the first flashes from paired experiments in the control condition (solid lines) and in the presence (dashed lines) of 2 μM BoNT/C1-LC (n = 7; control n = 6), 400 nM BoNT/D (n = 11; control n = 12) and 4 μM TeNT (n = 14; control n = 17). Flashes were triggered after 10 min of whole-cell dialysis, corresponding to 200 ms in the graph. Scaling bars, 200 fF.

five minutes, the cytosol concentration of the light chains of neurotoxins would reach 36% of the pipette concentration. Therefore, to test the role of SNAP-25 in exocytic processes, we used 400 nM BoNT/E-LC in the patch pipette and waited for ten minutes before elevation of $[Ca^{2+}]_i$ to ensure complete action of the toxin. As the action of clostridial neurotoxins are also steeply temperature dependent[35], the experiments were conducted at 32°C.

Under our experimental protocol, BoNT/E-LC not only blocked the slow phase of Cm increase, but also inhibited the exocytic burst (Fig. 4a; see also Table 1). Next, we wanted to assess the effects of BoNT/A, which cleaves the Q^{197}–R^{198} peptide bond very close to the C-terminus of SNAP-25 and thereby removes only nine amino acids, in contrast to BoNT/E, which cleaves the R^{180}–I^{181} peptide bond, 17 residues upstream. *In vitro* cleavage studies showed that BoNT/A has similar kinetics as BoNT/E (data not shown). However, even at 800 nM, which is twice the concentration used for BoNT/E, BoNT/A only partially inhibited secretion (Fig. 4b). BoNT/A reduced both the exocytic burst (Table 1) and the slow phase of secretion. The capacitance trace (Fig. 4b) seems to indicate complete block of the slow phase. However this result cannot be taken at face value, because endocytosis may compensate for ongoing exocytosis. Indeed, the amperometric trace indicates a small slow exocytic component after toxin treatment. By analyzing the amperometric trace, we concluded that the exocytic burst (approximately the integral response over the first two seconds) is reduced by a factor of 1.7, whereas the slow component (two to ten seconds) is reduced by a factor of 2.7. This strong reduction of the slow component suggests that BoNT/A affects a relatively early step in the secretory pathway by reducing the supply rate of release-ready granules. The complete block by BoNT/E (as compared to the partial one of BoNT/A) indicates that residues 181–197 at the C-terminus of SNAP-25 are essential for exocytosis, in accordance with previous studies[13,36].

Scrutinizing exocytic bursts revealed an additional change in BoNT/A-poisoned cells, which was that exocytic bursts were slowed down. The averaged Cm response from 50 control cells displayed an exocytic burst having two exponentials with time constants of 51.6 ms and 195.3 ms. However, the response of 46 BoNT/A-treated cells could be well fitted by a single exponential with a time constant of 207.8 ms, which is similar to the second component of the control cells (Fig. 4c). As a result, the initial maximal rate of rise of Cm was reduced 4.7-fold (see Fig. 4d). When Cm responses from BoNT/A-treated cells were analyzed individually, 33 out of 38 flash responses could be adequately fitted by a single exponential with an overall average amplitude of 398 ± 37 fF and a time constant of 281 ± 27.8 ms. This value is not different from that of the second component of exocytic bursts in control cells. Moreover, the time constant of this slow component was more or less constant in the $[Ca^{2+}]_i$ range between 15 and 70 μM. We therefore suggest that the truncated form of SNAP-25 cannot mediate a particularly rapid interaction between calcium-sensor and release machinery for catecholamine release (see Discussion). Alternatively, this suppression of the rapid phase of the exocytic burst by BoNT/A may represent the block of a

Table 1. Amplitudes of the exocytic burst in paired experiments

Experiment	Control (fF)	Treatment (fF)
AMP-PCP/NP-EGTA	242 ± 47 (n = 9)	21 ± 3 (n = 16)
AMP-PCP/DM-nitrophen	340 ± 82 (n = 4)	14 ± 7 (n = 10)
AMP-PNP/DM-nitrophen	496 ± 61 (n = 11)	79 ± 27 (n = 12)
BoNT/A (800 nM)	511 ± 38 (n = 50)	363 ± 27 (n = 38)
BoNT/E (400 nM)	196 ± 27 (n = 13)	37 ± 9 (n = 14)
BoNT/C1 (2 μM)	260 ± 37 (n = 6)	29 ± 4 (n = 6)
BoNT/D (400 nM)	423 ± 86 (n = 11)	43 ± 11 (n = 13)
TeNT (4 μM)	226 ± 28 (n = 13)	44 ± 8 (n = 12)

The capacitance values were measured at 800 ms after flashes. Values given are mean ± SE.

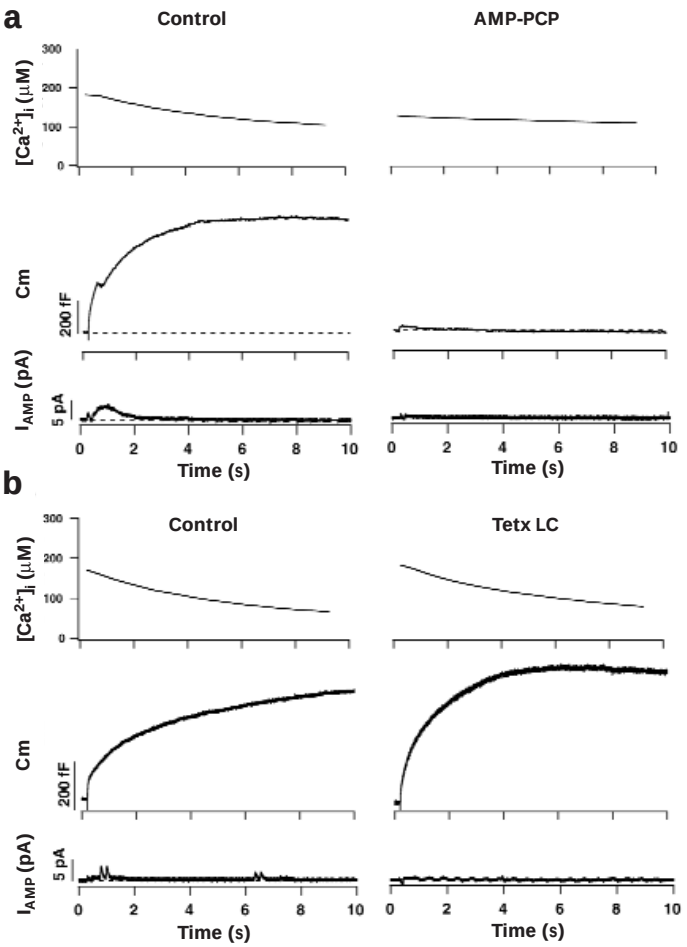

Fig. 6. Secretion at high $[Ca^{2+}]_i$ is sensitive to ATP but not to clostridial neurotoxins. **(a)** Comparison of secretory responses to high $[Ca^{2+}]_i$ after exhaustion of exocytosis at low $[Ca^{2+}]_i$. Averaged $[Ca^{2+}]_i$ levels, capacitance traces and amperometric currents from paired experiments in the presence of MgATP as control (n = 7) and in the presence of 2 mM AMP-PCP substituting for MgATP (n = 17) are displayed. The flashes were usually the third ones in a given cell. **(b)** The effect of TeNT on secretory responses to high $[Ca^{2+}]_i$ after exhaustion of exocytosis at low $[Ca^{2+}]_i$. Averaged $[Ca^{2+}]_i$ levels, capacitance traces and amperometric currents from paired experiments in control (n = 7) and in the presence of 4 μM TeNT-LC (n = 6) are displayed. The flashes were usually the third ones in a given cell.

small, more rapid and more toxin-sensitive component of exocytosis, not necessarily related to catecholamine release. In conclusion, BoNT/A has a dual effect, one at a relatively early step and another one modifying the exocytic burst, which is considered to be the final step in exocytosis.

BoNT/C1 cleaves both syntaxin and SNAP-25 in chromaffin cells[31]. This toxin cleaves SNAP-25 at a peptide bond (R^{198}–A^{199}) adjacent to the BoNT/A cleavage site (V.V. and H.N., unpublished results). To test the role of syntaxin in different components of secretion in chromaffin cells, we used 2 μM BoNT/C1-LC in the pipette solution. If an intact syntaxin were not essential for exocytosis, we would expect the same incomplete inhibition as seen with BoNT/A. However, BoNT/C1 completely blocked the secretion at low $[Ca^{2+}]_i$, suggesting that syntaxin is important in the final step of exocytosis (Fig. 5a).

Synaptobrevin is the substrate for TeNT and BoNT/B, D, F and G. In the presence of 400 nM BoNT/D-LC, capacitance and amperometric measurements (Fig. 5b) show that both components of secretion are blocked at low $[Ca^{2+}]_i$. Similar results were also obtained for 4 μM TeNT-LC (Fig. 5c), suggesting that synaptobrevin also is critical for the final steps of exocytosis. Comparing effective toxin concentrations shows that BoNT/D is much more potent in blocking secretion than TeNT. The inactive mutants of TeNT-LC (E234Q) and BoNT/C1-LC (E230A) had no effect on secretion, suggesting that the blockage of clostridial neurotoxins that we observed here was due to the toxins' metalloprotease activities.

It may be considered surprising that exocytosis is completely blocked within a time window of about ten minutes by many toxins that do not cleave their targets as long as those proteins are part of a SNARE complex, as many recent models envisage an intact SNARE complex as part of a release-ready fusion machinery. If this is the case, our findings imply that the respective proteins must cycle through a nonprotected state (which is the monomeric form) within a few minutes. Otherwise, we would expect the exocytic burst to be toxin resistant.

ATP-DEPENDENT, TOXIN-INSENSITIVE CAPACITANCE COMPONENT

We have shown that there is an additional large Cm increase at high $[Ca^{2+}]_i$ that is not related to catecholamine release. This Cm response is dependent on ATP. When we substituted ATP with AMP-PCP, this component vanished completely (Fig. 6a). This result gives us some confidence that the signal is not merely an artifact of Cm measurement.

We further tested this Cm increase for its sensitivity to clostridial neurotoxins. To do so, we first depleted the Cm responses by repetitive stimulations at low $[Ca^{2+}]_i$. Then, we elevated $[Ca^{2+}]_i$ to high levels in order to elicit this component. This way we could infer that this Cm increase does not represent the same pool as that measured at low $[Ca^{2+}]_i$. This protocol also provided the advantage that we could judge whether the neurotoxins were active in a given experiment. Even when the neurotoxins succeeded in blocking secretion at low $[Ca^{2+}]_i$, they failed to block the Cm increases at high $[Ca^{2+}]_i$. Figure 6b shows that TeNT-LC did not inhibit Cm responses at high $[Ca^{2+}]_i$. The same results were obtained with BoNT/E, D, A and C1. These results suggest the existence of an additional type of vesicles devoid of catecholamines, which are fusion competent at $[Ca^{2+}]_i$ over 100 μM. Exocytosis of these vesicles is not sensitive to clostridial toxins but is dependent on ATP. Above, we concluded that this intermediate component is elicited whenever $[Ca^{2+}]_i$ exceeded a certain threshold. This point is strengthened by the toxin results reported here, as no indication of this component was observed after toxin treatment when $[Ca^{2+}]_i$ increases were restricted to below 70 μM.

The existence of a Cm increase not related to catecholamine release that is triggered at $[Ca^{2+}]_i$ above 100 μM is somewhat puzzling. The presence of vesicles other than chromaffin granules may be an explanation. One possibility might be that low $[Ca^{2+}]_i$ mainly triggers the fusion of catecholamine-containing chromaffin granules, whereas high $[Ca^{2+}]_i$ preferentially triggers the exocytosis of synaptic-like microvesicles, which contain acetylcholine. This postulate agrees with the general view that

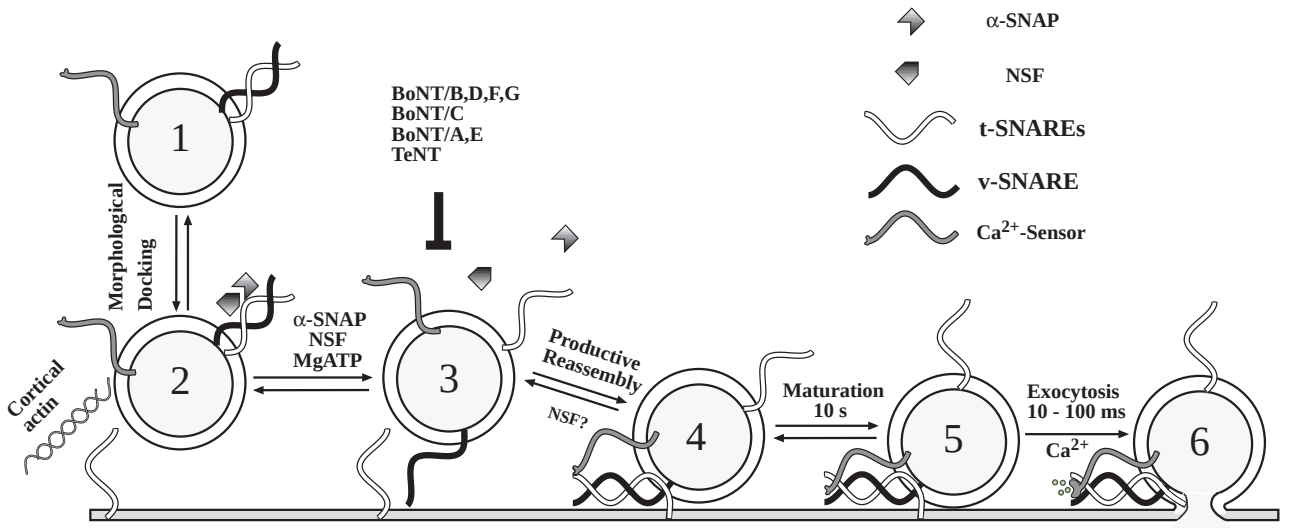

Fig. 7. Hypothetical model for the kinetic steps leading to exocytosis. Left, Morphological docking may require cortical actin, Munc18, Doc2 and similar proteins, but may not involve the interaction of v- and t-SNAREs. The 7S complex existing on the vesicle membrane prevents the v-SNAREs from interacting with plasma membrane t-SNAREs. The 20S complex is formed after the 7S complex binds to α-SNAP and NSF. It is disassembled by ATP hydrolysis, which makes v-SNAREs available to interact with plasma membrane t-SNAREs. For simplicity, 7S complexes are displayed as consisting only of one v-SNARE and one t-SNARE. In the productive reassembly of SNARE complexes between membranes, the energy released by complex formation is used to put the membranes under tension and to make the vesicle release ready. Calcium-sensor protein may interdigitate with the distal tail of the SNARE complex's coiled coil structure. A calcium-dependent conformational change might put additional torsion on the coiled-coil, forcing the two membrane anchors together and initiating fusion. The numbers represent functionally discrete pools of vesicles.

the $[Ca^{2+}]_i$ threshold for triggering small clear vesicles is higher than that for large dense-core vesicles. However, our data are inconsistent with this idea. Chromaffin cells express acetylcholine receptor channels. Therefore one might expect to observe a transient conductance following a flash if acetylcholine were released. This was not the case, however. Furthermore, the resistance of this non-catecholamine Cm increase to clostridial neurotoxins suggests that the involvement of SNAREs is not necessary for the fusion of the underlying vesicles. By exclusion, then, the explanation for this peculiar Cm increase seems to be that of an unspecific membrane fusion triggered by high level of $[Ca^{2+}]_i$, which the absence of ATP somehow prevents. Alternatively, this fusion event could represent a previously undetected process that involves a toxin-resistant set of SNARE proteins, as has been suggested for the apical route in polarized cells[37,38].

Discussion

In previous work, at least three late steps have been distinguished in the secretory pathway of neuroendocrine cells: morphological docking, priming, and exocytosis. Various properties have been assigned to the individual stages; thus, docking is ATP-dependent[28], priming is dependent on both ATP and calcium[20,39] and exocytosis shows a steep calcuim dependence[18,19]. Evidence for these properties has been obtained from different laboratories by very different techniques and by experiments on diverse time scales. It is a question of intense current research how features defined in such different ways correspond to each other, how they relate to molecularly defined states, and which of these states are susceptible to (or else protected from) the proteolytic action of clostridial neurotoxins.

Electrophysiological experiments showed that sufficiently strong stimuli elicit a so-called exocytic burst, which is interpreted to represent complete exocytosis of a pool of release-ready granules (the 'readily releasable pool', RRP). This size of this pool depends on $[Ca^{2+}]_i$ preceding the stimulus and on activation of PKC[4,40]. Following the exocytic burst, release proceeds at a much slower rate, which is believed to represent the combined process of recruiting vesicles to the RRP. Flash photolysis of caged calcium together with rapid assays of exocytosis, such as membrane-capacitance measurement and amperometry, allow a detailed kinetic analysis of secretory responses. Our goal was to establish links between the distinct kinetic components of such $[Ca^{2+}]_i$ jump experiments and molecular processes.

Five of the findings reported in our study bear on this issue. First, we eliminated one of the kinetic components as a candidate for a step in the release of catecholamines. Second, we showed that five minutes of neurotoxin action in the absence of stimulation are sufficient to eliminate the exocytic burst. This implies all of SNAP-25 or syntaxin or synaptobrevin must cycle through a toxin-sensitive state within that time period. Thus, any toxin-protected SNARE complex as part of a mature fusion machinery must have a lifetime shorter than five minutes. Third, cleavage of SNAP-25 by BoNT/A suppresses exocytosis only partially and manifests itself both at a slow (seconds to minutes) and at the fastest step by retarding the reactions in which SNAP-25 is involved. Fourth, the size of the exocytic burst is only partially affected by BoNT/A. Fifth, the time constant of the exocytic burst after BoNT/A action is not calcium dependent above 20 μM.

In the following, we explain these findings in the framework of a specific model of exocytosis control although we are aware that alternative interpretations are possible. We start with the recent proposal of a 'productive' reassembly of SNARE complexes between membranes, in which the energy released by complex formation is used to put the membranes under tension[41]. We incorporate this into a model similar to those previously pro-

posed[2,40], including the recent finding that v-SNARE and t-SNAREs are in parallel orientation in the complex[7] and arrive at Fig. 7. In this model, we allow for morphological docking, for NSF action to separate SNARE complexes[42], for SNARE complex formation (productive reassembly) and for fusion. For rapid action of calcium, the last step should be steeply dependent on calcium and fast, such that the readily releasable pool (state 5) can be depleted rapidly within the exocytic burst. In addition, a step of SNARE complex maturation is considered in the model to allow for the finding that this pool recovers from depletion (following strong stimulation) on the time scale of ten seconds[43] and that its size depends on PKC and $[Ca^{2+}]_i$[39,40]. This step (from state 4 to state 5) may correspond to the recruitment of other synaptic proteins to the complex such as the calcium-dependent activator protein for secretion (CAPS)[28] and the calcium sensor (possibly synaptotagmin). We also allow morphological undocking on the time scale of minutes[44]. Reversal of the productive reassembly step may involve NSF action. Such a model fits all of the findings summarized above. It explains why SNARE complexes are not protected from clostridial toxin action because NSF intermittently separates SNAREs within a time span of five minutes, which permits proteolytic action of the toxins. Action of most of the toxins, with the exception of BoNT/A, would completely prevent the productive reassembly and thereby prevent the formation of a release-ready pool of vesicles.

The model also provides a hint about why BoNT/A fails to eliminate secretion completely and seems to act both at an early and at a late step. BoNT/A cleaves off SNAP-25 the nine amino acids farthest from the plasma membrane, at the C-terminus. Truncated SNAP-25 still is able to form SNARE complexes that are disassembled by NSF[45,46], but the C-terminal end of the molecule is likely to be involved in initial contact during productive reassembly[41]. Lack of these nine amino acids may slow down rather than prevent this step. In our experiments, such a change would be reflected in a decrease of the rate of secretion at high $[Ca^{2+}]_i$, once the readily releasable pool is consumed. In other words, it would slow down the late component of secretion, as we observed. The same lack of nine amino acids may also explain the slowdown of the exocytic burst itself, if the calcium sensor for exocytosis (possibly synaptotagmin) interacts with the C-terminal end of the SNARE complex. Such interaction between the C-terminus of SNAP-25 and synaptotagmin was actually shown to occur *in vitro* (R.R.L. Gerona & T.F.J. Martin, personal communication). A specific proposal of how this might happen is shown in Fig. 7, referring to the finding that the ternary complex of SNAREs has a coiled-coil structure[41]. If the calcium sensor interdigitates with the tail of this structure, a calcium-dependent conformational change might put additional torsion on the coiled coil, forcing the two membrane anchors together and initiating fusion. Shortening of the SNAP-25 tail would most likely reduce the efficacy of the calcium sensor in transmitting the calcium signal to the SNARE complex. On the other hand, the affinity of the sensor itself is not expected to change (apart from possible allosteric coupling effects). In the model of Heinemann and colleagues[19], such a change would be most conveniently represented by a reduction of the rate constant γ (the rate constant of exocytosis from a triply calcium-bound state of the sensor) to a value of about five per second. Given the intrinsic dissociation constant of the calcium sensor of 13 μM[19] and a calcium-binding rate to the sensor of $8{\cdot}10^6$ per mole per second[47], a saturation of the calcium dependence of the release-rate constant would be expected for $[Ca^{2+}]_i$ in the range 20 to 70 μM, as observed after BoNT/A treatment.

Irrespective of the specific mechanism suggested here, the finding that removal of nine amino acids from SNAP-25 slows down the exocytic burst and does so more at high than at low $[Ca^{2+}]_i$ is strong evidence that this portion of the molecule is involved in mediating the action of a calcium sensor in rapid calcium-regulated exocytosis.

Methods

CELL PREPARATION AND SOLUTIONS. Chromaffin cells from bovine adrenal glands were prepared and cultured as described[48]. Cells were used one to four days after preparation. The external bathing solutions for experiments contained (in mM) 150 NaCl, 2.8 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 2 mg per 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 different concentrations of NP-EGTA (gift from Dr. Ellis-Davies, Philadelphia), fura-2 (Texas Fluorescence Labs, Austin, TX), furaptra (Molecular Probes, Eugene, OR), $CaCl_2$ or ATP 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 consisted of (in mM) 84.5 Cs-glutamate, 10 NP-EGTA, 8 $CaCl_2$, 1 $MgCl_2$, 2 MgATP, 0.3 GTP, 0.5 furaptra, 27 HEPES. For the DM-nitrophen experiment, internal solutions contained (in mM) 110 Cs-glutamate, 5 DM-nitrophen, 4 $CaCl_2$, 2 MgATP, 0.3 GTP, 0.5 furaptra, 35 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.

PHOTOLYSIS OF CAGED CALCIUM AND $[Ca^{2+}]_i$ MEASUREMENT. Flashes of ultraviolet light and fluorescence excitation light were generated as described[48]. To avoid any influence resulting from the 'loading transient'[4], we used the more calcium-selective caged compound Nitrophenyl-EGTA[49]. The flash photolysis efficiency was also measured as described[48] except for the following changes: we used 1 mM fura-2, 2 mM NP-EGTA and 2 mM $CaCl_2$ during flash experiments. Small flash intensities were applied by adding neutral-density filters. From measurements of $[Ca^{2+}]_i$ before and immediately after flashes, we calculated the photolysis efficiency for NP-EGTA. The photolysis efficiency of a 375 V discharge flash for NP-EGTA was determined to be 52%. As the $[Ca^{2+}]_i$ should decay significantly during 10 s of Cm measurement after flashes[48], we used the fluorescence excitation light to measure $[Ca^{2+}]_i$ and to simultaneously photorelease calcium 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 ref. 50. The calibration constants for furaptra measurements before and after 375 V discharge flashes were measured as described[48]. For 10 mM NP-EGTA, 0.5 mM furaptra, the changes of the calibration constants for a ratiometric measurement at 340 and 380 nm were as follows: R_{min} changed from 0.238 to 0.247, R_{max} from 6.554 to 4.708, K_{eff} from 2128 μM to 2053 μM.

WHOLE-CELL PATCH CLAMP AND CAPACITANCE MEASUREMENT. Conventional whole-cell recordings were done with sylgard-coated 2-3 MΩ pipettes. Series resistance ranged from 4–12 MΩ. An EPC-9 patch-clamp amplifier was used together with Pulse software (HEKA Electronics, Lambrecht, Germany). Capacitance measurements used the Lindau-Neher technique[17] implemented as the 'sine+dc' mode of the software lock-in extension of pulse software, which allowed long duration Cm measurement in single sweeps. A 800 Hz, 50 mV peak-to-peak sinusoid voltage stimulus was applied above 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). The analyses were conducted on a Macintosh computer using IGOR Pro. Unless otherwise stated, the data are given as mean ± SE.

AMPEROMETRY. Carbon-fiber electrodes were prepared from 10 μm diameter carbon fibers (Amoco performance products, Greenville, South Carolina) and were canulated 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. Arti-

facts 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.

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