# Molecular Identification and Reconstitution of Depolarization-Induced Exocytosis Monitored by Membrane Capacitance

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ABSTRACT    Regulated exocytosis of neurotransmitters at synapses is fast and tightly regulated. It is unclear which proteins constitute the "minimal molecular machinery" for this process. Here, we show that a novel technique of capacitance monitoring combined with heterologous protein expression can be used to reconstitute exocytosis that is fast ($<0.5$ s) and triggered directly by membrane depolarization in *Xenopus* oocytes. Testing synaptic proteins, voltage-gated $Ca^{2+}$ channels, and using botulinum and tetanus neurotoxins established that the expression of a $Ca^{2+}$ channel together with syntaxin 1A, SNAP-25, and synaptotagmin was sufficient and necessary for the reconstitution of depolarization-induced exocytosis. Similar to synaptic exocytosis, the reconstituted release was sensitive to neurotoxins, modulated by divalent cations ($Ca^{2+}$, $Ba^{2+}$, and $Sr^{2+}$) or channel (Lc-, N-type), and depended nonlinearly on divalent cation concentration. Because of its improved speed, native trigger, and great experimental versatility, this reconstitution assay provides a novel, promising tool to study synaptic exocytosis.

## INTRODUCTION

Regulated exocytosis is the $Ca^{2+}$-dependent fusion of cytoplasmic vesicles with the plasma membrane. When coupled to membrane depolarization, this process can achieve extreme speed and efficiency (1).

Understanding neuronal exocytosis requires both the identification of the presumably common "minimal machinery of membrane fusion" (2,3) and the specific molecular additions that knead generic membrane fusion into the rapid, voltage-operated process that eventually afford synaptic transmission (4).

Reconstitution assays of exocytosis offer a powerful experimental approach to study the general and the specifically neuronal aspects of exocytosis (5–8). Various such reconstitution assays of regulated secretion were reported, but the exocytosis process had in all cases lost the characteristic physiological traits of neuronal exocytosis: Reconstituted fusion occurs over minutes or hours instead of a few milliseconds, and it is not triggered by membrane depolarization (5).

To date, direct evidence from reconstituted systems regarding the molecular machinery of neuronal exocytosis is thus missing. On the other hand, simple extrapolation from slow, $Ca^{2+}$-regulated membrane fusion is discredited by the known complexity and diversity of exocytotic mechanisms as well as by persisting conflicts regarding the interpretation of the available data (2,9–11).

Several groups have used oocytes of the South African clawed frog *Xenopus laevis* in their attempts to reconstitute regulated exocytosis. For instance, $Ca^{2+}$-dependent release of ATP could be reconstituted in *Xenopus* oocytes by injection of total mRNA isolated from the electric lobe of *Torpedo marmorata* (12) or from rat cerebellum (13), and by injection of membrane vesicles (14,15) or of chromaffin granules (16). These experiments provided proof that exocytosis can be reconstituted in *Xenopus* oocytes. On the other hand, these assays do not provide an experimental platform that would allow one to study the molecular requirements of synaptic exocytosis: i), the molecular identity of the proteins was unknown and could not be manipulated individually; ii), exocytosis was not triggered by membrane depolarization; and iii), the methods used (e.g., luminometric ATP measurements or lectin secretion assays) had an unfavorable time resolution.

Here, we report a new approach to the use of *Xenopus* oocytes as a versatile testbed for the reconstitution and functional characterization of fast, depolarization-induced exocytosis, and data regarding the molecular requirements of this reconstituted process. As compared to other reconstitution assays of regulated exocytosis, our approach had the following advantages i), the assay affords selective expression of known proteins whose abundance can be varied and manipulated easily; this versatility allows one to delineate the minimal set of proteins needed to accomplish evoked release; ii), exocytosis is triggered directly via membrane depolarization under voltage-clamped conditions; and iii), exocytosis is detected with great convenience, precision and time resolution by monitoring of membrane capacitance. Our findings constitute the first data on regulated exocytosis that were obtained in a reconstituted system in which exocytosis was directly triggered by depolarization.

## MATERIALS AND METHODS

Plasmid cDNAs for full-length BoNT/A-light chain and TetX were generous gifts from H. Gaisano (Toronto Canada) and H. Niemann (Hannover, Germany) and GluR3 (L507Y) from Y. Stern-Bach (Jerusalem, Israel).

## Heterologous protein expression in *Xenopus* oocytes

*Xenopus* oocytes were injected with a mixture of cRNAs encoding the $Ca^{2+}$ channel subunits $\alpha_1 1.2$ (Lc-type; rabbit; 5 ng/oocyte), or $\alpha_1 2.2$ (N-type; rat; 10 ng/oocyte) together with $\alpha 2/\delta$ (rabbit; 5 ng/oocyte) and $\beta_2 A$ (rat; 10 ng/oocyte); sodium channel $Na_v 1.2$ (3 ng/oocyte), or the glutamate receptor GluR3 (3 ng/oocyte), and one day later either with water (for controls) or with a mixture of cRNAs encoding SNAP-25 (0.5 ng/oocyte), syntaxin 1A (0.5 ng/oocyte) and synaptotagmin I (1.0 ng/oocyte). Botulinum toxins cRNAs; Bot-A Bot-C1, and TetX (2 ng/oocyte) were injected at day 3 after channel cRNA injection.

Oocyte preparation, in vitro transcription, and cDNA constructs were as reported previously (17). Depolarization-induced exocytosis was studied after further incubation for 4 or 5 days.

## Capacitance monitoring in *Xenopus* oocytes

Membrane capacitance ($C_m$) was monitored in the two-electrode voltage-clamp configuration as published elsewhere (18). Briefly, $C_m$ was determined from the current response to a triangular, symmetrical voltage command. The up- and down-ramps ($\pm 20$ mV in 20 ms each) elicit membrane currents that are the sum of resistive and a capacitive current component. Switching from up- to down-ramp reverses the sign of the capacitive component but not that of the resistive component. Thus, subtraction of the down-ramp current integral from the up-ramp current integral eliminates the resistive component; the resulting pure capacitive charge allows one to compute—together with the known amplitude of the voltage stimulus—membrane capacitance. Continuous monitoring is achieved by applying this stimulus repetitively at a high rate (up to 10/s). Comprehensive tests in an electrical cell model as well as in *Xenopus* oocytes have demonstrated high precision, accuracy, and robustness of this technique (18). Starting from a holding potential of −80 mV, depolarizing stimuli were applied by clamping the cells to 0 mV for $2 \times 500$ ms, separated by 100 ms at −80 mV. Capacitance was monitored before and after the stimulus, together with membrane potential ($V_m$) and current ($I_m$).

## Biochemical assay of released ATP

Evoked secretion can be monitored after ATP release since vesicle content is rich in ATP (19). We used the highly sensitive luciferin-luciferase assay (ENLITTEN, Promega, Kibbutz Beit Haemek, Israel) to confirm depolarization-induced ATP release in oocytes. Oocytes were depolarized by incubation in 100 mM KCl for 60 s, and luminescence of the medium was determined directly in a luminometer before and after depolarization to lower basal secretion. Each assay contained 3 oocytes and the assay was repeated 5 times.

## Fluorescence measurements

Oocytes were injected as described in the electrophysiology section with GFP-synaptotagmin cRNA (20). Six days after cRNA injection oocytes were fixed in OCT (Miles, Pittsburg, PA) and cut into 50–100 $\mu$M slices using a cryostat. Slices were mounted on optical slides, and GFP-synaptotagmin was visualized by a Bio-Rad (Hercules, CA) confocal microscope. Confocal images were generated using the Confocal Assistant program (Bio-Rad).

## RESULTS

## Expression of a limited set of proteins conveys depolarization-evoked exocytosis to *Xenopus* oocytes

We first attempted to reconstitute depolarization-induced exocytosis in *Xenopus* oocytes (using membrane capacitance as a readout) by expressing SNARE proteins together with synaptotagmin 1A and a voltage-gated $Ca^{2+}$ channel. We used *Xenopus* oocytes expressing the Lc-type channel and the various synaptic proteins as indicated (Fig. 1). *Xenopus* oocytes (Fig. 1 *A*) were stimulated from a holding potential of −80 mV to 0 mV by two consecutive 500-ms pulses 100 ms apart (Fig. 1 *B*). Continuous monitoring of membrane capacitance showed the effect of depolarization on membrane capacitance ($C_m$) in oocytes expressing tSNARE proteins, syntaxin 1A, SNAP-25, which are key proteins in exocytosis (21,22) and Lc-type channel (Fig. 1 *C*). Representative original traces of voltage (*upper*), current (*middle*), and $C_m$ (*lower*) are shown (Fig. 1 *C*). The vertical dashed lines indicates the precise time at which $C_m$ was read for comparison with the baseline $C_m$ before considering depolarization values (Fig. 1 *C*). Uninjected oocytes did not exhibit depolarization-induced capacitance changes in response to a double 500 ms pulse (Fig. 1, *D* and *E*). Upon expression of the synaptic proteins syntaxin 1A, SNAP-25, synaptobrevin, and synaptotagmin I, without the $Ca^{2+}$ channel, the depolarizing stimuli produced no increases of $C_m$. Similarly, small $C_m$ changes could be elicited upon expression of a voltage-gated $Ca^{2+}$ channel alone (subunits $\alpha_1 1.2$, $\alpha$ $2\delta$, and $\beta 2A$) without expression of synaptic proteins ($0.44 \pm 0.1$ nF) (Fig. 1, *D* and *E*). However, simultaneous expression of the $Ca^{2+}$ channel, SNARE proteins and synaptotagmin resulted in much larger depolarization-induced step increases of $C_m$ ($\Delta C_m$) ($3.5 \pm 0.3$ nF) (Fig. 1, *D* and *E*). These $C_m$ changes correspond to $\sim$1–2% of an oocyte's total membrane surface area, or to fusion of $\sim 10^5$–$10^6$ cortical granules (assuming a diameter of 0.5–3 $\mu$m (23) with an individual capacitance of $\sim$5 fF; Fig. 1 *A*). In oocytes expressing $Ca^{2+}$ channel plus the SNAREs but not synaptotagmin I, depolarization elicited smaller $C_m$ increases ($1.3 \pm 0.2$ nF). In oocytes expressing $Ca^{2+}$ channel plus synaptotagmin I plus SNARE proteins except for synaptobrevin, depolarization elicited smaller $C_m$ increases ($2.8 \pm 0.2$ nF) than in oocytes expressing the same proteins plus synaptobrevin. Note that the $C_m$ change was completed during the 1.1 s of the depolarizing stimulus. A summary of the average $C_m$ change of oocytes ($n = 13$) is shown in Fig. 1 *E*. Amplitude of inward currents was only slightly affected by the presence of the various synaptic proteins (Fig. 1 *F*).

A fast transient component of the depolarization-induced capacitance change was observed in oocytes expressing the $Ca^{2+}$ channel alone (Fig. 1 *C*) or in combination with synaptic proteins (panels in the right columns of Fig. 1 *D*), in addition to a capacitance increase that was stationary or decaying only very slowly (panels in the right columns of Fig. 1 *D*). The nature of the fast transient is unclear. It is not correlated with the depolarization-induced current changes, which decay considerable faster. Whether this capacitance transient reflects genuine exocytosis is unclear (24). To avoid any confounding effects of current changes on the $C_m$ measurements, and not knowing the nature of the fast capacitance
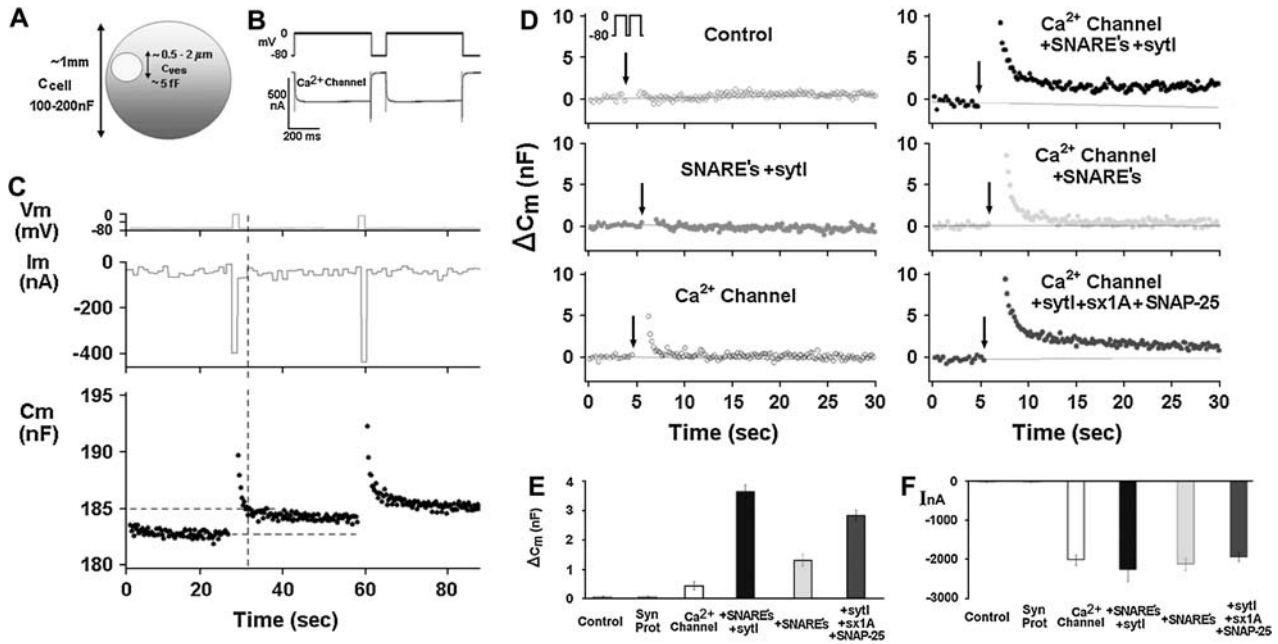
FIGURE 1 Reconstitution of depolarization-induced exocytosis in oocytes by expression of exogenous proteins. (*A*) Diameter and capacitance of plasma membrane and cortical granules in *Xenopus* oocytes. (*B*) Effect of depolarization on membrane capacitance in a *Xenopus* oocyte expressing a voltage-gated $Ca^{2+}$ channel. (*Upper trace*) Depolarizing voltage command (see Methods), consisting of depolarization from a holding potential of $-80$ mV to 0 mV for $2 \times$ 500 ms, separated by 100 ms at $-80$ mV. (*C*) Continuous monitoring of membrane capacitance showing the effect of depolarization on membrane capacitance ($C_m$) in an oocyte expressing, $Ca^{2+}$ *channel,* Lc-type $Ca^{2+}$ channel subunits $\alpha_1 1.2$, $\beta 2A$, $\alpha_2 \delta$; SNAREs: syntaxin 1A, SNAP-25, and synaptotagmin; representative original traces of voltage (*upper*) current (*middle*) and $C_m$ (*lower*). The vertical dashed line indicates the time at which $C_m$ was read for comparison with the baseline $C_m$ before depolarization. (*D*) Monitoring $C_m$ in oocytes expressing different proteins. Control, uninjected oocytes; other panels, oocytes expressing heterologously synaptic proteins: synaptic proteins, syntaxin 1A, SNAP-25, synaptobrevin, and synaptotagmin, $Ca^{2+}$ channel, Lc-type $Ca^{2+}$ channel subunits $\alpha_1 1.2$, $\beta 2A$, $\alpha_2 \delta$; SNAREs: syntaxin 1A, SNAP-25 and synaptobrevin; synaptotagmin I, sytI; $Ca^{2+}$ channel plus syntaxin 1A, SNAP-25 and syt I. (*E*) Summary: effect of depolarization on $C_m$. Groups as in *D*. $\Delta C_m$, depolarization-induced change of membrane capacitance; bars show mean $\pm$ SD ($n = 13$). (*F*) Effect of depolarization on membrane current. Depolarization-induced membrane current (InA; mean $\pm$ SD, $n = 13$ each). Data show that the $C_m$ changes (*C*) cannot be explained by membrane current, but rather reflect bona fide exocytosis.

transient (24), we determined the $C_m$ change just after the current had returned to its previous level (see vertical dashed line in Fig. 1 *C*). Assuming that the permanent $C_m$ changes reflect genuine exocytosis, this approach results in a measure of exocytosis that is specific, but potentially underestimates the extent of exocytosis.

That the observed $C_m$ changes indeed reflect exocytosis was supported by two further observations. Firstly, the amplitude of depolarization-induced current associated with the expression of the voltage-gated $Ca^{2+}$ channel was not significantly affected by coexpression of other synaptic proteins (Fig. 1 *F*). This observation rules out depolarization-induced mobilization of gating charges on the voltage-dependent $Ca^{2+}$ channel as a reason for the greater $C_m$ increases observed upon additional expression of synaptic proteins. Moreover, the lack of effect of the synaptic proteins on the $Ca^{2+}$ currents excludes improved membrane targeting of the $Ca^{2+}$ channel as an explanation of the enhanced exocytosis.

Secondly, similar results were obtained with ATP release as a second, independent approach to detect exocytosis (Fig. 2, *A* and *B*). ATP release from oocytes expressing synaptotagmin plus SNAP-25, syntaxin 1A, and

$Ca^{2+}$ channel was $>4$-fold higher than in oocytes expressing the $Ca^{2+}$ channel alone, and $>10$-fold higher than in uninjected oocytes.

Correct targeting of the heterolgously expressed synaptotagmin, on the other hand, was confirmed by experiments in which we expressed a GFP-synaptotagmin fusion protein in *Xenopus* oocytes (20). Labeling was localized to vesicles of 0.5–5 $\mu$m diameter (Fig. 2) the typical size of cortical granules (23).

Together, these experiments show that expression of synaptotagmin and the $Ca^{2+}$ channel along with SNAREs conveys depolarization-induced exocytosis to *Xenopus* oocytes, and this type of exocytosis is fast, robust, and can easily be studied quantitatively. The effect is readily distinguishable against a virtually zero background in native oocytes. Note, however, the small $C_m$ increase in oocytes expressing the $Ca^{2+}$ channel without the synaptic proteins; this effect could reflect the presence of endogenous homologs to the synaptic proteins that, together with the $Ca^{2+}$ channel, can mediate depolarization-induced exocytosis of limited magnitude. The contribution of these unknown native proteins is probably rather restricted given their lower level of expression relative
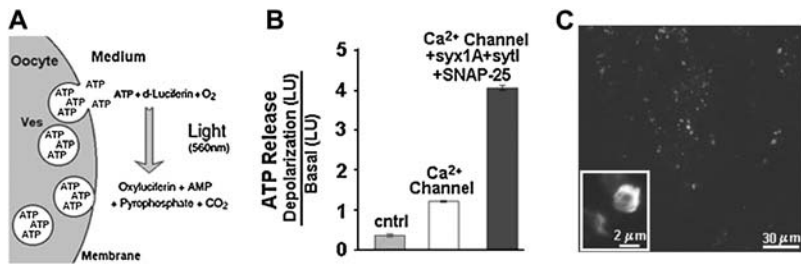
FIGURE 2 Effect of heterologous proteins on depolarization-induced ATP release. (*A*) A schematic presentation of ATP luminescence assay. (*B*) Oocytes were depolarized by incubation in high K$^+$-solution, and release of ATP was determined via a luminescence assay (see Methods). ATP release paralleled the $C_m$ changes (cf. Fig. 1, *A* and *B*), confirming that $C_m$ changes indeed reflect fusion of cytoplasmic vesicles with the plasma membrane. (*C*) Heterologously expressed synaptotagmin is localized in cortical granules. Confocal image of oocytes injected with cRNA for a GFP-synaptotagmin 1-fusion protein.

to the heterologously expressed proteins. As shown previously, the endogenous level of voltage gated Ca$^{2+}$ channels is rather low (>100-fold) compared to secreting cells (25).

## Role of individual synaptic proteins

We exploited the ability of our assay to establish the contribution of individual proteins to depolarization-induced exocytosis (Fig. 3). In principle, this could be done also with previously available reconstitution assays. Our assay however, has the considerable advantage, that it is sensitive specifically to effects on fast exocytosis, and screens out—by design—exocytotic processes with slower time courses and thus probably different molecular underpinnings than the forms of exocytosis that mediate synaptic transmission.

We used a panel of neurotoxins to address the individual contribution of the v-SNARE synaptobrevin and of the tSNAREs syntaxin 1A and SNAP-25, to reconstituted depolarization-induced exocytosis (Fig. 3, *A* and *B*).

When we coexpressed synaptobrevin together with the SNARE proteins, depolarization-induced $C_m$ changes were considerably larger than without synaptobrevin (VAMP-2). The tSNAREs, synaptotagmin and the Ca$^{2+}$ channel without synaptobrevin, were previously shown to form a complex with distinct kinetic properties, named excitosome (26) (Fig. 3 *A*; see also Fig. 1, *C* and *D*). This result is compatible with the notion that synaptobrevin increases either the number or the efficiency of the protein complexes between SNARE proteins, Ca$^{2+}$ channel, and synaptotagmin, or plays a role in targeting vesicles to the plasma membrane; alternatively, synaptobrevin may somehow modulate exocytosis independently from the excitosome. Coexpression of tetanus toxin (a protease specific for synaptobrevin; TetX) reduced the depolarization-induced $C_m$ increases to the level seen with the excitosome alone (rather than abolishing it completely). Partial inhibition is consistent with the lack of effect of TetX in oocytes expressing the excitosome; also, the inhibition confirms the successful functional expression of this neurotoxin. This finding shows that the reconstitution of depolarization-induced exocytosis in *Xenopus* oocytes was achieved without the expression of exogenous synaptobrevin, and also without a significant contribution of putative endogenous TetX-sensitive synaptobrevin. Likewise, complete cleavage of synaptobrevin by TetX in pancreatic acini caused

only a partial inhibition of exocytosis (27) and in a cell free Ca$^{2+}$-regulated exocytosis (28).

Coexpression of botulinum neurotoxin A (a protease specific for SNAP-25; BotA) together with the excitosome proteins abolished the depolarization-induced $C_m$ change (Fig. 3 *A*). A similar inhibition was observed upon coexpression of botulinum toxin C1 light chain (a protease specific for syntaxin 1A; BotC). Both neurotoxins had no significant effect on the depolarization-induced current amplitudes (Fig. 3 *B*). These results suggest that both syntaxin 1A and SNAP-25 are essential for depolarization-induced exocytosis. In all cases, current amplitudes were marginally affected by coexpression of neurotoxins (Fig. 3 *B*).

Next, we assembled the synaptic proteins step by step, adding one component after the other (Fig. 3 *C*). A small depolarization-induced $C_m$ change was seen with the Ca$^{2+}$ channel alone, and this effect was increased with additional coexpression of the Ca$^{2+}$ sensor protein synaptotagmin-I. Further coexpression of BotC light chain abolished this effect, suggesting the participation of endogenous syntaxin 1A (or a homolog) in the observed depolarization-induced exocytosis (29). Notably, SNAP-25 and syntaxin 1A together or individually with the channel failed to increase $C_m$ (data not shown), indicating the essential role of synaptotagmin.

Based on these results we chose to continue the $C_m$ studies in oocytes expressing syntaxin 1A, SNAP-25, synaptotagmin and the Ca$^{2+}$ channel, which appear to be the minimal set of proteins required for secretion.

We then established in a relatively simple yet informative experiment the fundamental fact that the three synaptic proteins comprised by the excitosome (synaptotagmin-I, syntaxin 1A and SNAP-25) are indeed required for and participate in the depolarization-induced exocytosis that we observed in *Xenopus* oocytes (Fig. 3 *A*). As demonstrated above (Figs. 1, *A* and *B*, and 3 *A*), only expression of these three proteins, but not expression of Ca$^{2+}$ channel alone, resulted in large depolarization-induced changes of $C_m$. Increasing the amount of injected cRNAs for the three synaptic proteins by a factor of two or four (at fixed cRNA amounts of channel subunits) resulted in roughly proportional increases of the $C_m$ changes (Fig. 3 *D*).

Recall the findings above that suggested the presence of some endogenous synaptic proteins, which might assemble with the heterologous Ca$^{2+}$ channel into functional
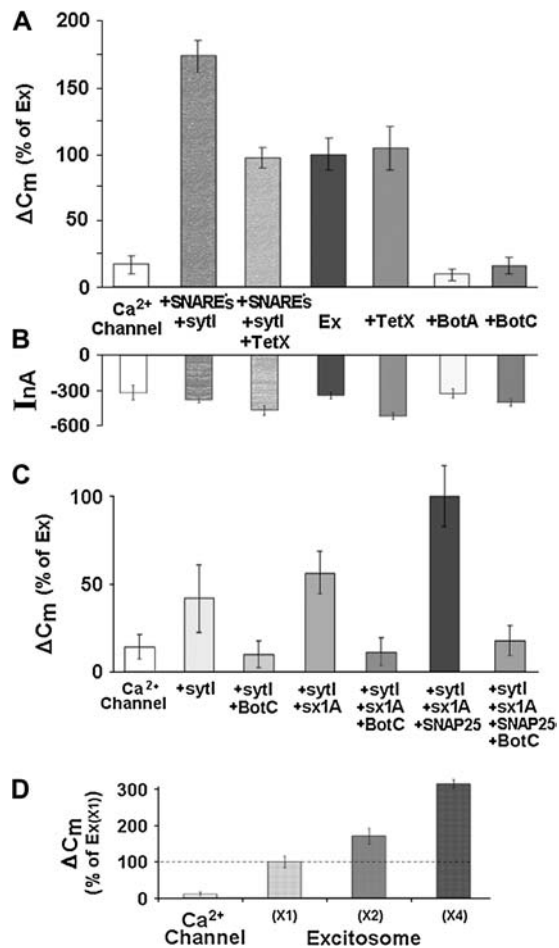
FIGURE 3 Dissecting out the role of synaptic proteins in reconstituted depolarization-induced exocytosis. (A) Effects of botulinum toxins (BotC and BotA) and tetanus toxin (TetX) on depolarization-induced capacitance changes. Lc-type $Ca^{2+}$ channel ($Ca_v1.2$); SNAREs (syntaxin 1A, synaptobrevin, and SNAP-25) and synaptotagmin I; Ex, $Ca^{2+}$ channel + syntaxin 1A + SNAP-25, were expressed at a level corresponding to "×1" in Fig. 3 D. Data show that SNAP-25 and syntaxin 1A are absolutely required for depolarization-induced exocytosis. In contrast, synaptobrevin is not required, although it increases exocytosis efficiency. (B) Effects of botulinum toxins and tetanus toxin on depolarization-induced currents. Current amplitudes and $C_m$ changes (A) are not correlated. (C) Stepwise molecular reconstitution of depolarization-induced exocytosis. Synaptic proteins as above, and Lc-type $Ca^{2+}$ channel ($Ca_v1.2$). Data show that syntaxin 1A and SNAP-25 are absolutely required for depolarization-induced exocytosis. The changes in $C_m$ in the presence of the toxins are similar to the step $C_m$ expressed by the channel alone. Data suggest that the synaptic proteins comprised by the excitosome are necessary and sufficient to specifically reconstitute depolarization-induced exocytosis against a very small background in Xenopus oocytes. (D) Quantitative correlation between reconstituted depolarization-induced exocytosis and expression of excitosome proteins. Coexpression of graded amounts of the synaptic proteins syntaxin 1A, synaptotagmin I, and SNAP-25 together with a fixed amount of Lc-type voltage-gated $Ca^{2+}$-channel subunits that comprise the excitosome (see Methods).

exocytotic units (Fig. 1, A and B). Our experiment (Fig. 3 D) shows that with increasing cRNA levels of excitosome one can obtain, with sufficiently strong expression of the exogenous proteins, such high numbers of correctly formed

exocytotic units (i.e., comprising exclusively exogenous channel and synaptic proteins) that all other exocytotic units (e.g., comprising exogenous channel and endogenous synaptic proteins) are outnumbered by far. With such a small background, the properties of depolarization-induced exocytosis can be attributed to the well-defined set of exogenous proteins.

Next, we characterized this reconstituted exocytosis in more detail, taking advantage of this assay system to control and vary stimuli, and to gather detailed, quantitative information from the elicited effects.

## Reconstituted depolarization-evoked secretion is nonlinear and is affected by the type of charge carriers

The effect of fixed-duration depolarization on $C_m$ depended strictly on the presence of either extracellular $Ca^{2+}$ or $Ba^{2+}$ and was concentration-dependent and saturable (Fig. 4 A). The concentration-dependence of $\Delta C_m$ was nonlinear with an estimated Hill coefficient of $n_H \approx 2.8$. This finding is in good agreement with the characteristic cooperativity of transmitter release in neuronal cells (30). Oocytes expressing the channel alone showed a very small change in capacitance, which slightly increased at 10 mM $Ba^{2+}$. When external $Ba^{2+}$ concentration was fixed at 5 mM, the effect of depolarization on $C_m$ depended on pulse duration, with a lower threshold at ~50 ms and saturated at ~400 ms (Fig. 4 B) (7,8). Thus, reconstituted depolarization-induced exocytosis is by at least 2 orders of magnitude faster than membrane fusion in previous reconstitution assays. The method of varying the depolarizing pulse to obtain the kinetics of exocytosis was first applied to capacitance measurements in nerve terminals by (31). Fitting a straight line to the data points between 50 to 400 ms resulted in a slope of 7.7 nF/s corresponding to initial rate of $1.2 \times 10^6$ vesicles/s and a pool of 600,000 vesicles at ~400 ms (Fig. 4 B). The data could also be fitted although less well, with a single exponential time constant of $101 \pm 12$ ms corresponding to initial rate of $6 \times 10^6$ vesicles/s (inset). The saturation in the capacitance jumps defines a readily releasable pool of ~$10^6$ to $10^7$ vesicles.

Both $Ca^{2+}$, $Sr^{2+}$, and $Ba^{2+}$ ions supported depolarization-induced $C_m$ changes, but with quantitative differences (Fig. 5 A). With either ion, a fast initial $C_m$ increase was followed by a slower $C_m$ relaxation; the latter probably reflects compensatory retrieval of the exocytosed membrane surface area. With $Ba^{2+}$, however, the fast initial $C_m$ increase became greater by a factor of ~2.5 as compared to $Ca^{2+}$, whereas the subsequent slow $C_m$ decay became slower by a similar factor. With $Sr^{2+}$, $C_m$ was similar to $Ca^{2+}$ showing an intermediate $C_m$ decay (Fig. 5 C). Similar to our findings in this reconstituted form of depolarization-induced exocytosis, the quantitative traits of native neuronal exocytosis are differentially affected by $Ca^{2+}$ versus $Ba^{2+}$ (32).

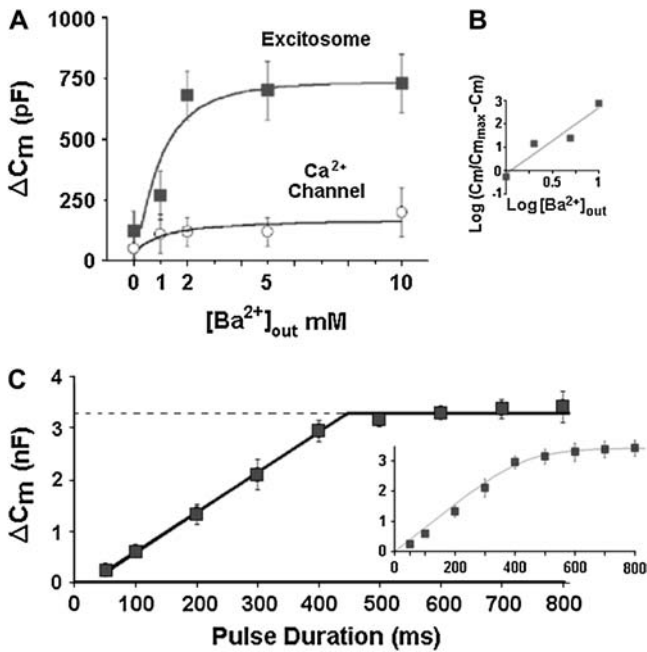Interestingly, when the depolarizing stimulus was applied repeatedly, the associated $C_m$ increase became progressively

FIGURE 4 Quantitative characterization of reconstituted depolarization-induced exocytosis. (*A*) Dependence of depolarization-induced $\Delta C_m$ on extracellular $Ba^{2+}$ concentration. Depolarization from $-80$ mV to 0 mV for $2 \times 500$ ms 100 ms apart in oocytes expressing Lc-type channel without (*lower trace*) or with (*upper trace*) the synaptic proteins syntaxin 1A, SNAP-25, and synaptotagmin I, as in Fig. 1. (*B*) Hill plot. Data (from *A*) show saturation of the $C_m$ effect $>2$ mM, and a nonlinear concentration-dependence of $\Delta C_m$ with an estimated Hill coefficient of $n_H \approx 2.8$. (*C*) Dependence of depolarization-induced $\Delta C_m$ on depolarization duration. Depolarization from $-80$ mV to 0 mV for indicated times in 5 mM $Ba^{2+}$. A linear correlation observed with pulse duration for longer depolarization periods, saturation at $\sim 400$ ms and half-maximal capacitance changes are reached at a depolarization time of $\sim 250$ ms. A straight line fitted to the data between 50 and 400 ms had a slope of 7.7 pF ms$^{-1}$ ($R^2 = 0.998$) corresponding to $\sim 600,000$ vesicles at saturation. The results could be fitted less well with a single exponent (*inset*) showing a time constant of $101.05 \pm 12$ ms corresponding to an initial rate of $6 \times 10^6$ vesicles/s (*inset*). The saturation in the capacitance jumps defines a readily releasable pool of $\sim 10^6$–$10^7$ vesicles (see 31).

smaller (Fig. 5 *C*). This exhaustibility is likely to reflect depletion of a pool of releasable vesicles that can be refilled after a period of minutes (33,34). Moreover, this phenomenon provides further evidence against a contribution of gating charges to the observed $C_m$ changes, which should not show exhaustibility.

## Role of the Ca²⁺ channel

Voltage-gated $Ca^{2+}$ channels (VGCC) are essential components of the molecular machinery that mediates depolarization-induced exocytosis at synapses. One obvious function herein is to transduce the electrical signal, the action potential, into VGCC activation ensued by a rise of cytoplasmic $Ca^{2+}$. On the other hand, we have shown by various approaches that the $Ca^{2+}$ channel is also physically
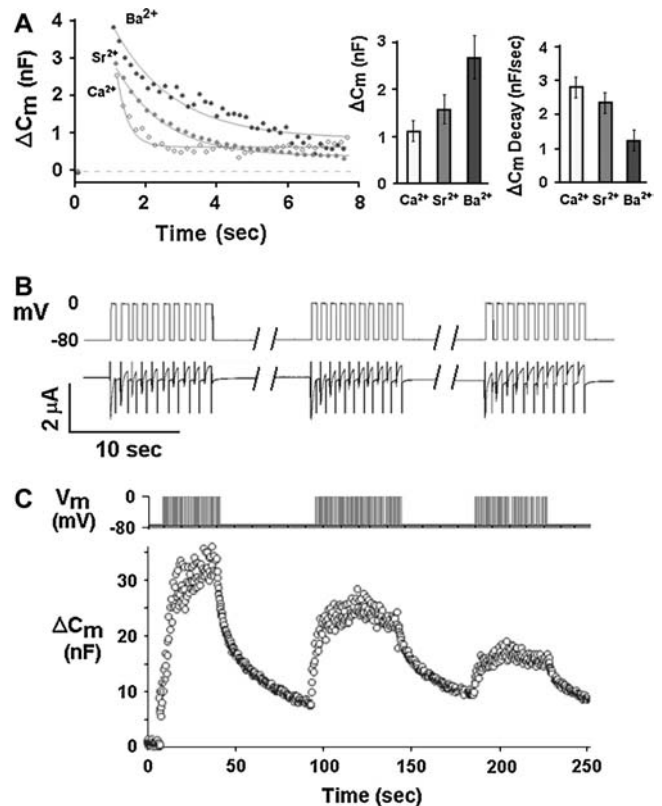


FIGURE 5 Differential effects of $Ca^{2+}$, $Sr^{2+}$, or $Ba^{2+}$ on depolarization-induced $C_m$ changes, and vesicle depletion by repetitive stimulation (*A*) Differential effects of $Ca^{2+}$, $Sr^{2+}$, or $Ba^{2+}$ on depolarization-induced exocytosis. Oocytes expressing $Ca_v1.2$, syntaxin 1A, syt-1, and SNAP-25 (excitosome). (*Left panel*) Original traces showing time course of $C_m$ upon depolarization, fitted by simple exponentials; (*middle panel*) instantaneous depolarization-induced $C_m$ increase in $Ca^{2+}$, $Sr^{2+}$, and $Ba^{2+}$ (mean $\pm$ SE, $n = 10$, each); and (*right panel*) rate of compensatory $C_m$ decrease (mean $\pm$ SE, $n = 10$, each). As compared to $Ca^{2+}$, the instantaneous $C_m$ increase was greater in $Ba^{2+}$ (*middle panel*), and the compensatory $C_m$ decrease was slower (*right panel*). In $Sr^{2+}$, the respective values were between those of $Ca^{2+}$ and $Ba^{2+}$. (*B*) Current amplitude during repeated trains of membrane depolarization. Oocytes were depolarized by three trains composed of 10 depolarizations from $-80$ to 0 mV 1 s each (*upper panel*). Depolarization-induced inward current inactivated during trains whereas no run-down of current amplitude was observed from one train to the other (*lower panel*). (*C*) Exhaustibility of depolarization-induced capacitance changes. Repeated depolarization pulses from $-80$ mV to 0 mV for 1 s each (*upper trace*) and the associated $C_m$ changes (*lower trace*). Data are consistent with depletion and only partial replenishing of a pool of releasable vesicles.

associated with synaptic proteins (20,25,26,35), forming a protein complex which we have termed the ''excitosome'' (26). Such a direct protein-protein interaction (36–41) could allow the $Ca^{2+}$ channel to affect the process of depolarization-induced exocytosis upstream to $Ca^{2+}$ entry (42,43). These potential additional roles are barely explored, mainly because this would require an experimental approach that simultaneously affords control of membrane potential and arbitrary variation of the particular $Ca^{2+}$ channel. Such

experiments are a unique strength of reconstitution in *Xenopus* oocytes.

We used two channel blockers to test for the requirement of a voltage-gated $Ca^{2+}$ channel in depolarization-evoked release (Fig. 6 *A*). Considerable depolarization-induced inward currents were recorded in oocytes expressing Lc-type $Ca^{2+}$ channel ($Ca_v1.2$; *right panel*), but these were
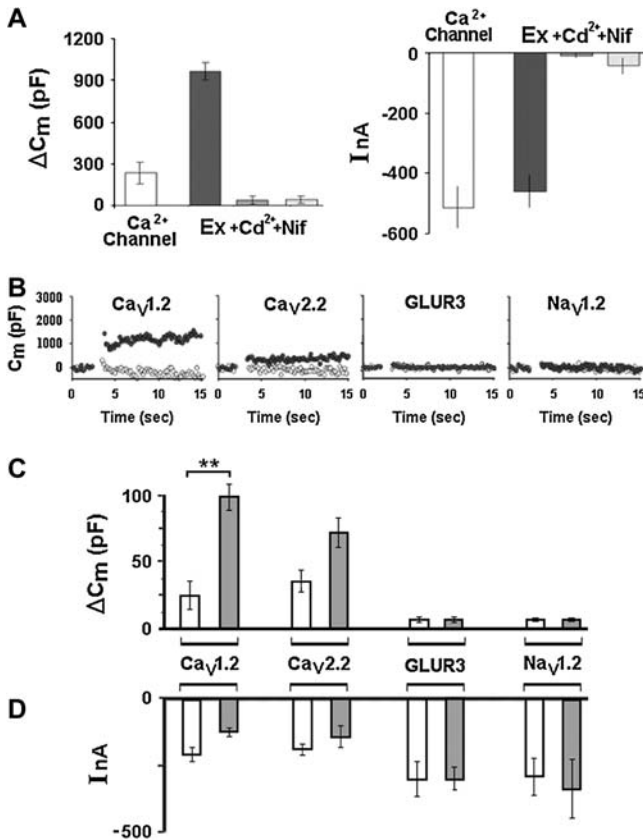
accompanied by only small $C_m$ changes (*left panel*). In contrast, coexpression of the entire excitosome resulted in much larger $C_m$ changes, whereas similar current amplitudes were not different from the currents observed with the channel alone (Fig. 6 *A*; cf. also Fig. 1, *D–F*). Both in the presence of either the pore blocker $Cd^{2+}$ (200 $\mu$M) or the selective Lc-type channel blocker nifedipine (10 $\mu$M), depolarization failed to induce any $C_m$ changes or inward currents.

Next, we tested how the particular type of voltage-gated $Ca^{2+}$ channel affects the extent and the kinetics of depolarization-induced $C_m$ changes (Fig. 6, *B–D*). Oocytes were injected with a fixed combination of syntaxin 1A, SNAP-25 and synaptotagmin I, and in addition with cRNA species encoding either Lc-type ($Ca_v1.2$), N-type ($Ca_v2.2$), or the neuronal voltage-gated sodium channel $Na_v1.2$ (Fig. 6 *B*; see Methods); assembly of these channels into excitosome complexes was shown previously (17,20,26,44) except for the $Na^+$ channel. The effect of depolarization on current and capacitance was tested in 5 mM $Ba^{2+}$ ($Ca^{2+}$ channels) or 50 mM $Na^+$ ($Na^+$ channel). In addition, we used the $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazol propionate (AMPA)-receptor GluR3, a glutamate-gated cation-selective channel that mediates the majority of fast excitatory synaptic transmission in the mammalian brain by transporting $Ca^{2+}$ into the cell. The ionotropic receptor was expressed in the oocyte and activated to introduce $Ca^{2+}$ by a hyperpolarizing step in the presence of 1mM glutamate (Fig. 6, *B* and *C*).

The particular channel type had a large effect on the magnitude of the depolarization-induced $C_m$ changes ($\Delta C_m$; value with versus without synaptic proteins (indicated in % of $\Delta C_m$ with $Ca_v1.2$): $Ca_v1.2$, 100 ± 20 vs. 24.5 ± 22; $Ca_v2.2$, 72 ± 23 vs. 35 ± 16; GluR3, 6 ± 3 vs. 6 ± 3 and $Na_v1.2$, 5 ± 1 vs. 5 ± 1, respectively. Thus as compared to the $C_m$ changes obtained with the respective channel only, the additional expression of the synaptic proteins enhanced depolarization-induced exocytosis by a factor of 4.16 ± 0.22 ($Ca_v1.2$) and 1.3 ± 0.2 ($Ca_v2.2$). The corresponding inward currents (Fig. 6 *D*) showed no correlation with $C_m$ changes. Hence differences in capacitance are due to factors other than the respective channel's ability to mediate $Ca^{2+}$ influx and maybe depolarization-evoked exocytosis requires the functionality of a voltage-gated $Ca^{2+}$ channel.

Although the activated ionotropic receptor GluR3 introduced $Ca^{2+}$ into the cells (hyperpolarization-induced inward currents) with GluR3 alone 300 ± 103nA, and with GluR3 plus syntaxin-1A, SNAP-25, and synaptotagmin, 300 ± 44; (Fig. 6 *D*) no increase in $C_m$ was detected (Fig. 6 *C*), consistent with the importance of a physical and functional interaction between channel and the synaptic proteins (25,26,43). Finally, no depolarization-induced capacitance increase was observed when a neuronal $Na^+$ channel was complementing the synaptic proteins. This is in keeping with the requirement for external divalent cations passing through a voltage-gated $Ca^{2+}$ channel.

FIGURE 6 Dissecting out the role of the $Ca^{2+}$ channel in reconstituted depolarization-induced exocytosis (*A*) Effect of $Ca^{2+}$ channel inhibitors on reconstituted depolarization-induced exocytosis. Oocytes were expressing either the Lc-type $Ca^{2+}$ channel alone (*leftmost bar*), or together with the synaptic proteins syntaxin 1A (sx1A), synaptotagmin I (syt I), and SNAP-25 (*other three bars*). Depolarization-induced changes of capacitance ($\Delta C_m$) and current amplitude (*I*) in 5 mM $Ba^{2+}$. The effects of the $Ca^{2+}$ channel blockers $Cd^{2+}$ (200 $\mu$M) and nifedipine (10 $\mu$M) show that depolarization-induced exocytosis absolutely requires the presence and functionality of a voltage-gated $Ca^{2+}$ channel. (*B*) Effect of channel type on depolarization-induced $C_m$ changes. Time course of $C_m$ in oocytes expressing various voltage-gated channels (L-, N-type $Ca^{2+}$ channels, GluR3 receptor, or brain-type II $Na^+$ channel; see Methods) without (*open circles*) or with SNAP-25, syntaxin 1A, and synaptotagmin 1 (*solid circles*); bath containing 5 mM $Ba^{2+}$ (with $Ca^{2+}$ channels and GluR3) or 50 mM $Na^+$ (with $Na^+$ channel). Depolarization from –80 mV to 0 mV for 2 × 500 ms. (*C*) Effect of channel type on size of depolarization-induced $C_m$ increase. Same protocol as in (*A*) (*open bars*) channel only; (*solid bars*) channel plus SNAP-25, syntaxin 1A, and synaptotagmin 1. Mean ± SD from 10 oocytes, each. $Ca_v1.2$ versus $Ca_v1.2$ + synaptic proteins (**), $P < 0.0015$; $Ca_v2.2$ versus $Ca_v2.2$ + synaptic proteins $P* < 0.1$ in Student's *t*-test. (*D*) Effect of channel type on depolarization-induced current changes. Same protocol as in *A* and *B*.

## DISCUSSION

The principle of our reconstitution assay is to combine heterologous expression of candidate proteins in *Xenopus* oocytes with monitoring of membrane capacitance as a readout of depolarization-induced exocytosis. Expression of heterologous proteins was accomplished in these large cells by established techniques that involve the injection of cRNA encoding the corresponding proteins (45,46). Control of membrane potential and application of a short depolarizing stimulus, was achieved by the similarly well-established two-electrode voltage-clamp technique (47). Continuous monitoring of membrane capacitance ($C_m$) by this technique has become feasible by a novel method developed recently by one of us (18). The powerful electrical approach of capacitance monitoring (in the sense of continuous measurements with high time-resolution and precision) was previously applied only in the patch-clamp configuration, precluding its use in large cells such as *Xenopus* oocytes.

Voltage-gated $Ca^{2+}$ channels are essential components of the molecular machinery that mediates exocytosis. Besides translating the action potential into a rise of cytoplasmic $Ca^{2+}$, the $Ca^{2+}$ channel is also physically associated with synaptic proteins. The physical association with other synaptic proteins provides multiple further points of interaction by which the channel may affect the process of depolarization-induced exocytosis (37–41). We have suggested that vesicle fusion in response to membrane depolarization is initiated by the "excitosome", a protein complex with distinct kinetic properties that comprises the voltage-gated $Ca^{2+}$ channel, syntaxin-1A, SNAP-25, and synaptotagmin. Such potential role is barely explored, mainly because this would require an experimental approach that simultaneously affords control of membrane potential and arbitrary variation of the particular synaptic protein and the $Ca^{2+}$ channel. Such experiments are a unique strength of reconstitution in *Xenopus* oocytes.

It is important to mention that *Xenopus laevis* oocytes do not respond to membrane depolarization and are not excitable cells, in contrast to mature eggs (49,50) in which capacitance changes were studied previously (51,52).

Reconstitution of depolarization-induced exocytosis in *Xenopus* oocytes was achieved by expressing synaptic proteins with $Ca_V1.2$, the voltage-gated $Ca^{2+}$ channel that supports fast release in bipolar cells (53) and neuroendocrine cells (54,55). Exocytosis was not observed when the $Ca^{2+}$ channel was either absent or blocked (by nifedipine or $Cd^{2+}$), or when synaptotagmin was not expressed. Similarly, the effect of neurotoxins that selectively cleave either syntaxin 1A (BotC) or SNAP-25 (BotA) showed that syntaxin 1A and SNAP-25 are strictly required for depolarization-induced exocytosis. In contrast, the only partial inhibition caused by TetX demonstrated that synaptobrevin is not absolutely required for depolarization-induced exocytosis although it enhances the process. Our data demonstrate directly for the first time that $Ca_V1.2$ together with syntaxin 1A, SNAP-25 and synaptotagmin, but not synaptobrevin, are sufficient and necessary for depolarization-induced secretion. Further studies are needed to explore the fact that the tSNAREs in combination with synaptotagmin can engender a depolarization-evoked secretion in the absence of synaptobrevin. Many further proteins are implied in depolarization-induced exocytosis (e.g., Munc-18 (56), Munc-13 (57), complexins (58), and others). Their exact roles can be assessed using our reconstitution assay, analogously to the approach taken in this study to characterize the $Ca^{2+}$ channel, syntaxin 1A, SNAP-25, and synaptotagmin I.

The features of the reconstituted process were similar to native depolarization-induced exocytosis: fast, robust, with nonlinear concentration dependence, modulated by the type of charge carrier and the type of $Ca^{2+}$ channel. The dissociation between the differential effects of these $Ca^{2+}$ channels on $\Delta C_m$ on the one hand, and on $Ca^{2+}$ currents on the other, implies that $Ca^{2+}$ channels have a role in depolarization-induced exocytosis beyond $Ca^{2+}$ transport into the cytoplasm. The existence of such additional roles was predicted from the excitosome hypothesis, and their experimental demonstration lends further support to this hypothesis.

Our reconstitution assay appears to mimic fast secretion rather faithfully. When interpreting the data, one should, however, keep in mind the differences compared to the native process at synapses, and to other reconstitution assays. Compared to neurons, the oocyte is clearly not an excitable cell in terms of geometry and organellar or cytoskeletal makeup. These properties may affect the kinetics of depolarization-induced exocytosis.

These shortcomings are seemingly inherent to other ex vivo systems such as cortices of sea urchin eggs, HEK293 cells and to in vitro systems. Compared to reconstitution assays that employ artificial lipid vesicles and purified proteins, the oocytes do provide a background that is neither completely "clean" nor completely defined. Rather, oocytes likely express endogenously multiple proteins that could to some extent affect depolarization-induced exocytosis. However, the contribution of these proteins can easily be unmasked by the significantly higher expression of the other heterologous proteins (which would make the putative contribution of the endogenous proteins rate limiting) or the use of specific neurotoxins (cf. the evidence for a putative endogenous syntaxin in this work provided by the finding that depolarization-induced exocytosis was sensitive to BOT-C even when no heterologous syntaxin was expressed; Fig. 2 *C*). Interestingly, we found that secretion was more efficient in $Ba^{2+}$ than in $Ca^{2+}$ whereas the ensuing compensatory endocytosis was significantly slower. Although this result is in line with certain data on secretion (59,60) it conflicts with reports that $Ba^{2+}$ ion was the least effective in both bipolar cells and in the squid giant synapse (61,62).

The higher efficacy of $Ba^{2+}$ with respect to depolarization-induced exocytosis could be explained by the depolarizing

effect of extracellular $Ba^{2+}$, its greater rates of entry through noninactivating $Ca^{2+}$ channels, by its poor intracellular buffering (largely arising from its weak affinity for plasmalemmal $Ca^{2+}$ extruders), and—finally—by the possible existence of fast and slow modes of secretion. On the other hand, data from bipolar cells suggested that the influx of $Sr^{2+}$ or $Ba^{2+}$ decreased the relative proportion of docked vesicles available for fast exocytosis as compared to $Ca^{2+}$, and thus $Ca^{2+}$ was more effective at triggering secretion (62). It appears that the contribution of cation selectivity to the efficacy of exocytosis in secreting cells is not fully resolved to date. Maybe, these aspects of the mechanism of secretion could be easier to investigate in a reconstituted system.

Clear advantages of the oocytes are that they allow one to use full-length proteins expressed with the corresponding signal peptide, targeted to the correct location and operating at the native membrane of a living cell, as opposed to reconstitution assays that employ truncated recombinant proteins and artificial membranes. In these experiments one has to consider that the extent of $\Delta C_m$ in different batches of oocytes varies, ranging from 1000–4000 pF, and most probably is dependent on the quality of the oocytes in the various batches.

Taken together, the data we could gather with this novel system of reconstituted depolarization-induced exocytosis is consistent with our previous proposed "excitosome" hypothesis. According to this hypothesis, the minimal molecular machinery that is sufficient and necessary for depolarization-induced vesicle fusion with the cell membrane is a protein complex comprising a voltage-gated $Ca^{2+}$ channel, syntaxin 1A, synaptotagmin, and SNAP-25. Synaptobrevin enhanced release efficiency but was not essential. These findings lend further support to the notion that the outcome of studies that aim to dissect the molecular basis of regulated exocytosis may vary widely depending on which of the many different exocytosis assays was employed. In this respect, our novel reconstitution assay in *Xenopus* oocytes offers the most direct experimental approach to depolarization-evoked exocytosis and could provide important clues to the further understanding of synaptic transmission.

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