Ca\textsubscript{\textit{v}}2.1 (P/Q channel) interaction with synaptic proteins is essential for depolarization-evoked release

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Abbreviations: VGCCs, voltage-gated Ca\textsuperscript{2+} channels; SNAP-25, synaptosome-associated protein of 25,000 daltons; t-SNAREs, target SNAREs syntaxin 1A and SNAP-25; VAMP-2, vesicle SNARE; BotC1, botulinum neurotoxin C1; BotA, botulinum toxin A

It is well-established that syntaxin 1A (Sx1A), SNAP-25 and synaptotagmin (Syt1) either alone or in combination, modify the kinetic properties of voltage-gated Ca\textsuperscript{2+} channels (VGCCs). The interaction interface resides mainly at the cytosolic II-III domain of the alpha 1 subunit of the channels, while Sx1A interacts with the channel also via two highly conserved cysteine residues at the transmembrane domain. In the present study, we characterized Ca\textsuperscript{2+}-independent coupling of the human neuronal P/Q-type calcium channel (Ca\textsubscript{v}2.1) with Sx1A, SNAP-25, Syt1 and synaptotobrevin (VAMP) in BAPTA-injected Xenopus oocytes. The co-expression of Ca\textsubscript{v}2.1 with Sx1A, SNAP-25 and Syt1, produced a multiprotein complex with distinctive kinetic properties analogous to the excitosome complexes generated by Ca\textsubscript{v}1.2, Ca\textsubscript{v}2.2 and Ca\textsubscript{v}2.3. The distinct kinetic properties of Ca\textsubscript{v}2.1 acquired by close association with Syt1 and t-SNAREs, suggests that the vesicle is tethered to the neuronal channel and to the exocytotic machinery independently of intracellular Ca\textsuperscript{2+}. To explore the relevance of these interactions to secretion we exploited a BotC1-and a BotA-sensitive secretion system developed for Xenopus oocytes not buffered by BAPTA, in which depolarization-evoked secretion is monitored by a change in membrane capacitance. The reconstituted release mediated by Ca\textsubscript{v}2.1 is consistent with the model in which the VGCC plays a signaling role in triggering release, acting from within the exocytotic complex. The relevance of these results to secretion posits the role of possible rearrangements within the excitosome subsequent to Ca\textsuperscript{2+} entry, setting the stage for the fusion of channel-tethered-vesicles upon the arrival of an action potential.

Introduction

Membrane depolarization causes the opening of voltage-gated Ca\textsuperscript{2+} channels (VGCCs) and subsequent Ca\textsuperscript{2+} influx, which by means of binding to intracellular proteins is linked to a variety of cellular responses, including the release of neurotransmitters from presynaptic sites.\textsuperscript{1}

The rapid temporal coordination of neurotransmitter release with membrane depolarization necessitates a close physical and functional proximity of the Ca\textsuperscript{2+} channel to the exocytotic machinery. This would enable tight coupling of depolarization to both vesicle fusion and calcium influx.

The VGCC, Ca\textsubscript{v}1.2 (Lc-type), Ca\textsubscript{v}1.3 (L\textsubscript{D}-type), Ca\textsubscript{v}2.2 (N-type), Ca\textsubscript{v}2.3 (R-type) and Ca\textsubscript{v}2.1 (P/Q-type) have all been shown to be both functionally and physically coupled to exocytotic proteins.\textsuperscript{2,3} This coupling has led to the postulate that the VGCCs serve this dual function of calcium influx and vesicle fusion. The relevance of synaptic proteins/channel interactions to evoked-secretion was established showing that the recombinant cytosolic II-III region of the \(\alpha\)1 subunit of Ca\textsubscript{v}2.2,\textsuperscript{2,5} and Ca\textsubscript{v}1.2,\textsuperscript{6,7} is able to effectively compete with the endogenous channel for binding to the synaptic proteins. The binary complex consists of two t-SNARE proteins (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) syntaxin 1A (Sx1A) and SNAP-25, and has been suggested to represent an intermediate step towards the assembly of the synaptic SNARE complex.\textsuperscript{8} Other studies have characterized putative exocytic units consisting of the t-SNARE’s, Syt1 and VGCC, such as Ca\textsubscript{v}1.2 (Lc-type), Ca\textsubscript{v}2.2 (N-type) or Ca\textsubscript{v}2.3 (R-type). The functional hetero-protein secretory complex, which is assembled in a Ca\textsuperscript{2+}-independent manner, has been termed excitosome.\textsuperscript{6} It might correspond to the 10 nm particles identified in pre-synaptic terminals.\textsuperscript{9,11} The assembly of the complex is a prerequisite for tethering the vesicles to be in a close contact with the channel. Using a reconstituted assay of depolarization-evoked release in Xenopus oocyte we have previously shown the relevance of these channel/synaptic proteins interactions to the excitation-secretion process.\textsuperscript{12} The excitosome model predicts that the channel is

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acting from within the complex, propagating a conformational signal capable of triggering fast vesicle fusion.\textsuperscript{2,6,13,14}

The neuronal P/Q-type Ca\textsuperscript{2+} channels, Ca2.1, is one of the main Ca\textsuperscript{2+} channel subtypes expressed in presynaptic nerve terminals, and its opening is linked to the rapid release of synaptic vesicles.\textsuperscript{13} Ca2.1 introduces large inward Ca\textsuperscript{2+} currents\textsuperscript{16,17} and its involvement in synaptic transmission has been confirmed by the use of selective channel blockers.\textsuperscript{18-22}

In the present study we explored (1) the possibility that the human P/Q type channel participates in a complex with Syt1 and the t-SNARE proteins when the internal Ca\textsuperscript{2+} concentration was clamped using BAPTA-injected Xenopus oocytes and (2) the feedback regulation of the human P/Q type channel by the t-SNARE proteins in combination with Syt1 and its significance to depolarization-evoked secretion in oocytes not buffered with BAPTA.

The results presented here show that Sx1A diminishes Ca2.1 current amplitude while SNAP-25 increases the peak current and accelerates both the activation and inactivation of the channel. Ca2.1 co-expressed with either one of the t-SNAREs or syt1 individually, displays a different kinetic profile. The co-expression of Ca2.1 with syt1, Sx1A and SNAP-25 generates Ca\textsuperscript{2+}-buffered conditions an excisosome complex. Using a reconstituted assay system, we have demonstrated that this complex corresponds to a minimal set of proteins that trigger a BotA- and BotC1-sensitive depolarization-evoked secretion.\textsuperscript{a}

**Results**

**Modulation of Ca2.1 by Sx1A.** The functional consequences Sx1A/Ca2.1 interactions were analyzed using whole-cell voltage-clamp recording in Xenopus oocytes. Expression of recombinant Ca2.1 was achieved by microinjection of the corresponding cRNA of the three subunits α,β2.1 (8.4 ng/oocyte), αβS1A (5.2 ng/oocyte), β3 (1.65 ng/oocyte). Twenty-four hrs later, the oocytes were divided into 5 groups. Four were injected with increasing Sx1A cRNA (0.6–2.5 ng/oocyte) and one group with DDW, in a final volume of 40 nl. At day 6, whole cell Ba\textsuperscript{2+} currents were recorded from each group of oocytes (n = 10–12) using the two-electrode voltage-clamp technique.\textsuperscript{2} Step depolarization from a holding potential of -80 mV to various test potentials activated inward currents, which peaked at +5 mV (Fig. 1A). Traces of inward currents (InA) evoked to +5 mV are shown in the upper panels of Figure 1A. A gradual inhibition of inward currents was correlated with increasing Sx1A concentrations at all voltages tested, as shown by the leakt- subtracted peak current-voltage relationship (Fig. 1A Lower; Table 1). The Sx1A effect on cardiac Lc-type Ca_{1.2} currents expressed in oocytes was compared using the same range of Sx1A concentrations and under the same experimental conditions (Fig. S1; Table 1). This negative modulation of current amplitude by Sx1A is dependent on the channel interaction with two highly conserved cysteine residues, Cy271 and Cys 272 within the Sx1A transmembrane domain.\textsuperscript{23} Sx1A appeared to be more effective at inhibiting the cardiac Ca_{1.2}, demonstrated by the percent inhibition of current amplitude (Fig. 1B). The increase in Sx1A expression was detected by western blot analysis using monoclonal anti-Sx1A antibodies (Fig. 1C).

**SNAP-25 alters current properties of Ca_{2.1}.** As opposed to Sx1A, the second t-SNARE protein, SNAP-25, caused a significant increase in current amplitude, and modified the kinetic properties of Ca_{2.1}, consistent with earlier studies of other VGCC, Lc-, N- and R-type channels (Fig. 2).\textsuperscript{24} It has been previously shown that the binary complex formed by SNAP-25 and Sx1A plays an essential role in the formation of the SNARE complex.\textsuperscript{25} Hence, current traces generated by Ca_{2.1}/SNAP-25 were compared to currents mediated by Ca_{2.1}/SNAP-25/Sx1A (Fig. 2). Unlike the Lc-type channel\textsuperscript{26} current traces of Ca_{2.1} were significantly increased by SNAP-25 (Fig. 2A, left; n = 12). When Sx1A was co-expressed along with SNAP-25, current amplitude of Cav2.1/SNAP-25/Sx1A was normal (Fig. 2A, right). Both the increase in current amplitude by SNAP-25 and the reversal to control level by SNAP-25/Sx1A (Fig. 2B, right; n = 12; Table 1) are depicted in the leak-subtracted inward currents. SNAP-25 also increased the channel activation (Fig. 2C) and the effect was nullified with the expression of the binary complex (Fig. 2C, right). The G/Gmax ratio was shifted to the left by SNAP-25, an effect that was reversed by Sx1A (Fig. 2D). The accelerated rate of Ca_{2.1} inactivation observed by SNAP-25 at most voltages (Fig. 2E, left), was significantly reversed in the presence of Sx1A (Fig. 2E, right). Hence, the reciprocity of Sx1A and SNAP-25 effects on Ca_{2.1} is consistent with the functional modification of other VGCCs.\textsuperscript{2,27}

The reversal of the positive regulation of [Ca_{2.1}/SNAP-25] by [Ca_{2.1}/SNAP-25/Sx1A] could result from competitive displacement of Sx1A from the II-III loop. Alternatively, the rearrangement of the binary complex vis a vis Ca_{2.1} could have prevented the interaction of the channel with the transmembranal Sx1A Cys271 and Cys272.\textsuperscript{2,6,23} The results clearly show that the binary complex interacts with the channel in a distinct way, which differs from the individual t-SNAREs.

**The effect of botulinum toxin C1 (BotC1) on Sx1A/channel interactions.** The functional consequences of Sx1A/Ca_{2+} channel interaction were analyzed using whole-cell voltage-clamp recording with the co-expression of the BotC1 light-chain. Previously it was shown that BotC1 reversed the Sx1A-negative regulation of Ca_{1.2} current amplitude.\textsuperscript{31} Sx1A decreased Ca_{2.1} inward currents elicited by a depolarization step from a holding potential of -80 mV to +10 mV, shown by the superimposed current traces (Fig. 3A, left; Table 2, see also Fig. 1). The Sx1A-inhibited Ca_{2.1} current was restored to normal amplitude when co-expressed with BotC1 (Fig. 3A upper right). The Sx1A-negative modulation of current amplitude and its reversal by BotC1 are illustrated also by the leak-subtracted current-voltage relationship, using data collected from groups of 10–12 oocytes (Fig. 3A, lower).

Since the recordings were performed in BAPTA-injected oocytes, the increase in current density by BotC1 was largely independent of intracellular Ca\textsuperscript{2+}, further confirming a direct interaction of the channel with Sx1A. Also the negative modulation of [Sx1A/SNAP-25]/Ca_{2.1}/Syt1 currents were abolished by BotC1, consistent with the importance of the channel/t-SNARE’s/Syt1-interaction for neurotransmission (data not shown). The Sx1A-modified steady-state voltage inactivation Figure 3B (left) (reviewed in ref. 2), was reversed by BotC1.
alpha-helical motifs, which generate a tight SNARE complex involved in vesicle fusion. Here we looked at whole cell Ca\(^{2+}\) currents recorded from oocytes injected with the three channel subunits together with either the SNARE complex, or the SNARE complex with Syt1 (Fig. 4). VAMP by itself increased current amplitude, slowed the rate of activation only at -5 mV (Fig. 4A–C, left), and shifted the steady-state inactivation toward more negative potential within the voltage range of -50 to -10 mV (data not shown). The kinetic parameters of the channel were affected neither by co expression of the SNARE proteins (Fig. 4, middle), nor with the SNARE proteins and syt1 (Fig. 4, right).

Modulation of Ca\(_{2.1}\) by Syt 1. Superimposed current traces elicited by a voltage step from -80 mV to +10 mV in response to a 500 ms pulse in oocytes expressing Ca\(_{2.1}\) alone or together with syt1, were similar (Fig. S2A). As shown by the leak-subtracted

(Fig. 3B, right). The data suggests BotCl-cleavage of Sx1A reinstated current amplitude, and the pre-assembly of Sx1A with Ca\(_{1.2}\) as indicated by the steady-state voltage inactivation.

The value of the midpoint voltage inactivation of the channel \(V_{1/2} = -22 \text{ mV} \pm 1.9\) is similar to the \(V_{1/2} = -17.2 \pm 1.2\) reported by Zhong et al. 1999, although other values were previously reported for HEK293 expressed without any \(\beta\) subunit\(^{28}\) or Xenopus oocytes expressed with \(\beta_3\) subunit.\(^{29}\)

The differences among the various experimental models mentioned above also depend on the expression level of the auxiliary subunits, which eventually determine the exact molar ratio among the channel subunits and the isoform used.

Ca\(_{1.2}\) (P/Q type-channel) interacts with Sx1A, SNAP-25, VAMP and Syt1. The t-SNAREs (Sx1A, SNAP-25) and v-SNARE (synaptobrevin; also known as VAMP) share common

alpha-helical motifs, which generate a tight SNARE complex involved in vesicle fusion.\(^{30}\) Here we looked at whole cell Ca\(^{2+}\) currents recorded from oocytes injected with the three channel subunits together with either the SNARE complex, or the SNARE complex with Syt1 (Fig. 4). VAMP by itself increased current amplitude, slowed the rate of activation only at -5 mV (Fig. 4A–C, left), and shifted the steady-state inactivation toward more negative potential within the voltage range of -50 to -10 mV (data not shown). The kinetic parameters of the channel were affected neither by co expression of the SNARE proteins (Fig. 4, middle), nor with the SNARE proteins and syt1 (Fig. 4, right).

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current-voltage relationship, Syt1 had no significant effect on the kinetics parameters at all voltages; data was collected from groups of 10–12 oocytes (Fig. S2B). Similarly, as previously shown for other types of VGCC, Syt1 affected neither the G/Gmax ratio nor the CaV2.1 activation (Fig. S2C).

Syt1 modulation of a channel assembled with t-SNARE complex: the kinetics of the excitosome complex. We sought to characterize the t-SNARE complex and syt1, which assemble with the channel to generate a kinetically distinct releasing unit, called the excitosome, as recorded in BAPTA-injected Xenopus oocytes. Excitosome kinetics was previously measured for the N-type channel the t-SNAREs/CaV1.2 elicited the largest Cm step (1.5 ± 0.25 nF) (Fig. 5C). A similar, to CaV1.2, a small depolarization-induced Cm change could be elicited upon expression of the channel subunits (CaV2.1) without the t-SNAREs (0.27 ± 0.08 nF; n = 10–15) (Fig. 5B and Table 3). The Cm step elicited by the CaV2.1 channel concurrently expressed with syt1 was larger, 0.68 ± 0.17 nF; n = 10–15, and increased upon adding Sx1A (0.93 ± 0.17 nF) while Syt1/t-SNAREs/CaV1.2 elicited the largest Cm step (1.5 ± 0.25 nF) (Fig. 5C and Table 3).

The role of individual synaptic proteins in reconstitution of depolarization evoked secretion determined by membrane capacitance. We reconstituted a CaV2.1-mediated depolarization-induced exocytosis, as previously demonstrated for the Lc-type channel.21,22 Although the preassembly of the excitosome complex is CaV2.1-independent, the priming of the vesicles prior to secretion needs CaV2.1 bound at the Syt1 C2 domains. Therefore, the reconstitution experiments were carried out in Xenopus oocytes that were not injected with BAPTA. The cells were stimulated from a holding potential of -80 mV to 0 mV by a single 500-ms pulse (Fig. 5A). Continuous monitoring of membrane capacitance was used to screen the effect of depolarization on membrane capacitance (Cm) in oocytes. Representative original traces of voltage (upper), current (middle), and Cm (lower) are shown (Fig. 5A). The Cm values were recorded from oocytes injected with CaV1.2/Syt1/Sx1A/SNAP-25 (see below). The vertical dashed line indicates the precise time at which Cm was read for comparison with the baseline Cm (horizontal dashed lines) (Fig. 5A). Next, we monitored Cm changes in oocytes in which the synaptic proteins were assembled step-by-step (Fig. 5B). Similar, to CaV1.2, a small depolarization-induced Cm change could be elicited upon expression of the channel subunits (CaV2.1) with SNAP-25 (2.4 ng/oocyte) -2447 ± 363 p 1A (0.93 ng/oocyte) 1A (2.5 ng/oocyte) -248 ± 87 * 4.6 ± 0.26 0.04 ± 0.34 61

| Current inhibition (%) | τs (ms) | V1/2 (mV) | Current inhibition (%) |
|------------------------|---------|-----------|------------------------|
| α,1.2/β2A/α2δ (CaV2.1) | -1282 ± 166 5.2 ± 0.22 -3.98 ± 0.50 - |
| +SNAP-25 (2.4 ng/oocyte) | -2447 ± 363 p 1A (0.93 ng/oocyte) 1A (2.5 ng/oocyte) -248 ± 87 * 4.6 ± 0.26 0.04 ± 0.34 61
| +SNAP-25 + Sx1A (1.7 ng/oocyte) | -1154 ± 191 5.2 ± 0.31 -2.82 ± 0.38 0

Currents were elicited from a holding potential of -80 mV to various test potentials in response to 500 ms test pulse. Peak current voltages ± standard deviation were obtained. 10 mM Ba2+ was used as the charge carrier.

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in oocytes injected with the mammalian CaV2.1 subunits, no further increase in Cm was observed. These results suggest either a lack of interaction of the endogenous synaptic proteins with the mammalian channel subunits, or a significantly lower level of expression.

The levels of synaptic proteins controls the extent of the reconstituted depolarization-evoked secretion. Representative traces of Cm observed in oocytes expressing the three channel subunits (α, β3 and α2δ) either alone (Fig. 6A, left) or with increasing amounts (two and three times) of cRNAs for the synaptic proteins, at fixed cRNA amounts of channel subunits) are shown. The quantification of the Cm signals showed roughly a progressive increase of the $C_m$ from 1.04 ± 0.12 nF, to 1.37 ± 0.08 nF and 1.9 ± 0.13 nF, respectively (Fig. 6B and Table 3; n = 10–15). There was no correlation between the levels of the cRNAs of the synaptic proteins and current amplitude (Fig. 6C, upper).

The reconstituted secretion is sensitive to BotC1 and BotA. We tested the sensitivity of the reconstituted depolarization-induced secretion to the botulinum toxin peptides, and determined the individual contributions of the t-SNAREs, Sx1A and SNAP-25, to the reconstituted exocytosis (Fig. 7).

Depolarization-induced Cm changes were considerably larger in oocytes expressing the excosome complex, than those monitored in oocytes expressing the channel subunits (α, β3 and β3) alone as shown in the Cm traces (Fig. 7A, upper). Co-expression of BotC1–light chain together with the excosome proteins abolished the depolarization-induced Cm change (Fig. 7A, lower B). A similar inhibition was observed upon the co-expression of botulinum toxin A (a protease specific for SNAP-25; BotA) as shown in Figure 7A, lower and C. These neurotoxins had no significant effect on the depolarization-induced current amplitudes (Fig. 7B and C, right). The results suggest that both Sx1A and SNAP-25 are essential for depolarization-induced exocytosis.

**Discussion**

The CaV2.1 (P/Q-type) and CaV2.2 (N-type) Ca^{2+} channels are the main Ca^{2+} channel subtypes expressed in presynaptic nerve terminals, which are involved in the rapid release of synaptic vesicles;^{15,33-36} the contribution of the CaV2.3 (R-type) Ca^{2+} channels to synaptic release is less obvious.^{37} CaV2.1,2, the L-type Ca^{2+} channel is involved in evoked-release mainly in neuroendocrine cells such as chromaffin and pancreatic beta cells.^{38,39}

All four Ca^{2+} channels, the N-type, P/Q-type, R-type and the L-type were shown to interact specifically through direct binding with synaptic proteins such as syntaxin 1, SNAP-25, Syt1 and other proteins such as cysteine string protein and Rim.^{2,31,40}

In the present study we have demonstrated functional interactions of CaV2.1 with proteins of the exocytotic machinery, and have reconstituted a CaV2.1-mediated depolarization-evoked secretion that is BotC1-and BotA-sensitive.^{12,23} Our results are
consistent with previous studies demonstrating that the VGCC serves as a major signaling component of the secretory unit called the excitosome complex, a multiprotein complex composed of a voltage-gated Ca\(^{2+}\) channel, Sx1A, SNAP-25 and Syt1.

Modulation of Ca\(_{\alpha 2.1}\) current kinetics by synaptic proteins and chlostridial neurotoxins. Here we characterized the Ca\(^{2+}\)-independent functional interactions of SNAP-25, Sx1A and Syt1 with Ca\(_{\alpha 2.1}\) in Xenopus oocytes, in a manner previously shown for Ca\(_{\alpha 1.2}\), Ca\(_{\alpha 2.2}\), Ca\(_{\alpha 2.2}\) and Ca\(_{\alpha 2.3}\).\(^{6,31,41}\)

Syntaxin 1A and SNAP-25 differently modify the kinetics of Ca\(_{\alpha 2.1}\) and Ca\(_{\alpha 1.2}\). In excitable cells the VGCCs are likely to be associated with the t-SNARE binary complex. Similar to previous transient expression systems we showed that the syntaxin 1A or SNAP-25, either alone, or in combination, altered Ca\(_{\alpha 2.1}\) current kinetics in a distinctive fashion when co-expressed in Xenopus oocytes. The decrease in Ca\(_{\alpha 2.1}\) current amplitude caused by Sx1A is consistent with its similar negative regulation of L\(-\), N- and R-type current amplitudes.\(^{26}\)

Ca\(_{\alpha 2.1}\) was significantly less sensitive to Sx1A than Ca\(_{\alpha 1.2}\), shown by the rightward shift in the concentration dependency curve.\(^{26}\) Also the kinetics of Ca\(_{\alpha 2.1}\) and Ca\(_{\alpha 1.2}\) were differently modified by SNAP-25.\(^{26}\)

With Ca\(_{\alpha 2.1}\) we showed that SNAP-25 accelerated the rate of activation and increased the current amplitude.\(^{26}\) The observed differences could result from sequence variation of the intracellular domains within the loop that connects segments II and III (synprint) of \(\alpha_{2.1}\) or \(\alpha_{1.2}\) subunits.\(^{26}\) It is well established that the II-III loop, which has poor sequence homology among the channels, constitutes the interaction interface of the channel with Sx1A, SNAP-25 and Syt1.\(^{6,42,43}\) This site determines the strength of the channel interaction with the synaptic proteins and could account for the differences in vesicle docking and the intensity of the stimulus-secretion signal. For example, in bovine chromaffin cells, Ca\(_{\alpha 1.2}\) appears to be tightly coupled to the secretory machinery, mediating ~90% of depolarization-evoked catecholamine secretion, and yet being responsible for only ~20–30% of total Ca\(^{2+}\) influx.\(^{44}\) In contrast, Ca\(_{\alpha 2.1}\) and Ca\(_{\alpha 2.2}\) mediate ~10% of the depolarization-evoked release in these cells, and are responsible for ~70% of the Ca\(^{2+}\) influx.\(^{45,46}\)

Previous results had shown that the rabbit rbA isoform of alpha1A was effectively modified by SNAP-25 when co-expressed in tsA-210 cells, and was not affected by Sx1A.\(^{47}\) In contrast, the human alpha1A clone used in the present study, was effectively modulated both by Sx1A and SNAP-25. These results are consistent with the identification of various \(\alpha_{2.1}\) gene isoforms\(^{48}\) that could differently interact with synaptic proteins,\(^{49}\) and support the

**Figure 3.** The Sx1A negative regulation of Ca\(_{\alpha 2.1}\) currents is abolished by Botulinum toxin C1. (A) Currents were elicited from a holding potential of -80 mV to various test potentials by a 500 ms test pulse in 5 mV increments. Upper, superimposed traces \(\alpha_{2.1}/\beta 3/\alpha\delta\) Ca\(^{2+}\) currents elicited in response to a 500 ms test pulse from a holding potential of -80 mV to +10 mV in oocytes injected either 24 hr later with Sx1A (2 ng/oocyte) (left) or additionally injected 24 hr later with BotC1 light chain (8 ng/oocyte) (right). The traces shown have been corrected for leakage and capacitive transients by off-line subtraction. Lower, leak-subtracted current-voltage plots for \(\alpha_{2.1}/\beta 3/\alpha\delta\) currents expressed without (open circle) or with Sx1A (open circle), or co-expressed with Sx1A (closed circle; left; \(V_{1/2} = -21.6 \pm 2\) mV) or Sx1A + BotC1 (closed circle triangle; right; \(V_{1/2} = -22.\pm 1.9\) mV) (n = 13–15). *p < 0.01; 10 mM Ca\(^{2+}\) was used as the charge carrier.

**Table 2.** BotC1 reverses the inhibitory effect of Sx1A on Ca\(_{\alpha 2.1}\) current amplitude

| Current (nA) | Current inhibition (%) |
|-------------|------------------------|
| \(\alpha_{2.1}/\beta 3/\alpha\delta\) (Ca\(_{\alpha 2.1}\)) | -1003 \(\pm\) 241 (10 mV) | - |
| +Sx1A | -421 \(\pm\) 147 (10 mV) | 58 |
| +Sx1A + BotC1 | -966 \(\pm\) 260 (5 mV) | 4 |

Currents were elicited from a holding potential of -80 mV to various test potentials in response to a 500 ms test pulse using 10 mM Ca\(^{2+}\) as the charge carrier. Peak current voltages are shown; current inhibition was determined at peak current and percent inhibition was calculated in relation to peak current obtained by the three-channel subunit. Two sample Student’s t-test assuming unequal variance was applied. *p values <0.01.
It was previously shown that the kinetics of the P/Q-, N- and R-type channels were significantly modified by botulinum toxin BotC1.28,51,55 In the case of the L-type channel, BotC1 relieved the inhibitory effect of Sx1A on current amplitude but not the negative modulation of the time constant of channel activation.56

Next we showed that BotC1 reversed the Sx1A-modified-steady-state voltage inactivation, and relieved the inhibition of current amplitude. These results are consistent with a previous study, in which the α2.1-induced Sx1A in HEK293 cells led to a steady state inactivation of the P/Q channel that was shifted to more positive potentials upon a direct exposure to Bot1C1.28 Other studies have shown that Sx1A cleavage by BotC1 did not reverse the shifted steady-state inactivation of the N-type channel.57

The positive modulation of CaV2.1 currents by VAMP (synaptobrevin) was reversed in the presence of either [Sx1A/SNAP-25], or [Sx1A/SNAP-25/Syt1], as recorded in BAPTA-injected oocytes. This Ca2+-independent kinetic modulation is most likely involved in vesicle docking prior to vesicle fusion.58,59

**Figure 4.** Functional modulation of CaV2.1 by VAMP, SNAP-25 and Syt1. (A) Current traces evoked by a 500 ms test pulse from a holding potential of -80 mV to 0 mV from oocytes injected with α2.1/β3/αδ2, or α2.1/β3/αδ2 with VAMP (0.5 ng/oocyte; left) 24 hr later, or α2.1/β3/αδ2 with SNAREs (VAMP, Sx1A and SNAP-25, middle), or excitosome (Sx1A/SNAP-25/Syt1) and VAMP (right). The traces shown have been corrected for leakage and capacitive transients by off-line subtraction. (B) Peak current-voltage relations of α2.1/β3/αδ2 (open circle) or with either VAMP (closed circle; left), SNAREs VAMP, Sx1A and SNAP-25 (middle; closed diamond), or excitosome plus VAMP (right; inverted triangle) as indicated (see also A). (C) The time constant of activation (τ act) of α2.3/β2A/αδ2 currents without (open circle), or with VAMP (right; closed circle), or with SNAREs (VAMP, Sx1A and SNAP-25; middle; closed diamond), or with excitosome plus VAMP (right; inverted triangle), as indicated (see A). The data points correspond to mean ± SEM, (n = 8–10); 5 mM Ba2+ was used as the charge carrier. cRNAs amounts as indicated in legends of Figures 2 and 3.
the endogenous SNAREs could have affected the channel kinetics, the exogenously expressed proteins eventually determined the functional modified kinetics.

Reconstitution of CaV2.1-mediated secretion. To provide further evidence for the relevance of the channel interaction with the synaptic proteins for secretion, we have reconstituted a CaV2.1-mediated depolarization-evoked release, monitoring capacitance changes by heterologous expression in Xenopus oocytes, as previously described for the Lc-type channel. Because the individual protein-protein interactions in excitable cells are difficult to dissect, and equally difficult would be an in vitro reconstruction of pre-associated complexes using recombinant transmembranal proteins, we chose to reconstitute depolarization-evoked secretion using the oocyte expression-system.

Figure 5. Reconstitution of depolarization-evoked secretion. (A) The effect of depolarization on membrane capacitance (Cm) in oocytes expressing the subunits α,2.1, β3, α2δ together with the t-SNARE complex and Syt1 (the excitosome); representative original traces of voltage (upper), current (middle) and Cm (lower) are shown. The vertical dashed line indicates the precise time at which Cm was read for comparison with the baseline Cm (horizontal dashed lines). (B) Representative capacitance traces elicited in oocytes by a single 500 ms pulse from a holding potential of -80 to 0 mV using 2 mM Ba2+ as the charge carrier. Oocytes were injected with cRNA of α,2.1 (4 ng/oocyte)/β3 (2 ng/oocyte)/α2δ (0.4 ng/oocyte) and 24 h later with Syt1, or a mixture of Syt1/syntaxin 1A, or a mixture of Syt1/Syntaxin1A/SNAP-25 and stimulated at day 5 (see Experimental Procedures) by a single 500 ms pulse. (C) Summary of depolarization effect on Cm observed in groups as in (B). The depolarization-induced change of membrane capacitance ∆Cm is shown as bars (mean ± SEM; n = 12–17). (D) The effect of synaptic proteins on the inward currents (InA), using the same protocol as in (A and B), is shown as bars. Each experiment was carried 3–5 times using different batches of oocytes. The data points correspond to mean ± SEM; n = 12–17 *p < 0.05; **p < 0.01; ***p < 0.005. cRNA injection, Syt1 0.77 ng/oocyte, Sx1A 1.2 ng/oocyte, SNAP-25 0.73 ng/oocyte.
non-releasable functional proteins was established in a manner previously minimal set of proteins, which does not preclude additional to reconstitute depolarization-evoked release. The proposed Syt1 in oocytes, which were not injected with BAPTA, was able strengthened when mutations at Syt1 C2A polylysine motif, type Ca2+ channel, could associate with Syt1, and the SNARE performed for reconstituting Lc-type-mediated release. 12 The shown to be a specific interface with the channel, abolished buffering of the intracellular Ca2+ (no BAPTA injection). We suggest that a rise of [Ca2+]i during an action potential would channels, and the fusion machinery. 12 This view was further supported by the ability of the two neurotoxins BotC1 and BotA to inhibit secretion.

Extrapolating from the oocyte expression system, we have previously proposed that the excitosome could coordinate secretion by providing a direct functional linkage between the channel, the vesicle, and the fusion machinery. 12 This view was strengthened when mutations at Syt1 C2A polylysine motif, shown to be a specific interface with the channel, abolished depolarization-evoked capacitance transients. 62 In summary. (1) Using Ca2+-independent interactions in BAPTA-injected oocytes we have demonstrated that the P/Q-type Ca2+ channel, could associate with Syt1, and the SNARE proteins to generate a distinct and kinetically active complex. We suggest that the long-winded Ca2+-independent channel interactions with the synaptic proteins lead to the tethering of the vesicle to the channel. Under resting calcium concentrations, these channel-associated docked vesicles are non-releasable vesicles, (2) We have reconstituted depolarization-evoked release in oocytes co-expressing P/Q-type with the t-SNARE's and Syt1. The BotC1- and BotA-sensitive reconstituted release was observed without buffering of the intracellular Ca2+ (no BAPTA injection). We suggest that a rise of [Ca2+]i during an action potential would prime the channel-tethered vesicle (non-releasable) to a releasable vesicle by Ca2+-binding to Syt1 C2 domains. These releasable vesicles will remain readily docked at the membrane and will fuse only in response to an oncoming action potential. This is consistent with a model 14 in which, a cycle of secretion begins by an incoming stimulus that releases vesicles from a non-releasable pool and at the same time, primes a fresh set of non-releasable vesicles, to be fused by the next incoming stimulus. This cycle of events could provide for the well-resolved sub-millisecond time-scale of depolarization-evoked synaptic transmission.

### Experimental Procedures

**cDNA constructs.** Human α2,1 (nm_000068) obtained from T. Snutch, rat β2A (m80545), β3 (M88751) and α1.2 subunit (x15539) were obtained from N. Qin and Dr. L. Birnbaumer (NC, USA); α2/δ rabbit skeletal (M86621) from A. Schwartz (OH, USA). Syt I, SNAP-25 and Sx1A were from M.L. Bennett (CA, USA). The in vitro transcription kit was from Stratagene (USA).

**Electrophysiological assays.** Stage V and VI oocytes were surgically removed from female *Xenopus laevis* and injected with cRNA mixtures encoding Ca2+ channel subunits α2,1, β3 and α2/δ or α1.2, β2A and α2/δ. One day later, cRNA of syntaxin1A, Syt1, SNAP-25 or water (control) was injected. Whole cell currents were recorded by applying a standard two-electrode voltage clamp in BAPTA-injected oocytes (5 mM) as previously described. 43 Recordings were made in Ba2+ solution (in mM): 5/10 Ba(OH)2, 50 N-methyl d-glucamine, 1 KOH and 5 HEPES (pH 7.5), titrated to pH 7.5 [(CH3)2SO4] or Ca2+ solution (in mM): 5/10 Ca(OH)2, 50 N-methyl d-glucamine, 1 KOH and 5 HEPES (pH 7.5), titrated to pH 7.5 [(CH3)2SO4]. Because of currents run-down, the protocol was applied to groups of oocytes (n > 80) that expressed the channel either alone or with synaptic proteins, subdivided for recording in various ions (n = 10–20). Although synaptic P/Q-type channels are most likely associated with β4 and/or β2a, many heterologus expression systems have studied P/Q type channel expressed with β3. 24, 29 The pulse duration of activation was for 500 ms in 10 sec intervals. Current traces were leak-subtracted on-line by the Clampex8.2 software, and channel activation rates were analyzed by applying a mono-exponential fit of the Clampfit 9 software (Axon instruments, Foster City, CA) to the current traces at the relevant ranges.

\[
\text{Fit} = A \exp \left[ t/\tau_{\text{act}} \right] + B
\]

where \(A\) = current amplitude, \(\tau_{\text{act}}\) = time constant, \(t\) = time to peak. Activation was determined from the beginning of the trace just after the capacitative transient to the peak-current region.

\[
\text{Inactivation} = \frac{1}{A \exp \left[ t/\tau_{\text{inact}} \right] + (A \exp \left[ t/\tau_{\text{inact}} \right] + B)}
\]

where \(\tau_{\text{inact}}\) is the fast component.

Conductance (G) was determined using equation

### Table 3. Reconstituted secretion in Xenopus oocytes

|                        | Peak current (nA) | ΔCm (nF) |
|------------------------|------------------|----------|
| α2,1/β3/α2δ (Ca2,1)   | -1441 ± 210      | 0.27 ± 0.08 |
| +Syt1                 | -2023 ± 376      | 0.68 ± 0.17 |
| +Syt1 + Sx1A          | -1208 ± 214      | 0.93 ± 0.17 |
| +Syt1 + Sx1A + SNAP-25 Excitosome (XI) | -1937 ± 310 | 1.50 ± 0.21 |
| α2,1/β3/α2δ (Ca2,1)   | -480 ± 37        | 0.43 ± 0.13 |
| Excitosome (x1)       | -685 ± 61        | 1.04 ± 0.12 |
| Excitosome (x2)       | -773 ± 96        | 1.37 ± 0.08 |
| Excitosome (x3)       | -606 ± 92        | 1.90 ± 0.13 |
| α2,1/β3/α2δ (Ca2,1)   | -532 ± 30        | 0.75 ± 0.08 |
| Excitosome            | -410 ± 28        | 1.60 ± 0.15 |
| Excitosome + BotC1    | -633 ± 38        | 0.80 ± 0.06 |
| α2,1/β3/α2δ (Ca2,1)   | -386 ± 34        | 0.74 ± 0.10 |
| Excitosome            | -346 ± 48        | 1.65 ± 0.12 |
| Excitosome + BotA     | -380 ± 43        | 0.80 ± 0.08 |

Xenopus oocyte injected with the corresponding cRNAs were depolarized by a 500 ms pulse from a holding potential of -80 mV to 0 mV. Recording were performed according to the Experimental Procedure using 2 mM Ba2+.
Capacitance monitoring in Xenopus oocytes. Membrane capacitance (Cm) was monitored in the two-electrode voltage-clamp configuration as published elsewhere.\(^{12,23}\) Briefly, Cm was determined from the current response to a triangular, symmetrical voltage command of “Paired ramps”.\(^6\) In a typical voltage stimulus, command voltage \(V_{com}\) increases by 40 mV within 20 ms, equivalent to a ramp slope of 2 V/s and with this slope, capacitance is obtained from the difference via simple division by 4:

\[ G = I/(V - E_{rev}), \]

where \( I \) = peak-current, \( V \) = voltage test pulse and \( E_{rev} \) = reversal potential of 5 or 10 mM Ba\(^{2+}\) obtained from linear regression at the X intercept of G-V curves plotted as G/Gmax versus voltage pulse and fitted according to Boltzmann.

Steady state inactivation curves were fitted by a single Boltzmann distribution with

\[ I/I_{max} = C/[1 + \exp \left(V_{1/2} - V_m/k\right)] + (1 - C), \]

where \( C \) = maximal steady state inactivating current, \( V_{1/2} \) = midpoint of inactivation, \( V_m \) is the conditioning voltage and \( k \) = slope parameter.

**Data presentation and statistical analysis.** Peak current and \( \tau_{app} \) were analyzed by Clampfit 9 and transferred to an Excel worksheet (Microsoft Inc., USA), and averaged for each group of oocytes and SE was determined. Data points correspond to means and error bars are ±SE. Statistical significance relative to the control group was calculated by Student’s t-test.

**Capacitance monitoring in Xenopus oocytes.** Membrane capacitance (Cm) was monitored in the two-electrode voltage-clamp configuration as published elsewhere.\(^{12,23}\) Briefly, Cm was determined from the current response to a triangular, symmetrical voltage command of “Paired ramps”.\(^6\) In a typical voltage stimulus, command voltage \( V_{com} \) increases by 40 mV within 20 ms, equivalent to a ramp slope of 2 V/s and with this slope, capacitance is obtained from the difference via simple division by 4:

\[ C = \left(\hat{i}_{up}^m - \hat{i}_{down}^m\right) \times \left(\frac{\Delta I}{2\Delta V}\right) \Rightarrow C_m = \frac{\left(\hat{i}_{up}^m - \hat{i}_{down}^m\right)}{4} \]

\(^{12,23}\)Data points correspond to mean ± SEM; \( n = 12–17; **p < 0.01; ***p < 0.005.\)
The up- and down-ramps (±20 mV in 20 ms each) elicit membrane currents that are the sum of a 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 \( \left( i_{m}^{up} - i_{m}^{down} \right) \) eliminates the resistive component; the resulting pure capacitive charge allows together with the known amplitude of the voltage stimulus the computation of the membrane capacitance. Continuous monitoring is achieved by applying this stimulus repetitively at a high rate (normally at 4/s). Comprehensive tests in an electrical cell model as well as in Xenopus oocytes have demonstrated the high precision, accuracy and robustness of this technique. Depolarizing stimuli were applied from a holding potential of -80 mV to 0 mV for 500 ms. Capacitance was monitored before and after the stimuli and simultaneously with the membrane potential \( (V_m) \). All Cm experiments were made in Ba\(^{2+}\) solution \((\text{mM}): 2\ \text{Ba(OH)}_2, 50\ \text{N-methyl d-glucamine}, 1\ \text{KOH} \) and 5 HEPES \((\text{pH}\ 7.5)\), titrated to pH 7.5. \((\text{CH}_3)_2\text{SO}_4\) and oocytes were used without BAPTA.

In these experiments the extent of DCm in different batches of oocytes varied, ranging from 1.0–2.0 nF, and probably was dependent on the quality of the oocytes in the various batches.

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Note
Supplementary materials can be found at: www.landesbioscience.com-supplement/CohenCHAN4-4-Sup. pdf
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