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Formation of the Full SNARE Complex Eliminates Interactions of Its Individual Protein Components with the Kv2.1 Channel[†]

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ABSTRACT: Previously, we have demonstrated physical and functional interactions of the voltage-gated potassium channel Kv2.1 with the plasma membrane protein components of the exocytotic SNARE complex, syntaxin 1A, and the t-SNARE, syntaxin 1A/SNAP-25, complex. Importantly, the physical interaction of Kv2.1 with syntaxin was shown to be involved in the facilitation of secretion from PC12 cells, which was independent of potassium currents. Recently, we showed that also VAMP2, the vesicular SNARE, interacts physically and functionally with Kv2.1. Here, we first set out to test the interaction of the full SNARE, syntaxin/SNAP-25/VAMP2, complex with the channel. Using the interaction of VAMP2 with Kv2.1 in *Xenopus* oocytes as a probe, we showed that coexpression of the t-SNARE complex with VAMP2 abolished the VAMP2 effect on channel inactivation and reduced the amount of VAMP2 that coprecipitated with Kv2.1. Further, *in vitro* pull down assays showed that the full SNARE complex failed to interact with Kv2.1 N- and C-termini in tandem, in contrast to the individual SNARE components. This suggests that the interactions of the SNARE components with Kv2.1 are abolished upon their recruitment into a full SNARE complex, which does not interact with the channel. Other important findings arising from the *in vitro* study are that the t-SNARE complex, in addition to syntaxin, interacts with a specific C-terminal channel domain, C1a, shown to mediate the facilitation of release by Kv2.1 and that the presence of Kv2.1 N-terminus has crucial contribution to these interactions. These findings provide important insights into the understanding of the complex molecular events involved in the novel phenomenon of secretion facilitation in neuroendocrine cells by Kv2.1.

Proteins of the SNARE[1] family, including the plasma membrane t-SNAREs, syntaxin 1A (Syx) and SNAP-25, and the vesicular partner, VAMP2, are crucial for transmitter and hormone release by exocytosis (*1*). In addition, voltage-gated Ca^{2+} channels (VGCC), which together with the putative Ca^{2+} sensor synaptotagmin mediate the Ca^{2+} influx, were also implicated in the regulated exocytosis (*2*). Importantly, t-SNARE proteins and synaptotagmin interact physically with VGCC, regulating their α_1 subunit availability and localization to nerve terminals (reviewed in ref *3*), and consequently affecting the efficacy of neurotransmitter release. Another class of ion channels that influence exocytosis is the voltage-gated K^+ (Kv) channels, whose function has classically been viewed to be inhibitory and exerted solely through their ion-conducting activities in presynaptic terminals (*4, 5*) and endocrine cells (*4*). Specifically, Kv2.1 channels constitute the majority of delayed-rectifier potassium currents in most mammalian central neurons (*5*), where they can influence the release of neuropeptides and neurotrophins, and in neuroendocrine cells, where they are well positioned to regulate hormone release. Recently, Kv2.1 has been identified as playing a novel role, facilitating transmitter release from PC12 cells. The facilitation was independent of ion-conducting function; instead it was mediated by a direct interaction with Syx (*6, 7*). These studies followed up studies conducted in *Xenopus* oocytes, in PC12 cells, in pancreatic β cells and in vitro (*8–12*, for review see *13*) that showed that the Kv2.1 channels interact with syntaxin, either alone or in a t-SNARE complex. However, the recent finding that VAMP2 can also interact with Kv2.1 in brain membranes and *Xenopus* oocytes (*14*) insinuated that the molecular mechanism underlying Kv2.1-induced facilitation of transmitter release is far from being resolved and immediately raised the question regarding the interaction of the channel with the full SNARE complex (Syx, VAMP2 and SNAP-25). To address the question, concomitant electrophysiological and biochemical assays of the interaction of the full complex with Kv2.1 in oocytes were conducted. The notion emerging from these studies was that the full SNARE complex does not interact with Kv2.1.

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[1] Abbreviations: BoNT/C, botulinum neurotoxin C; GST, glutathione *S*-transferase; Kv, voltage-gated potassium channel; Syx, syntaxin 1A; SNAP-25, synaptosomal-associated protein of 25 kDa; VAMP2, vesicle-associated membrane protein 2; SNARE, soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor, $Vi_{1/2}$, half-inactivation voltage.

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This notion was further corroborated in *in vitro* binding assays using constructs of Kv2.1 N- and C-termini in tandem, which provided a good model of the channel's integral cytosolic structure. Further, the *in vitro* study revealed structural data that provide important clues for the understanding of the molecular interactions underlying the Kv2.1-induced pore-independent regulation of secretion.

MATERIALS AND METHODS

Oocytes and Electrophysiological Recording. Xenopus laevis oocytes were prepared as described (*15*). Oocytes were injected with 0.05−0.25 ng/oocyte or 1.5 ng/oocyte of Kv2.1 mRNAs for electrophysiological and biochemical experiments, respectively. Syntaxin 1A mRNA was injected at 0.5−1.5 and at 5 ng/oocyte for electrophysiological and biochemical experiments, respectively. SNAP-25 mRNA was injected at 5−15 and at 15 ng/oocyte for electrophysiological and biochemical experiments, respectively. VAMP2 mRNA was injected at 1.5 ng/oocyte for both electrophysiological and biochemical experiments. BoNT/C mRNA was injected for both biochemical and electrophysiological experiments at 20−30 ng/oocyte. Two-electrode voltage clamp recordings were performed as described (*16*). To avoid possible errors introduced by series resistance, only current amplitudes up to 4 μA were recorded. Net current was obtained by subtracting the scaled leak current elicited by a voltage step from −80 to −90 mV. Oocytes with a leak of more than 3 nA/mV were discarded. Steady-state inactivation curves were obtained by maintaining the cells at various holding potentials, ranging from −60 mV to +15 mV (with an increment of 5 mV; totally 16 pulses), for 5 s before stepping to the 350 ms test depolarization of +50 mV. The raw data were analyzed with Clampfit software (MDS Inc., Toronto, Canada), and then steady-state inactivation curves were fit to the standard Boltzmann equation: $I/I_{max} = 1/(1 + (\exp(Vi_{1/2} - V)/a))$, using Sigma Plot software (Systat Software Inc., San Jose, CA), and mean values for half-inactivation voltage ($Vi_{1/2}$) were derived. The statistical significance of differences between two groups was calculated by the use of independent sample *t* test procedures assuming unequal variance (Mann−Whitney's rank-sum test). Statistical estimation of several groups comparison was performed using one-way analysis of variance (ANOVA) followed by Holm−Sidak or Tukey's tests. The data are presented as mean ± SEM (standard error of mean).

Immunoprecipitation in Oocytes. Oocytes were subjected to immunoprecipitation as described (*16*). Briefly, immunoprecipitates from 1% CHAPS oocytes homogenates were analyzed by SDS−PAGE (12% polyacrylamide). Digitized scans were derived by PhosphorImager (Molecular Dynamics), and relative intensities were quantified by ImageQuant.

cDNA Constructs. N^C and N^CdelC1a were created by amplification of the wild type rat Kv2.1 cDNA (which was in pcDNA3 vector) with two primers: sense primer corresponding to 5′- CAGCTAGCCAGTCTACGAACTTCTC-CGAGTTCTACAAG-3′ or 5′- CAGCTAGCCAGTCTACG-GAAGACATGTACAGTAAGATG -3′ (for N^CdelC1a) and antisense primer corresponding to 5′- GTGCTAGCCGTA-GACTGCAGATCCCAGAGTTTCTTCCT -3′. The sense primer encodes for the beginning of the C terminus (a. a. *N-411*) or for the beginning of the C1b domain (a. a. *E-526*), a three amino acids linker (a. a. *QST*) and a hanging domain complementary to the one in the antisense primer. The antisense primer encodes for the end of the N terminus (a. a. *L-174*), a three amino acid linker domain (a. a. *QST*) and a hanging domain complementary to the one in the sense primer.

In Vitro Binding of GST Fusion Proteins with ^{35}S-Labeled Proteins. The fusion proteins were synthesized and reacted as described (*17, 18*). Purified GST fusion proteins (∼150 pmol) immobilized on glutathione-Sepharose beads were incubated with 5 μL of the lysate containing ^{35}S-labeled N^C, N^CdelC1a or C (translated on the template of *in vitro* synthesized mRNA using a translation rabbit reticulocyte lysate kit (Promega) in 1 mL of phosphate buffered saline with 0.1% Triton X-100 or 0.5% CHAPS. The GST fusion proteins were eluted with 20 mM reduced gluthatione and then subjected to SDS−PAGE (12% polyacrylamide).

RESULTS

The Effect of VAMP2 on Kv2.1 Inactivation Is Abolished in the Presence of Coexpressed t-SNAREs and Is Rescued upon Knock down of Syx with Botulinum C BoNT/C in Oocytes. Functional effects of Syx, the t-SNARE complex (*11*) and VAMP2 (*14*) on the voltage-dependence of Kv2.1 inactivation were described previously in oocytes. Whereas coexpression of Syx or VAMP2 with Kv2.1 shifted the half-inactivation voltage, $Vi_{1/2}$, to hyperpolarized potentials, the coexpression of both Syx and SNAP25 together, forming the t-SNARE complex, shifted $Vi_{1/2}$ toward depolarized potentials. Here, we set out to assess the effect of the full SNARE complex on $Vi_{1/2}$, using the effect of VAMP2 as a probe. Whereas the effect of VAMP2 persisted in the presence of either Syx or SNAP-25, coexpressed at concentrations that do not affect $Vi_{1/2}$ (*11*) (Figure 1A, B), it was completely eliminated upon coexpression of both t-SNAREs (Figure 1C). Importantly, this elimination was reversed upon additional coexpression of BoNT/C (Figure 1D).

One possible scenario that could account for this result was that, upon coexpression of all SNAREs together, VAMP2 was recruited into the formation of the full SNARE complex that, in turn, did not interact with Kv2.1. The coexpression of BoNT/C that knocked down Syx (*11*) (see also Figure 2) prevented full SNARE complex formation and allowed VAMP2 to interact with the channel. However, one could not rule out the possibility that both VAMP2 and the t-SNARE complex each associated with the channel, but since their effects on $Vi_{1/2}$ were opposite, the net effect was precisely nil. The knock down of Syx by BoNT/C abolished the t-SNARE interaction, allowing the interaction of VAMP2 to dominate. To further elucidate the underlying scenario, the ability of VAMP2 to associate with the channel in the presence of t-SNAREs was evaluated using the coimmunoprecipitation in oocytes by an antibody against Kv2.1 C-terminus.

The Association of VAMP2 with Kv2.1 Is Reduced in the Presence of Coexpressed t-SNAREs and Is Rescued upon Knock down of Syx with BoNT/C. Complementary coimmunoprecipitations (Co-IP) in oocytes, using antibody against Kv2.1 C-terminus, demonstrated that the amount of VAMP2 that coprecipitated with the channel was reduced by about 55% upon coexpression of the t-SNAREs (see a representa-

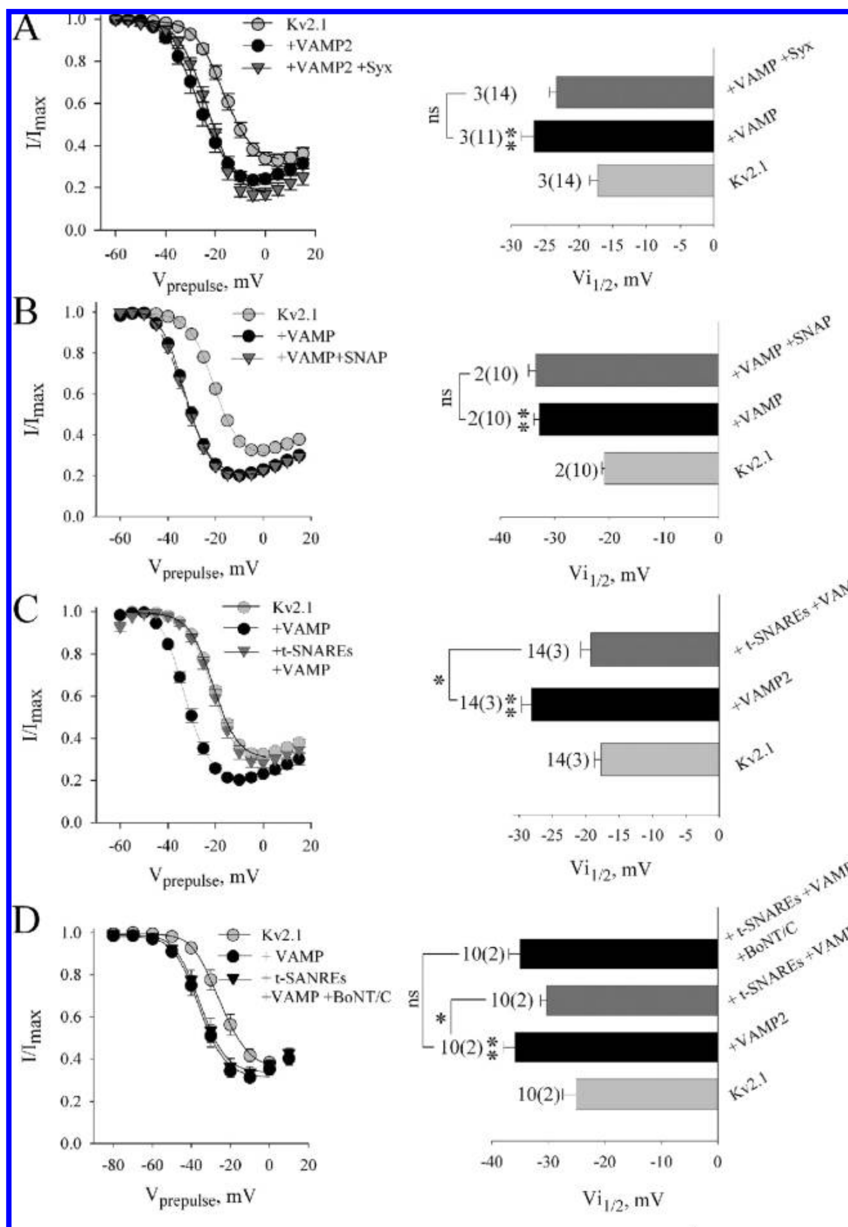

FIGURE 1: Effects of t-SNAREs on the interaction of VAMP2 with Kv2.1 in oocytes. (A−C) Representative experiments (5 oocytes per group) demonstrating the effect of Syx, SNAP-25 and their combination on VAMP2 induced leftward shift of Kv2.1 half-inactivation voltage ($Vi_{1/2}$). Oocytes were injected with Kv2.1 (Kv2.1) alone, with VAMP2 (+VAMP), or with combinations of VAMP2 with Syx (+VAMP+Syx; A), with SNAP-25 (+VAMP+SNAP; B) or with both Syx and SNAP-25 (+t-SNAREs+VAMP; C) mRNAs. (D) Knock down of Syx with BoNT/C restores the effect of VAMP2 on Kv2.1 inactivation. In all diagrams, numbers without and with parentheses denote total number of oocytes per group and number of batches. *, $p < 0.01$; **, $p < 0.001$.

tive experiment and a summary from several similar experiments in Figure 2A), although comparable amounts of VAMP2 were expressed in all reactions (verified using the antibody against the N-terminus of VAMP2; Figure 2B). The knock down of Syx with BoNT/C restored the association of VAMP2 with the channel (Figure 2A). When these results are taken together with the electrophysiological results presented above, a clear correlation between the effect of VAMP2 on Kv2.1 inactivation and its association with the channel emerges. Namely, the elimination of the VAMP2 effect upon coexpression with the t-SNAREs is correlated with reduced association of VAMP2, and the rescue of VAMP2 effect by BoNT/C coexpression is correlated with a parallel rescue of VAMP2 association.

One possible scenario that could account for the reduced VAMP2 binding in the presence of the t-SNAREs was a Kv2.1 binding competition between the t-SNAREs and VAMP2 (all coprecipitated with the channel; Figure 2A, lane 2) that reduced the availability of the channel for VAMP2 association. However, more plausible was a scenario in which VAMP2 was recruited into the formation of the full SNARE complex, which, in turn, cannot bind the channel, resulting in reduced availability of free VAMP2 for association with Kv2.1. This scenario was supported by the apparent massive recruitment of VAMP2 into ternary complexes. The amount of which was similar in the presence and absence of coexpressed Kv2.1 (compare lane 2 with lane 4 in Figure 2B), suggesting that the binding affinity of VAMP2 to the t-SNAREs overrides its affinity to Kv2.1. That BoNT/C, which impaired the formation of the ternary complex (see lane 3 in Figure 2B), completely rescued the association of VAMP2 with Kv2.1 (lane 3 in Figure 2A) supported the

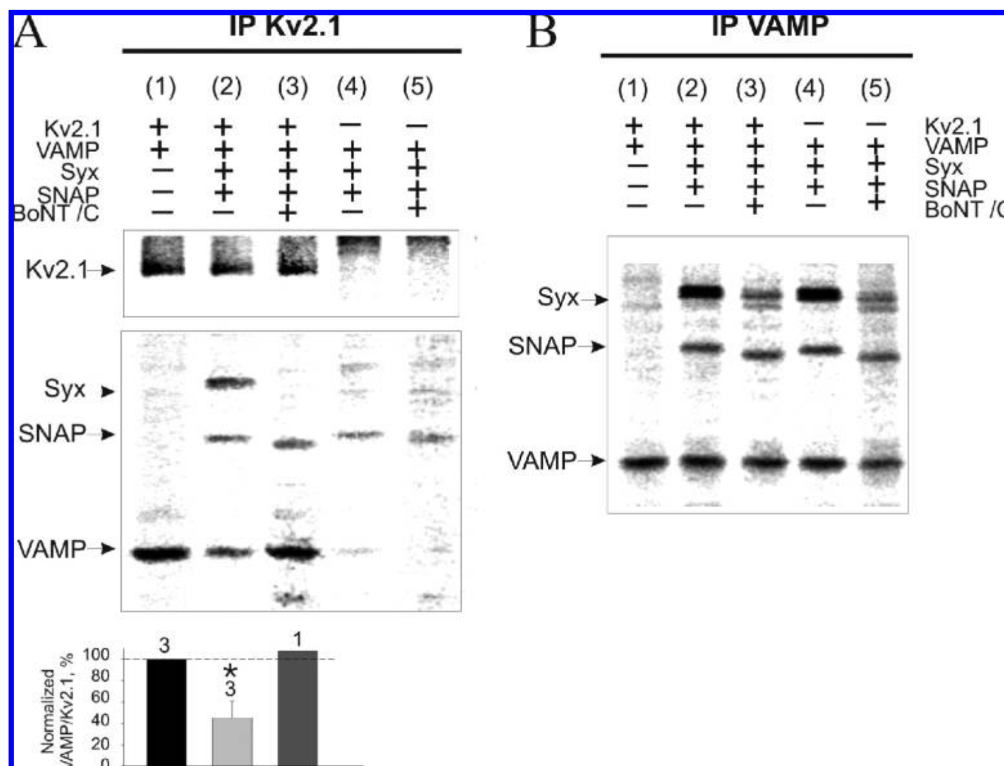

FIGURE 2: The amount of VAMP2 that coprecipitates with Kv2.1 is reduced in the presence of coexpressed t-SNAREs in oocytes. (A, B) Digitized PhosphorImager scans of SDS−PAGE analysis of the [^{35}S]Met/Cys-labeled SNARE proteins, immunoprecipitated with Kv2.1 by an antibody raised against the channel (IP by Kv: A), or with VAMP2 by an antibody raised against the VAMP2 (IP by VAMP: B). Bar diagram in A depicts the normalized VAMP2 to channel binding (averaged from 3 experiments as in the upper panel) in the presence of t-SNAREs. The amount of VAMP2 is reduced in the presence of coexpressed t-SNAREs (lane 2 in A); the knock down of Syx with BoNT/C restores the VAMP2 binding to the channel (lane 3 in A). The amount of VAMP2 in each of the reactions was similar (B). The presence of the channel does not affect the formation of full SNARE complexes (compare lane 2 with lane 4 in B). Note that BoNT/C is also able to proteolyze SNAP-25 (lanes 2 and 4 in A and B); however, as it occurs at the very distal part of SNAP-25 C-terminus (*36*), it does not affect the ability of SNAP-25 to bind its SNARE partners (lanes 2 and 4 in B).

notion that the full complex does not associate with Kv2.1. In an attempt to confirm rigorously this notion, we set out to evaluate the physical interactions of Kv2.1 with the SNAREs in an *in vitro*experimental system that could mimic the *in situ* setting satisfactorily. In contrast to oocytes where the levels of expression of a given protein vary among the different experimental settings (which affect the relative stability of a given protein, or the availability of the ribosomes for protein synthesis; e.g., see the uneven levels of Kv2.1 and VAMP2 among the different reactions in Figures 2A and 2B, respectively), the advantage of an *in vitro* system is that it allows the full control for equal amounts of participating proteins in each experimental setting (see below).

Kv2.1 Does Not Interact Physically with the Full SNARE Complex in Vitro. Previously, we evaluated the *in vitro* binding of Kv2.1 to the individual SNARE proteins using its isolated N- and C-termini. Yet, a tandem of both termini, which constitute the major cytosolic parts of Kv2.1, may provide a better structural integrity of the cytosolic portion of the channel, in view of the proposed role of physical interaction between N- and C-termini in the Kv2.1 gating (*19*), and may offer an *in vitro* binding analysis platform that represents more adequately the *in situ* setting. To this end, full Kv2.1 N- and C-termini were connected in tandem by a small flexible linker. The corresponding ^{35}[S]-labeled protein (N^C) was synthesized in reticulocyte lysate and used in pull down assays with GST-fused proteins corresponding to the various SNAREs. Figure 3 shows that the N^C protein interacted strongly with Syx alone and more weakly with the t-SNARE complex (t-com) and did not interact either with SNAP-25 alone or with the full SNARE complex (f-com). Importantly, the results concerning the t-SNAREs are in absolute agreement with previously documented interactions in *oocytes* (*11*) and thus vouch for the *in situ* relevance of the results concerning the full SNARE complex. Note that the residual apparent N^C which was pulled down by the full complex can be fully ascribed to pull down by the monomeric SNAREs that did not assemble into the full complex (see bands of the individual SNAREs in the reaction of f-com in Figure 3A and legend to Figure 3B). Taken together, the results of the functional (Figure 1) and the biochemical assays (Figure 2) in oocytes with the *in vitro* experiments (Figure 3) indicate that the full SNARE complex does not interact, either functionally or physically, with Kv2.1. These data further provide the order of the relative binding intensities of the various SNARE components: Syx > t-SNARE complex > VAMP2.

Kv2.1 N-Terminus Is a Crucial Factor in the Binding of Syx to Kv2.1 C1a Domain. Previously, we showed that Syx and the t-SNARE complex bind the isolated Kv2.1 C-terminus (*11, 12*). VAMP2 was shown to bind mainly the isolated N-terminus but also the isolated C-terminus, albeit to a smaller extent (Lvov et al., in preparation). The binding of SNAP-25 to either one of the isolated N- or C-termini was shown to be very weak (*11*). Here, on the basis of the

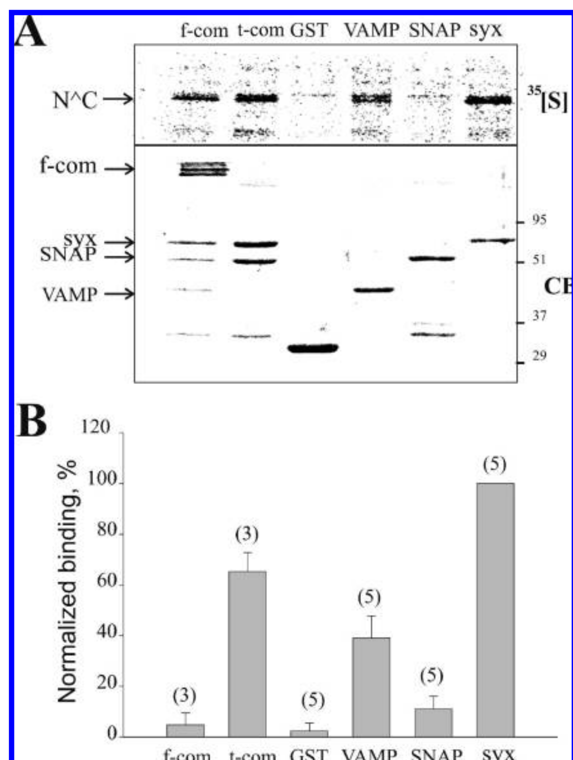

FIGURE 3: Tandem of Kv2.1 N- and C- termini does not interact physically with the full SNARE complex. (A) ^{35}S-labeled Kv2.1 N and C termini linked in a tandem (N^C) were incubated with ∼200 pmol of the indicated GST fusion proteins; Syx, SNAP-25, VAMP2, t-SNARE complex (t-com), full SNARE complex (f-com) and GST itself, immobilized on GSH-agarose beads in a 1 mL 0.1% Triton X-100 reaction volume for 1 h. The gluthatione-eluted proteins were analyzed by SDS−PAGE. Upper panel: digitized PhosphorImager scans. Lower panel: scans of Coomassie Blue (CB) staining. (B) Relative PhosphorImager signal intensity of pulled down N^C in each reaction was normalized to the amount(s) of the GST fusion proteins participating, derived from CB staining, and averaged over at least three experiments. Note that from the signal intensity of the full complex (f-com) reaction we subtracted calculated relative intensities, derived from the reactions of the t-complex (t-com) and VAMP2 (VAMP), which account for the pull down by the SNARE components that did not assemble into the full complex in this (f-com) reaction.

experimental data presented above, which suggests that the setting of Kv2.1 N- and C-termini together offers an *in vitro* binding analysis platform that represents adequately the *in situ* setting, we reassessed our previous *in vitro* binding results in an attempt to find out what is the contribution of the interaction between the intracellular termini to the bindings of the SNAREs. Comparison of pulled down N−C tandems (N^C) and isolated C-termini (C) by the GST-fused SNAREs allowed the assessment of the contribution of the N−C interaction to the binding of the SNAREs to the C-terminus. Since the C terminal binding domain for Syx (*11*) and VAMP2 (Lvov et al., in preparation) was mapped to the proximal quarter of the C-terminus, C1a, we used another N−C tandem in which the C-terminus was lacking the C1a domain (N^CdelC1a). This tandem was used as a control that monitors the binding to the N terminus itself.

Figure 4 shows results from representative experiments (Figure 4A) and summary of several experiments (Figure 4B) conducted with Syx, the t-SNARE complex, VAMP2 and SNAP-25. Syx interacted very strongly with N^C, as compared to N^CdelC1a, confirming our previous findings

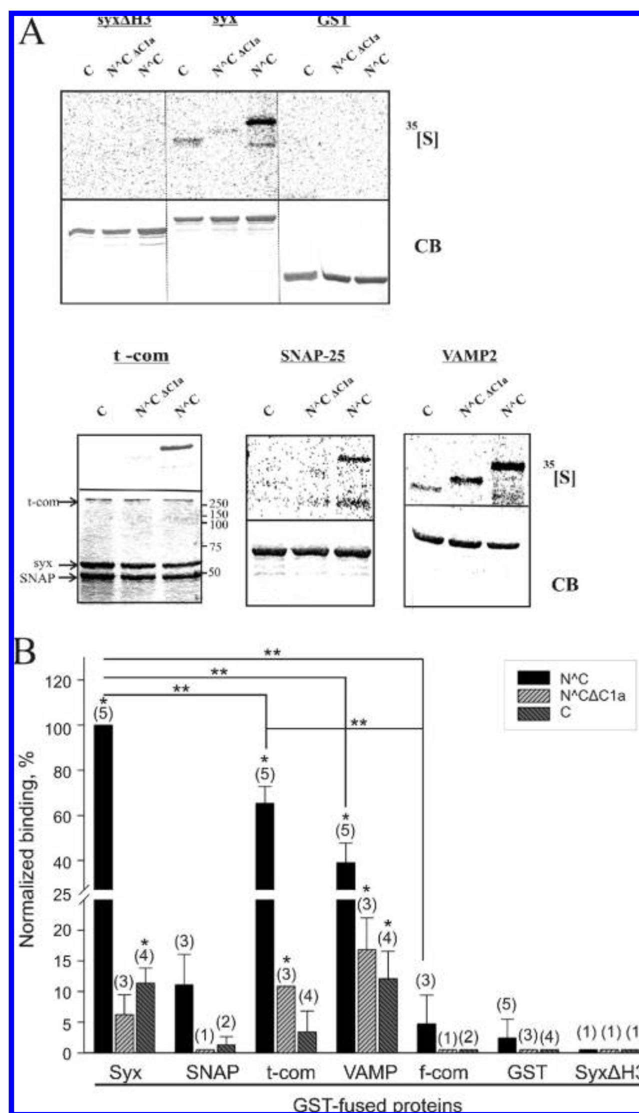

FIGURE 4: In the presence of Kv2.1 N-terminus the binding of syntaxin, either monomeric or in a t-SNARE complex, to the C-terminus is significantly enhanced; the C1a domain is crucial for the interactions. (A) *In vitro* synthesized ^{35}S-labeled Kv2.1 tandems of full length N with C termini (N^C), of full length N with C lacking C1a (N^CΔC1a), or the full length Kv2.1 C-terminus (C) were incubated with ∼200 pmol of the indicated GST fusion proteins: (syntaxin (Syx), SNAP-25, VAMP2, t-SNARE complex (t-com), syntaxin lacking its SNARE, H3, domain (SyxΔH3) and GST alone) and immobilized on GSH agarose beads in a 1 mL 0.1% Triton X-100 reaction volume for 1 h. The gluthatione-eluted proteins were analyzed by SDS−PAGE. Top panels show digitized PhosphorImager scans (^{35}S) from representative experiments. Bottom panels show the corresponding scans of Coomassie Blue staining (CB). (B) Relative intensities of bound N^C for each of the GST fusion proteins normalized to their amounts (derived from CB staining), averaged from several experiments (number of experiments are denoted above bars). Experiments are as in A.

that C1a is the target site for Syx binding. However, and most importantly, the interaction with the C terminus was relatively negligible, pointing out that the presence of the N-terminus potentiated dramatically the binding of Syx to C1a. Interestingly, the interaction of the t-SNARE complex with N^C was very strong, as compared to N^CdelC1a, pointing at the C1a domain as the target site for the t-SNARE complex also. Again, and most importantly, the binding of the t-SNAREs to the C-terminus was negligible, pointing out that the presence of the N-terminus potentiated dramati-

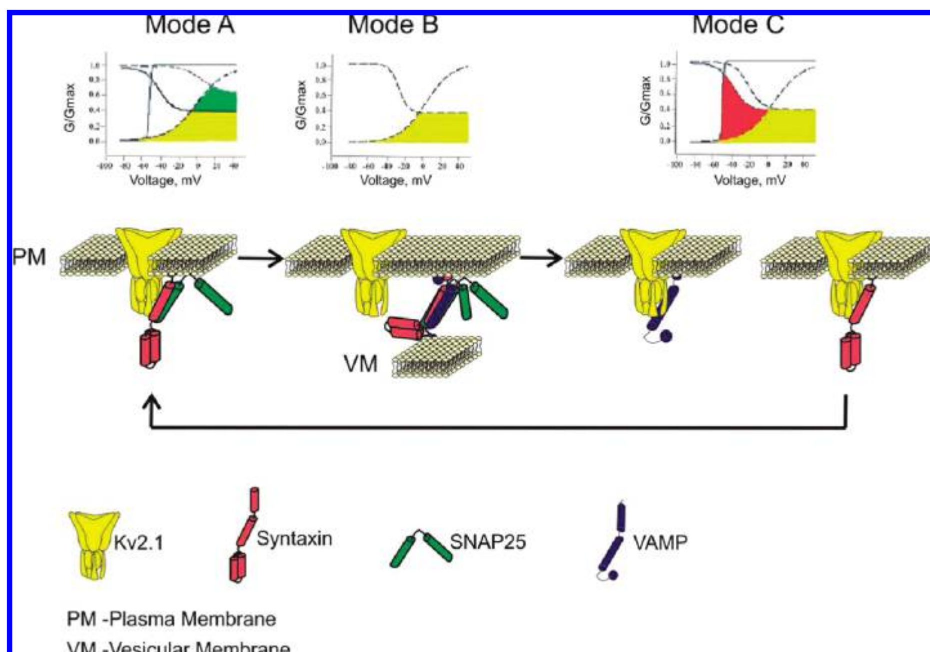

FIGURE 5: Schematic diagram illustrating postulated modes of the Kv2.1 channel, formed by interaction of the channel with the SNARE intermediates. Mode A, the channel is associated with the t-SNARE complex. Mode B, unoccupied channel, upon formation of the full SNARE complex. Mode C, the channel is associated with VAMP2 (a transient state until VAMP2 is endocytosed) or Syx. Upper panels, biophysical characteristics in each of the modes are represented by the "window" of voltages at which the channel conducts at the specific mode (colored areas), derived from superposition of the corresponding mean activation and inactivation curves (see text). For comparison, in mode A also shown are curves corresponding to mode C (solid lines), and in mode C also shown are curves corresponding to mode B (dashed lines). The yellow area in each mode is the window of voltages at which the unoccupied channel is open (mode B).

cally the binding of the t-SNAREs to C1a, albeit to a lesser extent than that of Syx. In contrast to Syx and the t-SNARE complex, the interaction of VAMP2 with the C-terminus was not potentiated by the N-terminus, it was rather additive to the interaction with the N-terminal site (as verified in N^CdelC1a). The interaction of SNAP-25 with the C-terminus, although being somewhat potentiated in the presence of the N-terminus, remained very weak in comparison to Syx and the t-SNARE complex, as previously suggested by us.

DISCUSSION

Kv2.1 is commonly expressed in the soma and dendrites of neurons (*20–22*) and in neuroendocrine cells (*9, 23*), where it could regulate the dense-core vesicle (DCV)-mediated release of neuropeptides, neurotrophins and hormones by affecting membrane potential.

Our previous studies concerning the characterizations of the t- SNAREs (*11*) and VAMP2 (*14*) interactions with Kv2.1 suggested that the channel function is being modulated by these interactions. While t- and v-SNAREs independently were shown to bind different domains within Kv2.1 N- and C-termini to regulate channel function, this study shows that the coming together of the three SNAREs to form the full SNARE complex kicks the complex off the channel altogether and the SNAREs cease to regulate Kv2.1. These findings, which emerged from studies integrating experiments performed in oocytes and *in vitro*, may have implications for the role of Kv2.1 along the process of DCV's release if one is aware of the fact that the oocyte can function as a common secretory cell and may provide a complete system for the analysis of the secretory process (*24*). Indeed, not only can the oocyte secrete selectively secretory proteins versus nonsecretory proteins (*24*), but the oocyte can support release induced by KCl depolarization and treatment with Ca^{2+} ionophore of both endogenous and heterologously expressed acetylcholine in a Ca^{2+}-dependent manner (*25*), and release of endogenous ATP induced by hypertonic solution (*26*). Importantly, the release process in oocytes was shown to involve endogenous v- and t-SNARE proteins (e.g., refs *26, 27,). Finally, depolarization and Ca^{2+}-evoked secretion could be reconstituted in oocytes upon overexpression of protein components of the exocytotic machinery including the SNARE proteins (28).*

Taken together, our findings raise an interesting sequential function of the SNAREs on Kv2.1 channel regulation along the process of DCV-mediated release. Subject to its interaction with the different molecular SNARE intermediates, the Kv2.1 is proposed to exist in three conducting modes, sketched in Figure 5. The biophysical characteristics of the channel in each of the modes are presented by the window of voltages at which the channel conducts (conductivity window), derived from superposition of the determined mean steady-state activation and inactivation curves at the specific mode (*11, 14*; Figure 5, upper panels). Prior to vesicle fusion, Kv2.1 associates with the t-SNARE complex and resides in mode A in which the conductivity window is set at depolarized potentials and channel conductance is high. Such behavior of the channel, while not interfering with the generation of small membrane depolarizations, is expected to impair the summation of graded depolarizing potentials in dendrites and neuroendocrine cells by shortening of the depolarizations (due to shortening of their decay phase) and by decreasing the membrane input resistance (shunting; known to reduce summation due to reduction in membrane time constant (*29*)). Consequently, summation of only high

frequency depolarizations will occur, enhancing inward Ca^{2+} ($[Ca^{2+}]_i$) transients to trigger SNARE complex-mediated vesicle fusion. Once fusion per se is taking place, upon the formation of the full SNARE complex the channel becomes empty and shifts to mode B in which its conductance is decreased, prolonging the decay phase of the graded potentials and alleviating their summation to produce stronger depolarizations and larger $[Ca^{2+}]_i$ transients. Such a positive feedback mechanism on $[Ca^{2+}]_i$ that coincides with fusion complex formation contributes to efficient release. SNARE complex dissociation, upon termination of fusion, increases the local plasma membrane level of dissociated VAMP2 and syntaxin, and both interact with Kv2.1 (SNAP-25 does not interact with the channel). These interactions shift the channel to mode C in which the conductivity window is set to hyperpolarized voltages and conductance is increased. Consequently, small membrane depolarizations are suppressed, contributing to membrane potential stabilization and preventing large $[Ca^{2+}]_i$ transients which are futile prior to initiation of subsequent vesicle fusion cycle. In all, we suggest a modal behavior of a Kv channel that is sensitive to the assembly status of the t-SNARE complex and contributes to metabolically efficient secretion reinforcing the requirements for high frequency firing for exocytosis of DCVs in neuroendocrine cells and in dendrites.

We have recently put forward the notion that Kv2.1 plays a novel role of facilitating DCV-mediated release, which is independent of its classical role that involves its K^+-conducting pore (as describe above), and is mediated through a specific protein domain, C1a (*6, 7*). The present study provides additional important insights into the molecular mechanism underlying this novel role. That C1a was previously shown to strongly interact with Syx (*12, 30*) it was suggested that direct physical interaction of the channel with Syx is involved in this novel role of Kv2.1. However, it becomes now evident that the C1a domain mediates interaction not only with monomeric Syx but also with Syx in the context of a t-SNARE complex. This suggests that Kv2.1 stabilizes complexes of plasma membrane t-SNAREs, the concentration of which determines the rate of association with the vesicular SNARE, VAMP2, to form full SNARE complexes that drive fusion (*31*). Notably, the finding that the full SNARE complex does not interact with the channel suggests that Kv2.1 releases the bound t-SNARE complexes and does not interfere with their association with VAMP2. This suggests that Kv2.1, via its pore-independent function, catalyzes DCV-mediated release by stabilizing the transition state of the t-SNARE complex formation.

An additional finding of this study is that the interaction between N- and C-termini is crucial for both Syx and the t-SNARE interactions with the C1a domain of Kv2.1. That Syx and the t-SNARE complex affect Kv2.1 gating (*11*) and that recent studies (*19, 32*), using fluorescence resonance transfer (FRET) microscopy (*19*), suggested voltage-gated relative rearrangement between the Kv2.1 N and C termini, which seem to affect both activation and inactivation gating of Kv2.1 (*33–35*), may suggest that the physical interactions of the t-SNAREs with Kv2.1 affect channel gating by changing the relative orientation of the N- and C-termini. Scrutinizing this hypothesis will provide insights into the mechanism underlying the effect of the SNAREs on channel gating and will enhance our understanding of the role of N−C interaction in channel gating.

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