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Kv2.1 Channel Activation and Inactivation Is Influenced by Physical Interactions of Both Syntaxin 1A and the Syntaxin 1A/Soluble *N*-Ethylmaleimide-Sensitive Factor-25 (t-SNARE) Complex with the C Terminus of the Channel

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ABSTRACT

Kv2.1, the prevalent delayed-rectifier K^+ channel in neuroendocrine and endocrine cells, was suggested previously by our group to be modulated in islet β-cells by syntaxin 1A (Syx) and soluble *N*-ethylmaleimide-sensitive factor attachment protein-25 (SNAP-25). We also demonstrated physical interactions in neuroendocrine cells between Kv2.1, Syx, and SNAP-25, characterized their effects on Kv2.1 activation and inactivation in *Xenopus laevis* oocytes, and suggested that they pertain to the assembly/disassembly of the Syx/SNAP-25 (t-SNARE) complex. In the present work, we established the existence of a causal relationship between the physical and the functional interactions of Syx with the Kv2.1 channel using three different peptides that compete with the channel for binding of Syx when injected into oocytes already coexpressing Syx with Kv2.1 in the plasma membrane: one peptide corresponding to the Syx-binding region on the N-type Ca^{2+} channel, and two peptides corresponding to Syx-binding regions on the Kv2.1 C terminus. All peptides reversed the effects of Syx on Kv2.1, suggesting that the hyperpolarizing shifts of the steady-state inactivation and activation of Kv2.1 caused by Syx result from cell-surface protein-protein interactions and point to participation of the C terminus in such an interaction. In line with these findings, the effects of Syx were dissipated by partial deletions of the C terminus. Furthermore, the t-SNARE complex was shown to bind to the Kv2.1 C terminus, and its effects on the inactivation of Kv2.1 were dissipated by partial deletions of the C terminus. Taken together, these findings suggest that physical interactions of both Syx and the t-SNARE complex with the C terminus of Kv2.1 are involved in channel regulation.

The soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor proteins (SNARE proteins) comprise three conserved families of membrane-associated proteins, syntaxin, synaptobrevin/vesicle-associated membrane protein, and SNAP-25, that participate in the fusion of internal membranes in eukaryotic cells (Bennett and Scheller, 1993; Rothman and Warren, 1994; Jahn and Sudhof, 1999; Gerst, 2003) and thus play a crucial role in transmitter and hormone release (Sudhof, 1995). They interact with a wide range of proteins, some of which (such as synaptotagmin) are associated with vesicular membranes or with plasma membranes (e.g., voltage-gated Ca^{2+} channels) (Bajjalieh and Scheller, 1995; Bennett, 1995; Sudhof, 1995; Sheng et al., 1996; Linial, 1997). Recent studies by our group have suggested a physical and functional coupling of the SNARE proteins with the voltage-gated K^+ (Kv) channel Kv1.1 (Fili et al., 2001; Michaelevski et al., 2002); for example, it is suggested that Kv1.1 in a complex with the auxiliary Kvβ1.1 subunit coprecipitates with syntaxin 1A (Syx), SNAP-25 (SNAP), and synaptotagmin from brain synaptosomes in a manner that de-

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ABBREVIATIONS: SNARE, soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor; Syx, syntaxin 1A; RCF, residual current fraction; $Vi_{1/2}$, half-inactivation voltage; $Va_{1/2}$, half activation voltage; Kv, voltage-gated potassium channel; aa, amino acid; PAGE, polyacrylamide gel electrophoresis; SNAP, soluble *N*-ethylmaleimide-sensitive factor attachment protein; t-SNARE, syntaxin 1A/soluble *N*-ethylmaleimide-sensitive factor attachment protein-25; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; His_6-$N_{718-963}$, the synprint peptide; C1, the proximal half of the Kv2.1 C terminus; C2, the distal half of the Kv2.1 C terminus; N, N terminus.

pends on the physiological state of the synaptosomes. As a result of the physical interaction with Syx, the rapid inactivation of Kv1.1 in complex with Kvβ1.1 in *Xenopus laevis* oocytes is enhanced. G-protein $\beta\gamma$ subunits also participate in this interaction. It is interesting that similar characteristics were also exhibited by the interaction of N-type Ca^{2+} channels with Syx (Sheng et al., 1996; Tobi et al., 1998; Bezprozvanny et al., 2000; Catterall, 2000; Jarvis et al., 2000; Jarvis and Zamponi, 2001). Taken together, the above findings suggested that the Kv1.1 channel participates in the fine-tuning of presynaptic release processes. It is worth noting that interaction of Kv1.1 with SNAP was also observed in pancreatic β cells (Ji et al., 2002).

The voltage-gated K^+ channels Kv2.1, which are mainly distributed postsynaptically in neurons (Trimmer, 1993; Lim et al., 2000) but are also abundant in neuroendocrine and endocrine tissues (Sharma et al., 1993; Barry et al., 1995), were also shown by our group to interact with Syx and SNAP in PC12 cells (Michaelevski et al., 2003) and in pancreatic β cells (MacDonald et al., 2002; Leung et al., 2003), in which they participate in repolarization of action potentials and hence in insulin secretion (MacDonald et al., 2001). Detailed biochemical and electrophysiological analyses in *X. laevis* oocytes revealed that Syx and the target membrane SNARE (t-SNARE) complex, Syx/SNAP, had different effects on both the activation and the slow inactivation of Kv2.1 (Michaelevski et al., 2003), suggesting that the Kv2.1 function might be fine-tuned by the assembly/disassembly status of the complex. In this work, we establish a causal relationship between the physical and the functional interactions of both Syx and the t-SNARE complex with the Kv2.1 channel and suggest that the C terminus of the channel participates in both interactions.

Materials and Methods

Constructs and Antibodies. The primary antibodies used were Kv2.1-C terminus (Alomone Labs, Jerusalem, Israel), polyclonal syntaxin 1A (Alomone), and monoclonal anti-HPC-1 (Sigma Israel, Rehovot, Israel). Syx and SNAP (kindly donated by E. Isacoff, Berkeley, CA) cDNAs were cloned in pGEMHE. Kv2.1, its C-terminal truncation mutants ΔC318 and ΔC416, missing the last 318 and 416 amino acids, respectively (VanDongen et al., 1990), its C-terminal construct [amino acids (aa) 414–853], and N-terminal construct (aa 1–184) (Bentley et al., 1999) cDNAs were cloned in pBluescript. mRNAs were prepared as described previously (Jing et al., 1999). DNAs of Kv2.1 fragments for production of GST fusion proteins were constructed as described previously (Michaelevski et al., 2003). Materials and enzymes for molecular biology and biochemistry were purchased from Roche Diagnostics (Mannheim, Germany), Promega (Madison, WI), and MBI Fermentas (Vilnius, Lithuania).

Oocytes and Electrophysiological Recording. *X. laevis* oocytes were prepared as described previously (Levin et al., 1996). Oocytes were injected with Kv2.1 mRNA (0.05- 0.25 ng/oocyte), Syx mRNA (0.5 ng/oocyte), or SNAP mRNA (5 ng/oocyte). Two-electrode voltage-clamp recordings were performed as described previously (Levin et al., 1996). To avoid possible errors introduced by series resistance, we recorded current amplitudes of up to 4 μA only. Net current was obtained by subtracting the scaled leak current elicited by a voltage step from -80 to -90 mV. Oocytes with a leak current of more than 3 nA/mV were discarded. Experimental protocols and data analyses are described in the figure legends.

Immunoprecipitation. ^{35}S-labeled proteins translated on the template of in vitro-synthesized mRNAs using a translation rabbit reticulocyte lysate kit (Promega, according to the manufacturer's instructions) were solubilized in 1 ml of 0.1% Triton X-100 (or 1% CHAPS) solution containing 50 mM Tris-HCl, pH 7.4, 150 mM KCl, 1 mM EDTA, 1 mM EGTA, and supplemented with protease inhibitor cocktail (Roche Diagnostics). Proteins were immunoprecipitated as described previously for oocytes (Levin et al., 1996). Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (12% polyacrylamide). Digitized scans were derived by PhosphorImager, and relative intensities were quantified by ImageQuant (both from Amersham Biosciences, Piscataway, NJ).

In Vitro Binding of GST Fusion Proteins with Syx and SNAP. The fusion proteins were synthesized and reacted with Syx as described previously (Fili et al., 2001). In brief, purified GST fusion proteins (150 pmol) immobilized on glutathione Sepharose beads were incubated either with 8.5 μl of lysate containing ^{35}S-labeled Syx or with 180 pmol recombinant Syx peptide prepared from a GST fusion construct (aa 1–264) cleaved by thrombin (molar ratio, 1:500) in 1 ml of phosphate-buffered saline with 0.1% Triton X-100 for 1 h at room temperature with gentle rocking. After washing, the GST fusion proteins were eluted with 20 mM reduced glutathione in 40 μl of elution buffer (120 mM NaCl and 100 mM Tris-HCl, pH 8), separated by SDS-PAGE (12% polyacrylamide), and subjected to Western blot analysis using the ECL Detection System (Pierce, Rockford, IL).

Statistical Analysis. Data are presented as means $\pm$ S.E.M. The statistical significance of differences between two groups was calculated by the use of independent sample *t* test procedures assuming unequal variance (Mann-Whitney rank sum test). One-way analysis of variance was used to calculate the statistical differences when several groups were compared.

Results

The C Terminus of Kv2.1 Mediates Binding of Syx. A previous study by our group, using reciprocal coimmunoprecipitation analysis in plasma membranes of *X. laevis* oocytes coexpressing Syx and Kv2.1, showed that Syx binds strongly to Kv2.1 (Michaelevski et al., 2003). In addition, we quantitatively determined in vitro a high-affinity, saturable, physical interaction of Syx with the GST fusion peptide, corresponding to the proximal half of the Kv2.1 C terminus (C1) and not to the distal half (C2) or the N terminus (N) (Fig. 1B, top shows the peptides) (Leung et al., 2003; Michaelevski et al., 2003). The aim of the present study was to further substantiate the physical interaction of the channel with Syx. First, we used an in vitro assay of competitive binding between immobilized C1 and hexahistidine-tagged (His_6) protein-expressing segment II- III (aa 718–963) of the N-type Ca^{2+} channel (His_6-$N_{718-963}$, the "synprint" peptide). This domain of the Ca^{2+} channel interacts strongly with Syx and was reported to be physiologically relevant (Sheng et al., 1996; Rettig et al., 1997). As a control, we used His_6-$N_{718-859}$, corresponding to a shorter II–III segment that is unable to interact with syntaxin (Sheng et al., 1996). In this experiment, binding of the recombinant cytoplasmic part of Syx (corresponding to aa 4–264) to C1 was allowed to proceed in the presence of two concentrations of His_6-$N_{718-963}$. With increasing molar concentration of this peptide, a significant decrease was observed in the amount of bound Syx, whereas no such decrease was seen with His_6-$N_{718-859}$ even when its molar concentration was doubled. When the molar ratio of $N_{718-963}$ to C1 was equal to 1, the bound Syx was reduced to ~25% of its amount in the absence of the competitor. Thus, the interaction of C1 with Syx was inhibited by $N_{718-963}$ (Fig. 1A).

We then further narrowed down the region of the C terminus that interacts with Syx. Using immobilized GST fusion proteins corresponding to two halves of C1 (aa 411–522 and aa 523–632; C1a and C1b, respectively), we showed that Syx bound strongly to C1a and only very weakly, if at all, to C1b (Fig. 1B).

The C Terminus of Kv2.1 Mediates Binding of the t-SNARE Complex. In a previous study, using a reciprocal coimmunoprecipitation analysis, our group demonstrated weak binding of SNAP to Kv2.1 channels in the plasma membranes of oocytes coexpressing SNAP and Kv2.1. Likewise, the in vitro binding of SNAP alone to the cytosolic domains of the channel was rather weak and was not clearly confined to a specific domain. It is noteworthy that reciprocal coimmunoprecipitation analysis showed that the binding of SNAP to Kv2.1 became substantial in oocytes that also coexpressed Syx. This was probably caused by the formation of stable t-SNARE complexes, which bind the channel with higher affinity than that of SNAP alone (Michaelevski et al., 2003).

In this study, we attempted to locate the binding site of the complex. To this end, using antibodies against the Kv2.1 C terminus, we performed coimmunoprecipitation analysis in reticulocyte lysates expressing the whole C terminus (aa

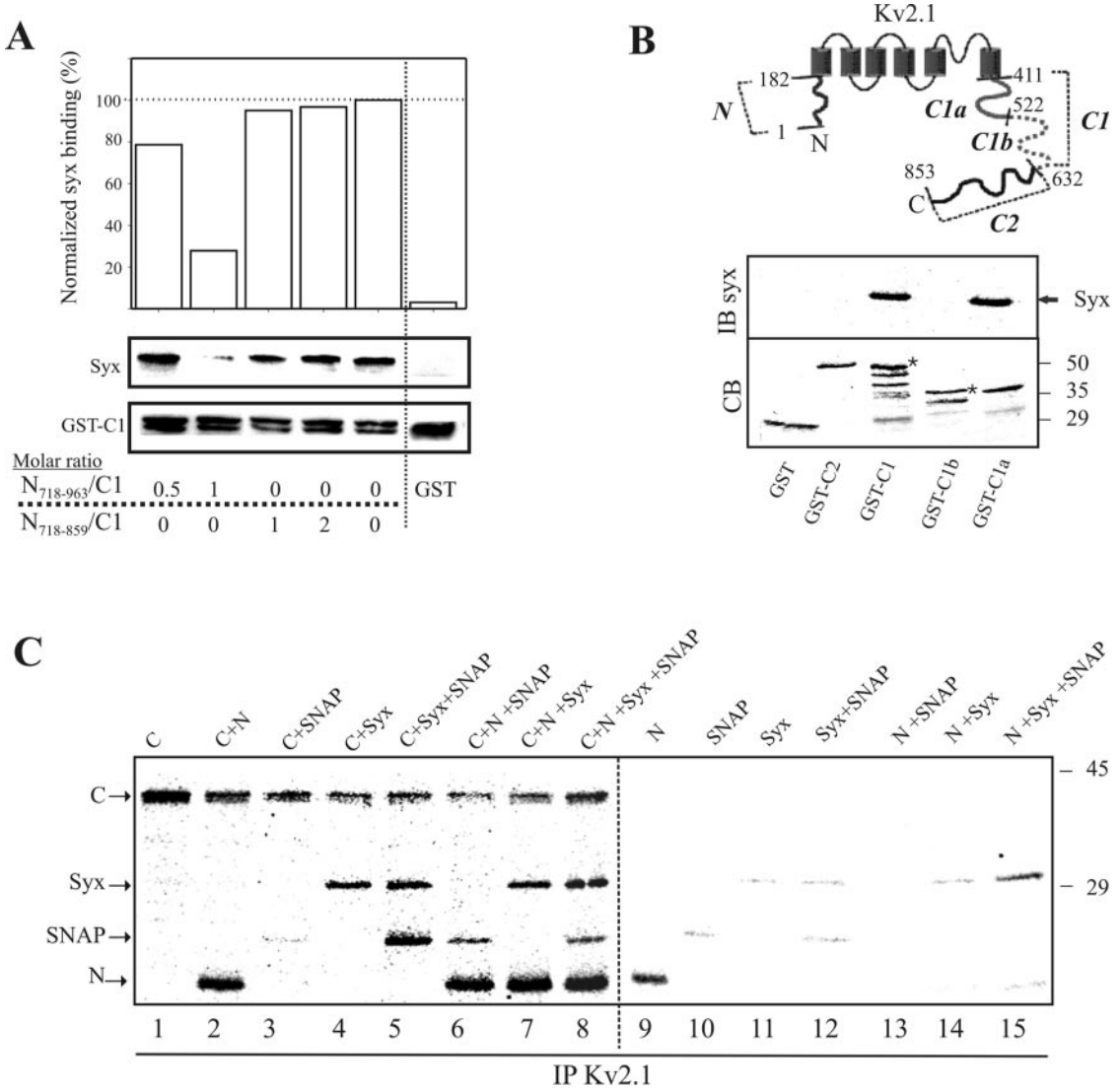

Fig. 1. In vitro interactions of Syx and SNAP with the C terminus of Kv2.1. A, binding of Syx to the proximal half of Kv2.1, C1, is inhibited by the synprint peptide $N_{718-859}$. Thrombinized cytoplasmic Syx was bound to immobilized GST-C1 in the presence of either increasing concentrations of His_6-$N_{718-963}$ or His_6-$N_{718-859}$. GST itself was used as a control, as indicated. The bar diagram shows the normalized Syx binding values according to the intensity of immunostaining for Syx and GST-C1 (beneath the bars). The molar ratio in each of the reactions between GST-C1 and the His_6 peptide is indicated below the corresponding bars (bottom). B, top, schematic presentation of Kv2.1 showing fragments generated as GST-fusion proteins. Bottom, in vitro synthesized ^{35}S-labeled Syx was incubated with 200 pmol of the indicated GST fusion proteins 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. The top shows digitized PhosphorImager scans, and the bottom shows scans of Coomassie Blue staining. Asterisks denote relevant protein products. Numbers on the right refer to the mobility of prestained molecular mass standards. C, left, coimmunoprecipitation of SNAP and Syx with the C terminus of Kv2.1. ^{35}S-labeled proteins were precipitated by an antibody directed against the C terminus of Kv2.1 from 0.1% Triton X-100 reticulocyte lysates expressing the proteins indicated above the lanes: Kv2.1 C terminus, aa 416 to 485 (C), Kv2.1 N terminus (N), syntaxin 1A (Syx), and SNAP-25 (SNAP). Right, control reactions are as shown in the left but in the absence of the C terminus. The precipitated proteins were analyzed by SDS-PAGE. A digitized PhosphorImager scan of one of three independent experiments is shown. Numbers on the right refer to the mobility of prestained molecular mass standards.

414–853) and SNAP in the presence and absence of coexpressed Syx. Substantial binding of SNAP was observed in the presence of Syx (Fig. 1C, compare lanes 3 and 5), presumably in the context of the t-SNARE complex, suggesting that the binding domain of the complex is the C terminus of Kv2.1. In light of the evidence from functional assays that the C and N termini of the channel interact (VanDongen et al., 1990; Ju et al., 2003) and the demonstration of physical interaction between them (Ju et al., 2003) (Fig. 1C, lane 2), it was of interest to determine how this interaction would affect the binding of Syx or the complex to the C terminus. To this end, we repeated the experiment in the presence of coexpressed N terminus (aa 1–184; Fig. 1C, lanes 6–8). The binding of SNAP alone was then increased to some extent (compare lanes 3 and 6), reflecting either the binding of SNAP to both the C and N termini concomitantly (in vitro binding assay using GST fusion proteins corresponding to the N and C termini showed that SNAP alone binds weakly to each one of the constructs) (Michaelevski et al., 2003) or else reflecting enhanced SNAP binding to the N and/or C termini caused by the interaction between the termini. This binding was not enhanced in the presence of Syx (compare lanes 8 and 6), suggesting that the interaction between the termini of the channel had somewhat impaired the binding of the t-SNARE complex to the C terminus. This interaction did not affect the binding of Syx to the C terminus (compare lanes 4 and 7).

Deletions at the C Terminus of Kv2.1 Dissipate the Effects of Syx and the t-SNARE Complex Observed in the Wild-Type Channel. Having established that the C terminus interacts physically with Syx and the t-SNARE complex, we were interested in determining whether the C terminus is involved in their functional interactions. To examine this possibility, we used two C-terminal deletion mutants, one lacking the last 318 amino acids (ΔC318) but including the C1a region (which binds Syx in vitro with high affinity) and the other lacking the last 416 amino acids (ΔC416), representing approximately one third of the C1a region.

Using two-electrode voltage-clamp analysis, our group characterized previously the effect of SNAP alone when coexpressed with Kv2.1 in oocytes on the delayed-rectifier K^+ current and showed that it resembled the effect of coexpressed SNAP and Syx together. We further showed that the effect of SNAP alone was mediated by endogenous Syx and suggested that it reflects the effect of the t-SNARE complex composed of exogenous SNAP and endogenous Syx (Michaelevski et al., 2003). The t-SNARE complex was shown to affect three parameters of the slow inactivation of Kv2.1: the half steady-state inactivation voltage ($Vi_{1/2}$) was shifted to depolarized potentials (values of $\Delta Vi_{1/2}$ were positive); the sustained current fraction (RCF) remaining at the end of a depolarizing voltage pulse to 10 mV was increased (values of ΔRCF were positive); and the time constant of the onset of inactivation at −10 mV (τ) was increased (Michaelevski et al., 2003) (Fig. 2, see legend for experimental protocols).

Here, we assessed the effects of t-SNARE on the C-terminal deletion mutants in oocytes coexpressing SNAP alone with the mutant channels and compared them with the effects of SNAP alone coexpressed with the wild-type channel in the same batches of oocytes (Fig. 2). In both ΔC318 and ΔC416 mutants, the depolarizing shift of $Vi_{1/2}$ was reduced (and became statistically insignificant) by ~38 and ~75%, respectively, and the increase in RCF was completely abolished (RCF even decreased in the ΔC416 mutant) (Fig. 2, A and B). In addition, in ΔC416, the increase of τ was abolished and replaced by a small decrease (Fig. 2C). Thus, the effects of SNAP observed in the wild-type channel were abolished in both mutants, indicating that the C terminus is involved in the effect of the t-SNAREs on the wild-type channel inactivation to shift $Vi_{1/2}$ to depolarized potentials and to increase both RCF and τ. The apparent effects of SNAP on ΔC416 inactivation that are different (and smaller) from those on the wild-type channel may reflect interactions of SNAP with parts of the channel, other than the C terminus, that are either masked in the wild-type channel or else are unique to the mutant channel (see *Discussion*). It should be mentioned that in both deletion mutants, the fraction of current that is inactivated after depolarization was initially significantly smaller than that in the wild-type channel (VanDongen et al., 1990); in particular, in some batches of oocytes, inactivation of ΔC416 was too small for rigorous steady-state inactivation analysis.

We showed previously that Syx coexpressed with Kv2.1 affects both steady-state activation and steady-state inactivation by shifting the half-activating ($Va_{1/2}$) and the half-inactivation ($Vi_{1/2}$) voltages to hyperpolarized potentials (negative values) (Michaelevski et al., 2003) (Fig. 3). Here, we assessed the effects of Syx on the deletion mutants and compared them with the effects on the wild-type channel in the same batches of oocytes. We found that the hyperpolarizing shift of $Vi_{1/2}$ was abolished in ΔC318 (and replaced by a statistically insignificant depolarizing shift; Fig. 3A) and that in both mutants, the hyperpolarizing shift of $Va_{1/2}$ was eliminated (Fig. 3B). Syx caused a significant decrease (13.82 ± 3.12% in 20 oocytes of 4 batches per group) in the RCF of ΔC318 but not in the wild-type channel. Thus, the effects of Syx observed in the wild-type channel to shift $Vi_{1/2}$ and $Va_{1/2}$ to hyperpolarized potentials were eliminated in the mutant channels. The apparent small effects of Syx on ΔC318 inactivation that are different form those on the wild-type channel (Fig. 3A) may reflect interactions of Syx with parts of the channel, other than the C terminus, that are either masked in the wild-type channel or else are unique to the mutant channel (see *Discussion*). These results led us to conclude that the Kv2.1 C terminus mediates the functional effects of both the t-SNARE complex and Syx on the wild-type channel.

The effects of Syx on both the activation and the inactivation observed in wild-type channels were noticeably abolished in ΔC318, which contains the high-affinity Syx-binding site, C1a. The simplest interpretation of this result is that there is no causal relationship between the binding of Syx to C1a (determined in vitro) and its functional effects on the wild-type channel. Alternatively, low-affinity Syx-binding site(s) at other parts of the channel (e.g., at the distal part of the C terminus, C2) (Leung et al., 2003; Michaelevski et al., 2003) could be relevant for function. We reasoned, however, that a causal relationship could not be ruled out if it is assumed that, although Syx binds C1a itself, the functional interaction requires other parts of the channel. Our next aim, therefore, was to determine whether a causal relationship exists between the binding of Syx to C1a and its functional interaction with Kv2.1.

Physical Interaction between Kv2.1 and Syx in Plasma Membranes of Oocytes Mediates the Functional Effects of Syx. To examine the possible existence of

a causal relationship between the physical and the functional interactions of Syx with Kv2.1, two approaches were used. First, we took advantage of the finding that the $N_{718-963}$ (synprint) peptide competed successfully with the C terminus of the Kv2.1 channel for binding to Syx (Fig. 1A). We tried to rescue the channel from the functional effects of Syx on both activation and inactivation by microinjecting this peptide 20 to 40 min before electrophysiological assay of Kv2.1 currents into oocytes already expressing both the channel and syntaxin proteins in the plasma membrane. The injected peptide should exert an effect by competing with the channel for syntaxin binding. Therefore, Syx should dissociate from the functional channel and its effect on the channel reversed. As a control, we used the $N_{718-859}$ peptide, which does not compete for Syx binding (Fig. 1A). As shown in Fig. 4A, the hyperpolarizing shift of $Vi_{1/2}$ caused by Syx could indeed be reversed by $N_{718-963}$. In the presence of the control peptide, the hyperpolarizing shift was statistically significant, confirming that $N_{718-963}$ attenuates the effect of Syx on the steady-state inactivation by disrupting its interaction with the Kv2.1 channel. Likewise, the hyperpolarizing shift of $Va_{1/2}$ caused by Syx was reversed by the synprint peptide and not by the control peptide (Fig. 4B). These results indicate that there is a causal relationship between the physical and functional interactions of Syx with the channel; however, they do not identify the site of interaction on the channel. The second approach took advantage of the two recombinant peptides, C1 and C1a (fused to GST), which were shown to bind strongly to Syx in vitro (Fig. 1B). Again, we tried to rescue the channel from the effects of Syx by microinjecting these two peptides 20 to 40 min before the electrophysiological assay into oocytes coexpressing Kv2.1 with Syx. As a control, we used GST itself. Figure 5A shows that the left shift of $Vi_{1/2}$ was abolished by both C1 and C1a but remained statistically significant in the presence of GST. In addition, the left shift of $Va_{1/2}$ was abolished by injection of C1a but not of GST itself (Fig. 5B). The results of this approach point to a link between the functional effects of Syx both on activation and inactivation and its physical interaction with the C terminus of the channel.

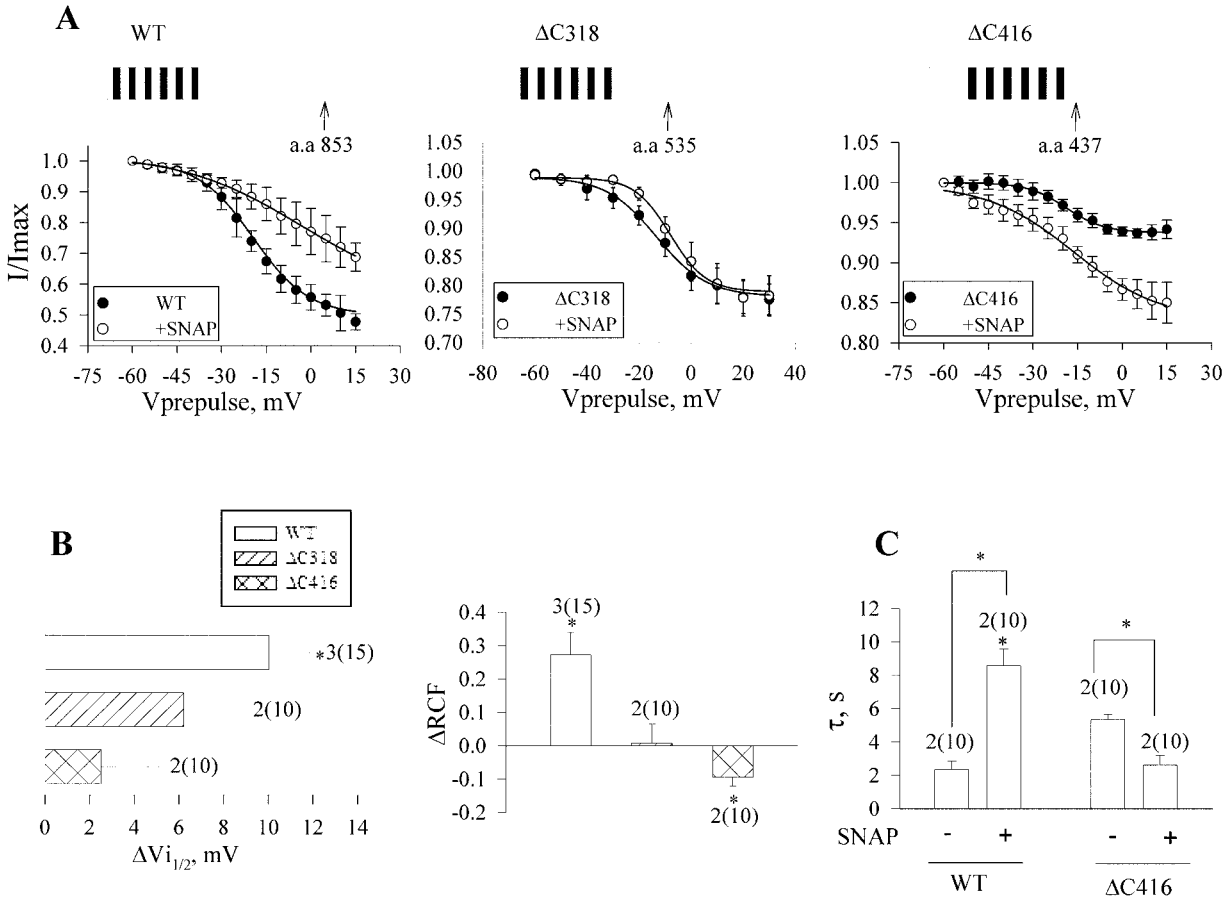

Fig. 2. Partial deletions of the C terminus of Kv2.1 dissipate the effects of SNAP on inactivation. A, effects of SNAP on the steady-state inactivation of wild-type (WT, left), Δ318 (middle), and Δ416 (right) channels derived by 5-s depolarizing prepulses to the indicated voltages ($V_{prepulse}$), followed by a 250-ms test pulse to +50 mV. The time between episodes was set to 30 s to eliminate accumulation of inactivation. Currents were leak-subtracted, and the mean fractional currents (I/I_{max}) were plotted versus $V_{prepulse}$. Each panel shows a representative experiment in a single batch of oocytes with five oocytes per group. B, data from each oocyte were fitted to a Boltzmann equation: I/Imax = 1/(1+exp ($Vi_{1/2} - V_{prepulse}$)/a) + RCF, and mean values for $Vi_{1/2}$ and RCF remaining after a 5-s depolarizing prepulse to +15 mV were derived from oocytes (numbers in parentheses next to each bar) tested in two to three batches (as indicated next to each bar) corresponding to each panel in A. Bar diagrams show the mean effects of SNAP on $Vi_{1/2}$ (rightward shifts; $\Delta Vi_{1/2}$, left; *, $p < 0.05$) and on RCF (ΔRCF, right; *, $p < 0.001$). C, effects of SNAP on rates of inactivation onset of wild-type and Δ416 channels. Mean rate constants (τ) at −10 mV derived from one exponential decay fits to peak normalized currents elicited by 250-ms test pulses to +50 mV after −10-mV prepulses of increasing duration in the absence (−) and presence (+) of SNAP (two batches of oocytes, each with 10 oocytes per group). The same oocyte batches were tested for all groups. *, $p < 0.001$.

Discussion

The notion that Kv channels might interact directly with proteins of the exocytotic machinery was formulated from work done by our group over the last few years. At first, we focused on the presynaptic Kv1.1 channel and showed that it interacts physically with SNARE proteins in fresh brain synaptosomes, depending on their physiological state (Fili et al., 2001). Its interactions with Syx and SNAP were shown to feed back on the channel function in oocytes and in pancreatic β cells, respectively (Fili et al., 2001; Ji et al., 2002).

More recently, our focus was shifted to Kv2.1, the prevalent Kv channel in neuroendocrine and endocrine cells. We showed that it interacts physically with Syx and SNAP in PC12 cells (Michaelevski et al., 2003) and that the interactions of the channel with Syx and SNAP feed back on the channel function in pancreatic β cell (MacDonald et al., 2002; Leung et al., 2003) and in oocytes (Michaelevski et al., 2003). Biochemical and electrophysiological analyses carried out in oocytes in which Kv2.1 was coexpressed with Syx alone, SNAP alone, or Syx and SNAP together showed that the channel interacts both physically and functionally either with Syx alone or with the t-SNARE complex. The channel does not interact functionally with SNAP unless SNAP is in a complex with Syx, the latter being either endogenous to the oocyte or exogenously expressed. On the basis of the correlation between the physical and the functional interactions, we proposed a model describing the modes of interaction of Kv2.1 with Syx and the t-SNARE complex (Michaelevski et al., 2003). In vitro binding assays identified the proximal half of the Kv2.1 C terminus (the C1 peptide) as the target of Syx binding; the binding target of the t-SNARE complex was not identified (Michaelevski et al., 2003).

The present work yielded two significant findings that enhance our understanding of the modes of interaction of the channel with Syx and with the *t*-SNARE complex. First, we present sound evidence that a causal relationship exists between the physical and functional interactions of Syx with Kv2.1: binding of Syx to functional channels in the plasma membrane was found to affect both activation and inactivation gating of the channel. The reliability of this finding could be inferred from the use of three different peptides that can compete with the channel for Syx binding. Upon injection of the peptides into oocytes in which Syx was already coexpressed with Kv2.1 in the plasma membrane, half activation and inactivation voltages could be reverted to their former values (Figs. 4 and 5). One peptide was the synprint peptide, corresponding to the Syx-binding domain of the N-type Ca^{2+} channel, which was shown here in an in vitro binding assay to compete efficiently with the binding of Syx to the Kv2.1 C terminus (Figs. 1A and 4). The result of this experiment strongly suggested that the synprint peptide reversed the effect of Syx on the extent of inactivation by disrupting the interaction between Syx and the channel, meaning that the hyperpolarizing shifts of steady-state inactivation and acti-

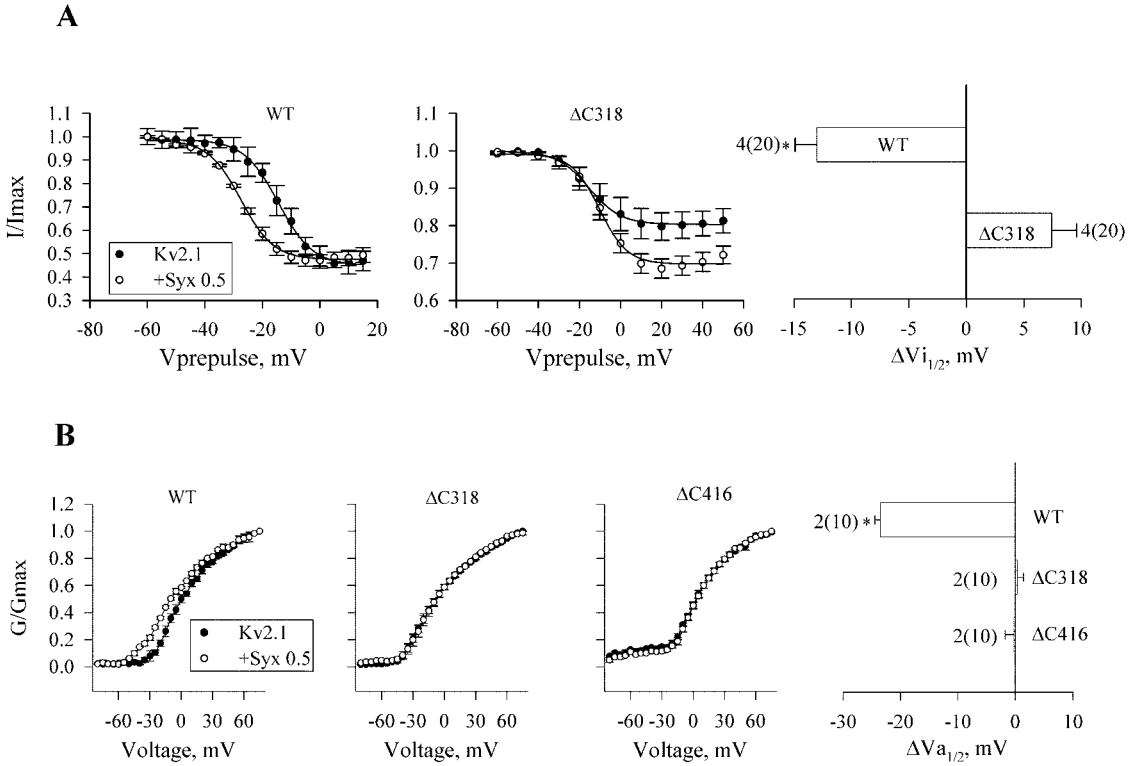

Fig. 3. Partial deletions of the Kv2.1 C terminus dissipate the effect of Syx on both steady-state activation and steady-state inactivation. A, effects of Syx on steady-state inactivation of wild-type (WT, left) and Δ318 (middle) channels in representative experiments, each in a single batch of oocytes with five oocytes per group; right, mean effects of Syx on $Vi_{1/2}$ ($\Delta Vi_{1/2}$) from four batches of oocytes with 20 oocytes per group. The same batches of oocytes were tested for all groups. Analyses and presentations are as in Fig. 2, A and B. *, $p < 0.001$. B, effects of Syx on steady-state activation of wild-type, Δ318, and Δ416 channels. Normalized conductance (G/G_{max})-voltage relationships were derived from current-voltage relationships obtained from leak-subtracted peak currents elicited by 250-ms steps to the denoted potentials (with intervals of 1 s between episodes). Three left panels show representative experiments, each in a single batch of oocytes with five oocytes per group. Right, data from each oocyte were fitted to a Boltzmann equation: $G/G_{max} = 1/1 + (\exp(Va_{1/2} - V)/a)$, and values for $Va_{1/2}$ were derived from two batches of oocytes with 10 oocytes per group. Bar diagram shows mean effects of Syx on $Va_{1/2}$ ($\Delta Va_{1/2}$). *, $p < 0.01$.

vation of Kv2.1 caused by Syx were the result of protein-protein interactions at the cell surface. The two other peptides that reversed the effects of Syx were C1 and C1a, which correspond to domains of the C terminus of Kv2.1 itself (Fig. 5). C1 peptide, the proximal half of the C terminus of Kv2.1, was previously identified (by the use of an in vitro binding assay) as the Syx-binding domain (Michaelevski et al., 2003). In this study, the binding domain was localized to C1a, the proximal quarter of the C terminus (Fig. 1B), narrowing down the Syx-binding site to ~100 amino acids. Thus, the results of these experiments not only strongly suggested a causal relationship between the physical and the functional interactions of Syx with Kv2.1 but actually validated the model describing the mode of interaction of Kv2.1 with Syx that was proposed previously (Michaelevski et al., 2003) merely from correlations between binding and functional interactions. Furthermore, these experiments implied that the C terminus might participate in the interaction between Syx and the functional channel. This notion was substantiated by the finding that the effects of Syx alone, as observed in the wild-type channel, were practically eliminated in the two C-terminal deletion mutants (Fig. 3; see considerations below). The observation that one of the deletion mutants, ΔC318, contains the high-affinity Syx-binding site, C1a, may indicate that although Syx binds C1a, its effect on channel gating is conveyed via a "gating modulator" located further down the C terminus. The second significant finding of this study was the identification of the Kv2.1 C terminus as the target for the t-SNARE complex binding (Fig. 1C) and functional interaction, because the effects of SNAP were practically eliminated in the two C-terminal deletion mutants (Fig. 2).

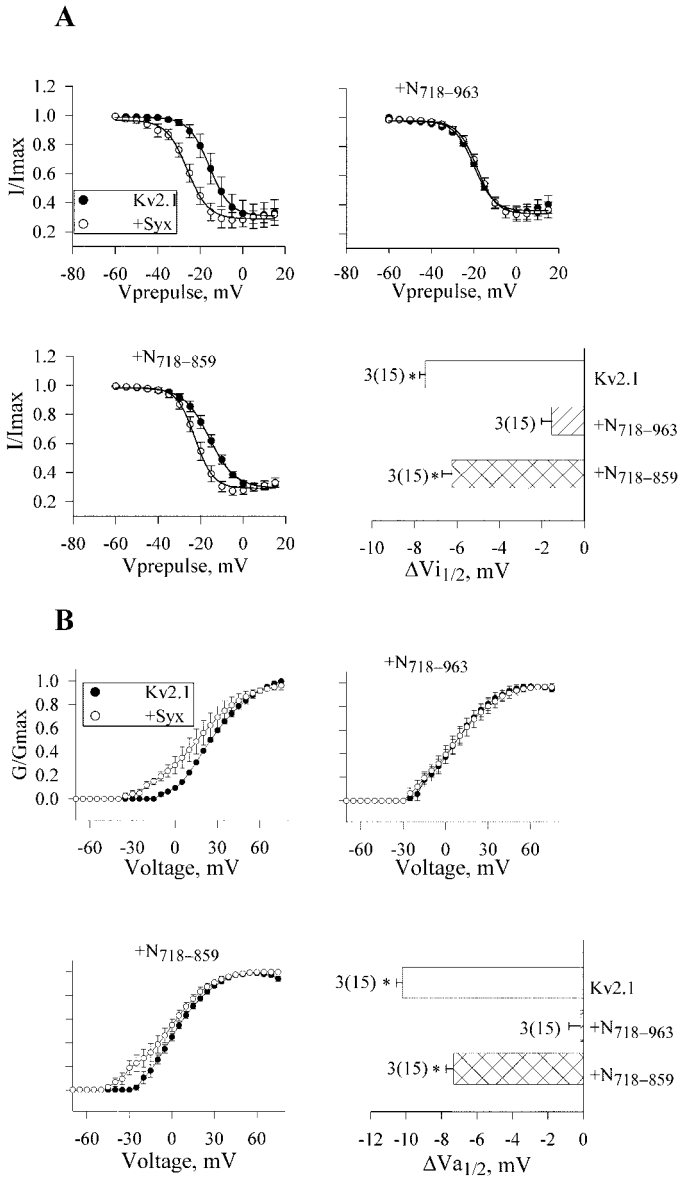

Fig. 4. The synprint $N_{718-963}$ peptide, injected 30 to 40 min before electrophysiological assay of K2.1 currents, reverses the effect of coexpressed Syx on both steady-state inactivation (A) and steady-state activation (B) of Kv2.1. Before the assay, oocytes expressing Kv2.1 alone or together with Syx (left) were injected with 1 μM (final concentration in oocytes, assuming a volume of 1 μl) of either His-tagged $N_{718-859}$ (control) or His-tagged $N_{718-963}$, as indicated. Analyses of inactivation and activation and their presentations are as in Fig. 3. *, $p < 0.001$ (A) or $p < 0.05$ (B).

It should be noted that Syx and SNAP had small effects on the C-terminal deletion mutants that were different from those on the wild-type channel. These effects could reflect interactions with parts of the channel other than the C terminus. Several possibilities may account for these interactions. One possibility would be that the interactions are unique to the mutant channel, which undergoes conformational changes that do not occur in the wild-type channel. Note that in the wild-type channel, the bulky C terminus seems to wrap around the N terminus (Wray, 2004) so that there might be no possibility for the N terminus to interact with Syx or SNAP. However, in the deletion mutants, the C terminus is extensively deleted and may render the N terminus accessible for interaction with SNAP itself (and not the t-SNARE complex) or with Syx (both agents were shown to weakly bind the N terminus in vitro) (Michaelevski et al., 2003). Another possibility would be that the interactions outside the C terminus occur in the wild-type channel; however, they are overridden by the simultaneous interaction at the C terminus.

The model describing the modes of interaction of Kv2.1 with Syx and the t-SNARE complex suggested previously by our group (Michaelevski et al., 2003) postulates that the channel has two potential binding sites, site I for Syx and site II for the t-SNARE complex. Depending on the nature of occupancy of these sites, the channel can exist in one of three gating modes, characterized by the specific activation and inactivation characteristics determined in the earlier study (Michaelevski et al., 2003). In mode A, in the absence of Syx or SNAP, neither of the sites is occupied. In mode B, in the presence of Syx and absence of SNAP, site I is occupied by Syx. In mode C, in the presence of both Syx and SNAP, the t-SNARE complex binds to site II, and site I becomes practically vacant. The results of the present study suggest that both binding sites may be localized at the C terminus; site I is probably located at the proximal quarter of the C terminus (C1a) (VanDongen et al., 1990; Ju et al., 2003).

Our studies demonstrated the interaction of syntaxin 1A with two Kv channels: Kv2.1, which is abundant in endocrine and neuroendocrine cells, and Kv1.1, which, either alone or in complex with its Kvβ auxiliary subunits, locates to presynaptic terminals (see references in Introduction and this study). Likewise, syntaxin 1A has been shown to interact with voltage-gated Ca^{2+} channels of the L-type, which is

abundant in endocrine cells, and its neuronal presynaptic counterparts, the N and P/Q types. It is interesting that the interactions of syntaxin with the Ca^{2+} and with the K^+ channels share several similar features (see references in Introduction). First, a physical interaction of syntaxin 1A with a cytosolic domain of each one of the Kv and Ca^{2+} channels has been demonstrated and shown to feed back on the channel's function. Such interaction primarily affects the voltage dependence of steady-state gating characteristics, leading to altered availability of the channels. Second, the interaction of syntaxin with both the N-type Ca^{2+} and the Kv1.1 channels was shown to involve G-protein $\beta\gamma$ subunits. Third, the concomitant presence of syntaxin 1A and SNAP-25, each interacting with the same domain of the channel, reverses the effect of syntaxin 1A on the N-type Ca^{2+} and Kv2.1 channels.

Despite the similar features of interaction shared by these channels, the intracellular channel domains that participate in the interaction differ. In the case of the Ca^{2+} channels, which have only one N and one C terminus but have long interdomain loops that are probably exposed to the intracellular milieu (Wang et al., 2004), the loop connecting the

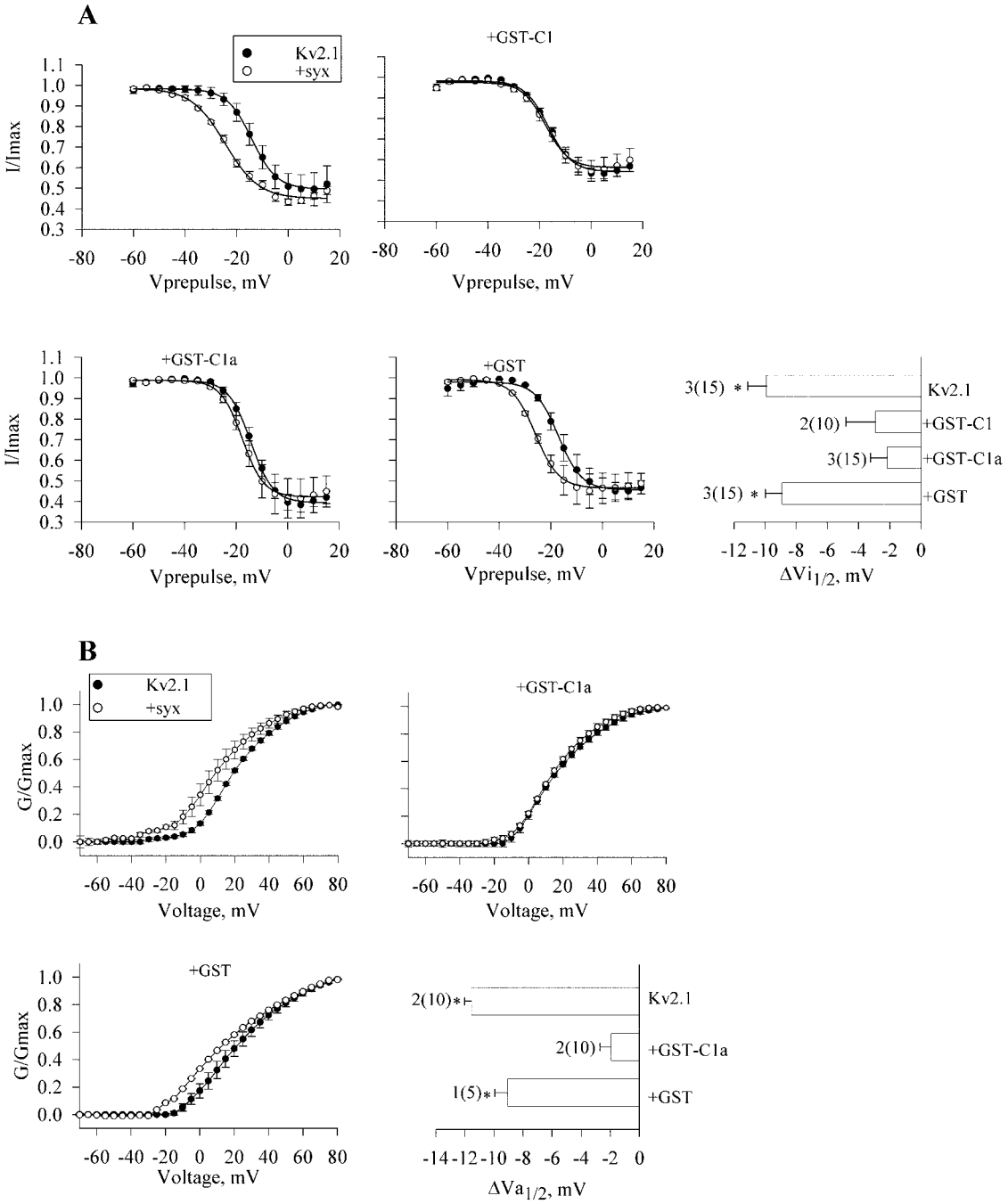

Fig. 5. The C1 and C1a Syx-binding peptides, injected 30 to 40 min before electrophysiological assay of K2.1 currents, reverse the effects of coexpressed Syx on both steady-state inactivation (A) and steady-state activation of Kv2.1 (B). Before the assay, oocytes expressing Kv2.1 alone or together with Syx (left) were injected with 0.5 μM GST-fused peptides corresponding to either C1 or C1a domains of Kv2.1, or with GST alone (control), as indicated. Analyses of inactivation and activation and their presentations are as in Fig. 3. *, $p < 0.001$ (A) or $p < 0.05$ (B).

transmembrane domains II and III has been shown to mediate the interaction of both syntaxin 1A and SNAP-25 (Rettig et al., 1996). In contrast, the Kv channels lack interdomain loops and instead contain four N and C termini. In addition, the intracellular loops in both Kv channels are quite short and are probably buried deep in the C- and N-terminal bulk so that they cannot be accessed for intracellular protein-protein interactions. Cryoelectron microscopy of the Shaker channel, closely related to Kv1.1, showed deep protrusion (tens of angstroms long) into the cytosol of the intracellular part, which is composed of the N and C termini of the channel (Sokolova et al., 2003). Syntaxin 1A and SNAP-25 were indeed both shown to interact with the N terminus of Kv1.1 (Ji et al., 2002; Michaelevski et al., 2002). Syntaxin 1A was shown also to interact with the cytosolic Kvβ1.1 subunit (Fili et al., 2001). In the case of Kv2.1, however, the huge C terminus (~440 amino acids long) probably wraps around the N terminus of the channel (Wray, 2004) and is therefore the only accessible intracellular domain for protein-protein interactions. Syntaxin 1A and SNAP-25 (the latter in the form of a t-SNARE complex) were indeed both shown in the present study to interact with the C terminus of Kv2.1. It is not surprising that protein interactions with the N terminus of Kv1.1 and the C terminus of Kv2.1 affect channel gating, because gating was shown to be affected by mutations in these regions (Wray, 2004). Taken together, it seems that the interactions of syntaxin 1A and SNAP-25 with the voltage-gated channels are mediated in each of the channels by a different cytosolic structure that is accessible for protein-protein interaction, resulting, however, in quite similar functional impact.

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