Previously we suggested that interaction between voltage-gated K⁺ channels and protein components of the exocytotic machinery regulated transmitter release. This study concerns the interaction between the Kv2.1 channel, the prevalent delayed rectifier K⁺ channel in neuroendocrine and endocrine cells, and syntaxin 1A and SNAP-25. We recently showed in islet β-cells that the Kv2.1 K⁺ current is modulated by syntaxin 1A and SNAP-25. Here we demonstrate, using co-immunoprecipitation and immunocytochemistry analyses, the existence of a physical interaction in neuroendocrine cells between Kv2.1 and syntaxin 1A. Furthermore, using concomitant co-immunoprecipitation from plasma membranes and two-electrode voltage clamp analyses in Xenopus oocytes combined with in vitro binding analysis, we characterized the effects of these interactions on the Kv2.1 channel gating pertaining to the assembly/disassembly of the syntaxin 1A/SNAP-25 (target (t)-SNARE) complex. Syntaxin 1A alone binds strongly to Kv2.1 and shifts both activation and inactivation to hyperpolarized potentials. SNAP-25 alone binds weakly to Kv2.1 and probably has no effect by itself. Expression of SNAP-25 together with syntaxin 1A results in the formation of t-SNARE complexes, with consequent elimination of the effects of syntaxin 1A alone on both activation and inactivation. Moreover, inactivation is shifted to the opposite direction, toward depolarized potentials, and its extent and rate are attenuated. Based on these results we suggest that exocytosis in neuroendocrine cells is tuned by the dynamic coupling of the Kv2.1 channel gating to the assembly status of the t-SNARE complex.

The soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)[1] proteins syntaxin, SNAP-25, and VAMP are crucial factors in processes of transmitter and hormone release (1). They interact with a wide range of proteins, some of them (such as synaptotagmin) associated with vesicular membranes or with plasma membranes (for example, voltage-gated Ca²⁺ channels) (1–5). We suggested previously (6) that SNARE proteins interact with a member of the Kv subfamily of the voltage-gated K⁺ (Kv) channels and that these interactions may play a role in synaptic efficacy and neuronal excitability. Our results showed that in brain synaptosomes the presynaptic Kv1.1 channel (7) interacts with some of the protein components of the exocytotic apparatus, including syntaxin 1A, SNAP-25, and synaptotagmin, in a manner that is sensitive to the exocytotic state of the synaptosomes. We also showed that Kv1.1 in complex with the auxiliary Kvβ subunits (8) interacts directly with syntaxin 1A, and the feedback effect of this interaction on the channel function enhances its fast inactivation in Xenopus oocytes. Involvement of G protein βγ subunits was found to be a requirement for this interaction (9). These characteristics of the interaction of presynaptic Kv channels with syntaxin 1A are reminiscent of the interaction of the presynaptic N-type voltage-gated Ca²⁺ channels (10–12).

Recently, we focused on endocrine cells, and we showed that Kv1.1 interacts with SNAP-25 in islet β-cells (13) and that in these cells also the Kv2.1 channel, a member of the Kv2 subfamily of Kv channels, is modulated by SNAP-25 and syntaxin 1A (14). Interestingly, in β-cells the interaction of exocytotic proteins with the L-type voltage-gated Ca²⁺ channels has also been described (15).

Kv2.1, a slow-inactivating delayed rectifier channel (16), although being widely distributed in the central nervous system, mainly on postsynaptic structures (17, 18), is the prevalent Kv channel in neuroendocrine and endocrine cells (19, 20). It was shown that the Kv2.1 current repolarizes β-cell action potentials during a glucose stimulus to limit Ca²⁺ entry and insulin secretion (21). In the present work we show that the Kv2.1 channel interacts directly with the two plasma membrane-associated SNARE proteins, syntaxin 1A and SNAP-25 in neuroendocrine cells. We further show that these interactions have functional implications observed in Xenopus oocytes. Both activation and inactivation of the channel are affected, depending on the assembly/disassembly of the binary complex SNAP-25/syntaxin 1A. On the basis of our results, we suggest a physiological relevance for these interactions in the release processes described in neuroendocrine cells.
**Interaction of Kv2.1 with Syntaxin 1A and SNAP-25**

**EXPERIMENTAL PROCEDURES**

**Constrasts and Antibodies**—The primary antibodies used were Kv2.1-C terminus (Alomone Labs, Jerusalem, Israel), polyclonal syntacin 1A (Alomone Labs), monoclonal aplysia, leech, and sea urchin homologs as well as to rat syntaxin 1A. The sequence ODN, 5′-GGA(CU)AUG-3′ and MBI Fermentas (Vilnius, Lithuania). The degenerate phosphorothioate antisense oligodeoxynucleotides (AS-ODNs) (including 5′-ATCGTTTGTGAGCGCTTCG-3′) was used as a non-sense oligomer.

**Oocytes and Electrophysiological Recording—**An expression system was described (24). Oocytes were injected with 15 ng/oocyte for biochemical or electrophysiological experiments. BoNTA or BoNT/C mRNA was injected at 15–50 ng/oocyte for biochemical experiments and at 15 or 5 ng/oocyte for electrophysiological experiments. BoNTA or BoNT/C mRNA was injected for both biochemical and electrophysiological experiments. AS-ODN at 0.05 ng/oocyte was injected 2 days before the electrophysiological assay, which was done 3 days after the mRNA injection. Two-electrode voltage clamp recordings were performed as described (25). To avoid possible errors introduced by series resistance, only current amplitudes up to 4 μA were recorded. Net current was quantified by the use of independent sample t tests. All electrophysiological results were analyzed by the use of independent sample t tests. Analyses described in the figure and table legends.

**Immunoprecipitation in Oocytes**—Oocytes were subjected to immunoprecipitation as described (25). Briefly, immunoprecipitates from 1 Triton X-100 homogenates of either plasma membranes (PM) or internal fractions were separated mechanically, as described in Ref. 26, and analyzed by SDS-PAGE (8% polyacrylamide). Digitized scans were derived by PhosphorImager (Amersham Biosciences), and relative intensities were quantified by ImageQuant.

**Immunoprecipitation and Immunoblotting in PC12 Cells**—Immunoprecipitation (IP) has been described in detail for synaptosomes (6). In the following, only the changes relevant for PC12 cells are described. Cells (5–15 × 10^6) were suspended in 5 ml of lysis buffer (20 mM Tris, pH 7.5, 5 mM EDTA, 100 mM EGTA, 100 mM NaCl, freshly prepared 1% CHAPS (Roche Applied Science) supplemented with protease inhibitor mixture (Roche Applied Science), incubated for 1 h at 4 °C, and centrifuged at 10 000 × g at 4 °C, 14 000 rpm. After overnight incubation of the supernatant with antibodies at 4 °C, protein A-Sepharose beads (Zymed Laboratories, Inc., South San Francisco, CA) were added, and the bound proteins were thoroughly washed (in phosphate-buffered saline with only 0.1% Triton X-100), separated by SDS-PAGE, and subjected to Western blot analysis using the ECL detection system (Amersham Biosciences).

**Confocal Microscopy in PC12 Cells**—One day after the PC12 cells were seeded on a plastic coverslip (Thermanox, Nunc, Naperville, IL), the cells were washed twice with TBS solution (10 mM Tris and 135 mM NaCl) containing 2 mM CaCl₂, fixed for 30 min in 4% paraformaldehyde, washed, permeabilized with 0.01% Triton for 10 min, and washed twice with TBS buffer containing 2 mM CaCl₂. Nonspecific sites were blocked with donkey immunoglobulin G (IgG, whole molecule, Jackson Immunoresearch Laboratories, West Grove, PA) for 30 min. Each coverslip was incubated for 2 h with mouse antibodies (Abs) against syntacin 1A (1:2000; Sigma) and rabbit Abs against Kv1.1 (1:5; Alomone Labs). Residual Abs were removed by washing 3 times, each time for 5 min, with TBS containing 2 mM CaCl₂ and 2% bovine serum albumin. This was followed by incubation for 30 min with the secondary Abs Cy3 donkey anti-rabbit IgG (1:1000, The Jackson Laboratory) or Alexa Fluor 488 goat anti-mouse IgG (1:500, Molecular Probes, Eugene, OR). Free secondary Abs were then washed three times with TBS containing 2 mM CaCl₂ and 2% bovine serum albumin, and the coverslips were mounted on a glass slide. The fluorescent labeling was examined by the use of a confocal laser-scanning microscope (LSM 410 invert, Zeiss, Oberkochen, Germany) equipped with a 25-milliwatt krypton-argon laser (488 and 586 maximum lines). A 40 NA/1.2 C-apochromat water-immersion lens (Axiovert 135 M, Zeiss) was used for imaging. Cy3- or Alexa-conjugated Abs were excited at 488 or 568 nm, respectively, and the emitted light was collected using BP 515-540 or LP 590 filters, respectively. The fluorescent signals were analyzed using the microscope manufacturer's program (LSM, Zeiss).

**In Vitro Binding of GST Fusion Proteins with Syx and SNAP**—The fusion proteins were synthesized and reacted with Syx as described (6, 27). Briefly, purified GST fusion proteins (150 pmol) immobilized on glutathione-Sepharose beads were incubated either with 5 μl of the lysate containing 25S-labeled syntaxin or SNAP (translated on the template of in vitro synthesized mRNAs using a translation rabbit reticulocyte lysate kit (Promega) or with 200-pmol of recombinant syntaxin peptide prepared from a GST fusion construct (amino acids 1–264) cleaved by thrombin, as described, 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 μl reduced glutathione and then subjected to SDS-PAGE (12% polyacrylamide).

**Statistical Analysis—**Data are presented as means ± S.E. The statistical significance of differences between the two groups was calculated by the use of independent sample t tests. One-way analysis of variance was used to estimate the statistical differences in experiments comparing several groups.

**RESULTS**

The Voltage-gated K⁺ Channel Kv2.1 Interacts Physically with Syntaxin 1A and SNAP-25 in PC12 Cells—In view of the wide distribution of the Kv2.1 channel in neuroendocrine cells, we were interested in finding out whether it interacts in PC12 cells with syntaxin 1A (Syx) and SNAP-25 (SNAP), which are the target SNARE (t-SNARE) partners of the exocytotic machinery (Fig. 1A). By using an antibody against Kv2.1, we found that both Syx and SNAP co-precipitate with the Kv2.1 protein. The co-precipitation of both Syx and SNAP could be reduced substantially by preincubation of the antibodies with the peptide against which the antibodies were raised. To verify the specificity of the co-precipitation, we performed the reciprocal experiments, in which Kv2.1 was co-precipitated with Syx and SNAP, using antibodies against Syx or SNAP.

**Kv2.1 Co-localizes with Syx in PC12 Cells**—The co-immunoprecipitation results indicated an interaction between the channel protein and Syx (the interaction with SNAP was suggested to be prevalently via Syx, see below). To evaluate the extent of the interaction, we carried out an immunocytochemical study in PC12 cells. A monoclonal antibody against syntaxin 1A and a polyclonal antibody against Kv2.1 were used for double staining of the corresponding proteins. The confocal fluorescence microscopic images are shown in Fig. 1B. Kv2.1 was found to be distributed both at perinuclear regions and at plasma membranes, whereas Syx was mostly at the plasma membranes. The overlay image (Fig. 1B, right panel) shows co-localization of the channel at the plasma membranes with Syx.

**Syx and SNAP Directly Bind Cytosolic Domains of Kv2.1**—Previously, we have shown in an in vitro binding assay that Syx and SNAP bind to the Kv2.1 channel (14, 28). In this study we carried out a more comprehensive in vitro binding assay to substantiate the physical interaction of the channel with these proteins. First, immobilized glutathione S-transferase (GST) fusion proteins corresponding to the major cytoplasmic parts of the channel: the N terminus (amino acids 1–182; N), and the proximal and distal halves of the C terminus (amino acids 411–632 and 633–853; C1 and C2, respectively) (Fig. 2A).
These fusion proteins and GST itself were incubated in the presence of 0.1% Triton X-100 with 35S-labeled full-length Syx or SNAP synthesized in reticulocyte lysate (Fig. 2, B and C). Both Syx and SNAP bound to the channel, Syx bound preferentially to the C1 domain, whereas SNAP bound to the N- and the C-terminal domains with some preference for C2. Notably, the binding of Syx was stronger by about 1 order of magnitude than that of SNAP which was rather weak. The binding of Syx to C1 was further evaluated by the use of the recombinant cytoplasmic part of syntaxin (corresponding to amino acids 4–264) cleaved by thrombin from its corresponding GST fusion protein in two different detergents (0.1% Triton X-100 and 0.5% CHAPS) (Fig. 2D). By using different concentrations of Syx, we estimated that under our binding conditions, the binding of Syx was stronger by about 1 order of magnitude than that of SNAP which was rather weak. The binding of Syx to C1 was further evaluated by the use of the recombinant cytoplasmic part of syntaxin (corresponding to amino acids 4–264) cleaved by thrombin from its corresponding GST fusion protein in two different detergents (0.1% Triton X-100 and 0.5% CHAPS) (Fig. 2D). By using different concentrations of Syx, we estimated that under our binding conditions, the binding of Syx to the channel was devoid of detergent artifacts, strong, targeted to a defined region on the channel (C1), dose-dependent, and saturable. However, the binding of SNAP-25 was less conclusive, being weak and not defined to a distinct region on the channel.

Syx and SNAP Affect the Voltage Dependence of Inactivation of Kv2.1 Expressed in Xenopus Oocytes—To study the modulation of the Kv2.1 currents by Syx and SNAP, we used Xenopus oocytes. Oocytes injected with Kv2.1 mRNA exhibited outward K⁺ currents, which inactivated slowly upon 25-s membrane depolarizations to different voltages (Fig. 3A, upper left panel). Co-injection of Syx or SNAP mRNAs affected the Kv2.1 current traces (Fig. 3A). The effects of Syx and SNAP on the voltage dependence of the Kv2.1 current inactivation were studied using depolarizing pulses. We started with pulses of 25-s duration, which induces full inactivation, but for practical reasons subsequent experiments were done with 5-s pulses, which yielded qualitatively similar results. Co-injection of Syx mRNA with Kv2.1 resulted in a negative shift of the half-inactivation voltage (\(V_{1/2}\)) by \(20\) mV, with no effect on the residual current fraction (RCF), defined as the fraction of current remaining after a 5-s pulse to \(+15\) mV (Fig. 3B and Table I).

Co-injection of SNAP with Kv2.1 mRNAs shifted \(V_{1/2}\) to membrane potentials that were more depolarized by \(9.5\) mV and enhanced the RCF by \(\sim 55\%\) (Fig. 3C and Table I). Neither Syx nor SNAP had a significant effect on the slope factor (a) of the inactivation curve (Table I).

Effect of a Combination of Syx and SNAP on Kv2.1 Voltage Dependence of Inactivation—The target membrane SNAREs syntaxin 1A and SNAP-25 have been shown to form a complex on presynaptic plasma membranes (29–32). We therefore assessed the functional interaction of a combination of Syx and SNAP with Kv2.1. Concomitant expression of Syx and SNAP resulted in elimination of the leftward shift of \(V_{1/2}\) by Syx. Rather, the combination of Syx and SNAP yielded effects similar to those observed with SNAP alone, namely a rightward shift of \(V_{1/2}\) and an increase in RCF (Fig. 3D and Table I). The rightward shift in \(V_{1/2}\) was smaller than in the case of SNAP alone.

SNAP Affects the Kinetics of Kv2.1 Channel Inactivation—The kinetics of inactivation was characterized by the use of two experimental protocols. At potentials above the level where current activation became significant (above \(10\) mV), inactivation was measured as the ratio of current decay during 25-s depolarizing pulses (Fig. 4A). At \(-10\) mV, the onset of inactivation was determined by imposing pulses of different duration to \(-10\) mV, followed by a fixed test pulse to elicit the remaining current (Fig. 4B). In both protocols, the membrane was maintained at a negative potential between successive trials to allow complete recovery from inactivation before each measurement. The rate of inactivation was estimated by the inactivation time constant (\(\tau\)) derived from a single exponential decay fit. At all voltages tested, increases of \(85–90\%\) in \(\tau\) were observed in the presence of SNAP. Syx did not affect \(\tau\) at any of the voltages tested. Recovery from inactivation was studied by a classic two-pulse procedure (Fig. 4C). Following a 25-s depolarizing test pulse to inactivate the current, recovery was assessed by application of a test pulse at a variable interval. The rate of recovery was estimated by the fast and slow time constants derived from a two-exponential fitting. Neither Syx nor SNAP had any significant effect on the rate of recovery (Fig. 4C, legend).
Syx Affects the Voltage Dependence of Activation of Kv2.1—As shown in Fig. 5, Syx affected the activation of a fraction of the channels. The activation curve of Kv2.1 co-expressed with Syx fitted well to a two-component Boltzmann function: one component resembled the conductance ($G$)-voltage curve of Kv2.1 expressed alone (with the same half-activation voltage ($V_{1/2}$) and slope factor ($a$)) and the other component had a negative shift of $-55$ mV in $V_{1/2}$ and a much smaller slope factor (1 compared with 16, indicating much steeper slope) (Fig. 5A and Table II). The two components probably represent two distinct fractions of channels, the one not affected and the other affected by Syx. The channel activation was not affected at all by SNAP (Fig. 5B and Table II). The combination of SNAP and Syx completely abolished the effect of Syx on channel activation (Fig. 5C and Table II). The maximal channel conductance ($G_{\text{max}}$) was not affected significantly under any of the above conditions (not shown).

Association of Syx and SNAP with the Kv2.1 in Oocytes Is Interdependent—Concomitantly with the functional experiments, we examined the physical interactions of Syx and SNAP with the Kv2.1 channel in the oocyte PM (manually dissected (26)) and IF comprising the rest of the cell. Reciprocal co-immunoprecipitation analysis in the PM of oocytes from a single batch (Fig. 6A), using antibodies against Kv2.1, Syx, and SNAP, showed strong Syx binding and weak SNAP binding to the channel when Syx and SNAP were each expressed on their own with the channel. Their combined expression resulted in significant reduction in the binding of Syx to the channel (a decrease of $\sim$2-fold, as indicated by intensity ratios shown in the bar diagrams below the corresponding lanes in the middle panels of Fig. 6A). At the same time, the binding of SNAP to the channel was enhanced in the presence of Syx. In contrast, binding of Syx to the channel was increased in oocytes co-expressing the light chain of Botulinum neurotoxin A (Fig. 6B, BoNT/A), which reduces the level of endogenous SNAP (by cleaving SNAP (33)). It should be noted that a prominent feature of the PM was the presence of Syx:SNAP complexes (Fig. 6A, two right panels). It is evident from Fig. 6A (two left panels) that the relative amount of channel protein in the PM (but not in the IF) of oocytes that express the channel alone was much smaller than in the other groups of oocytes. This suggests that either trafficking of the channel or its stability in the PM was enhanced by either SNAP or Syx. This phenomenon was observed in two of a total of three similar experiments.

Working Hypothesis—The results of the electrophysiological (Figs. 3–5) and biochemical (Fig. 6) experiments in oocytes were consistent with the following scenario. Syx binds strongly to the channel (Fig. 6A) and consequently shifts both $V_{1/2}$ and $V_{\text{int}}$ of the currents to the left (Figs. 3B and 5A). SNAP binds relatively weakly to the channel (Fig. 6A) and consequently induces a right shift of $V_{\text{int}}$ and enhancement of RCP (Fig. 3C). The combined co-expression of Syx and SNAP weakens the binding of Syx and enhances the binding of SNAP (Fig. 6A). As a consequence, the shift of $V_{\text{int}}$ of the inactivation that is
determined by the opposing effects of Syx and SNAP is biased toward the latter (Fig. 3D), and the shift of the $V_{1/2}$ of activation by Syx is practically abolished (Fig. 5C). Moreover, the effect of SNAP on RCF is even increased (Fig. 3D).

The above postulated scenario yields a prediction that is based on the observation that the binding of SNAP is enhanced upon co-expression of Syx. Thus, it may be assumed that upon expression of SNAP alone, its binding to the channel and hence its effects on the currents are due to the presence of endogenous Syx and will be reduced upon knock down of the latter. This prediction was tested as described below.

**Knock Down of Endogenous Syx Reverses the Effects of SNAP**—To test the prediction that the effect of exogenous SNAP is mediated by endogenous Syx, we knocked down the latter using two approaches. First we used an AS-ODN, which is directed against highly conserved stretches of the linker domain separating the H2B and H3 helixes of Syx (ASx) and was previously shown to be an efficient tool to reduce endoge-

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**TABLE I**

| Effect of Syx | Kv2.1 (Control) | +Syx | +SNAP | +Syx+SNAP |
|---------------|-----------------|------|-------|-----------|
| $V_{1/2}$     | -17.32 ± 0.9    | -37.43 ± 6.15  | -15.14 ± 1.96  | -17.86 ± 1.54 |
| a             | -5.08 ± 0.83    | -7.38 ± 1.4    | -4.61 ± 0.28   | -6.94 ± 0.99  |
| Normalized RCF | 100             | 99.28 ± 2.09   | 155.26 ± 8.06 | 155.09 ± 5.63 |

**TABLE II**

| Effect of SNAP | Kv2.1 (Control) | +SNAP |
|----------------|-----------------|-------|
| $V_{1/2}$      | -17.72 ± 0.79   | -8.95 ± 2.49  |
| a             | -6.57 ± 1.36    | -8.33 ± 1.45  |
| Normalized RCF | 100             | 155.26 ± 8.06 |

$^a p$ values denoting statistically significant differences from control were obtained by $t$ test in two group experiments and by one-way analysis of variance in multigroup experiments. $p < 0.001$.

$^b p < 0.05$. 

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**Fig. 3. Effects of Syx, SNAP, and the combination of Syx and SNAP on the inactivation of Kv2.1.** A, representative current traces (leak subtracted) from single oocytes of the same batch injected with Kv2.1 alone (Kv2.1), with Syx (+Syx), or with SNAP (+SNAP) mRNAs. Currents were elicited by 25-s voltage steps to values ranging from −70 to +30 mV with 10-mV increments from a holding potential of −80 mV. B–D, effects of Syx (B), SNAP (C), or the combination of Syx and SNAP together (D) on the inactivation of Kv2.1 currents derived by 5-s depolarizing prepulses ($V_{prepulse}$) applied from −80 mV in ascending order, followed by a 120-ms test pulse to +50 mV. Mean fractional currents ($I/I_{max}$) were plotted as functions of $V_{prepulse}$. Each panel shows a representative experiment in a single batch of oocytes with 5 oocytes per group. Data from each oocyte was fitted to a Boltzmann equation (see legend to Table I) and mean parameters of inactivation drawn from several experiments corresponding to each panel are shown in Table I.
nous Syx level (6). A non-sense ODN of scrambled nucleotide sequence was used as control. Analysis of the effects of ASx (Fig. 7, A and B, and Table III) showed that injection of 50 pg into oocytes co-expressing SNAP and Kv2.1 abolished the effect of SNAP on RCF and on $V_{1/2}$, but did not affect $V_{1/2}$. The effect of the non-sense ODN injection was statistically insignificant (not shown). ASx exhibited no significant effect when the channel was expressed alone (Table III).

The second method we used to knock down endogenous Syx was to express the light chain of Botulinum neurotoxin C (BoNT/C) which cleaves Syx (33). The advantage of this approach over the antisense approach is that whereas the antisense targets the mRNA and hence only the newly synthesized protein, the toxin targets the whole protein pool. First we verified the potency of BoNT/C on the Syx effect and showed that its co-expression with Syx completely abolished the left shift of both the $V_{1/2}$ (Table III) and $V_{1/2}$ (not shown). ASx exhibited no significant effect when the channel was expressed alone (Table III).

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**DISCUSSION**

**Physical Interaction of Kv2.1 with Syx and SNAP Occurs in Neuroendocrine Cells with Functional Consequences in Xenopus Oocytes**—Recently we showed that the Kv2.1 channel, which is the principal delayed rectifier $K^+$ channel of islet $\beta$-cells and controls membrane repolarization and downstream insulin exocytosis, interacts functionally with $\beta$-cell SNAP and Syx (14). In the present study we report for the first time in neuroendocrine cells the existence of a physical interaction of the Kv2.1 protein with Syx (33). The advantage of this approach over the antisense approach is that whereas the antisense targets the mRNA and hence only the newly synthesized protein, the toxin targets the whole protein pool. First we verified the potency of BoNT/C on the Syx effect and showed that its co-expression with Syx completely abolished the left shift of both the $V_{1/2}$ (Table III) and $V_{1/2}$ (not shown). Next we examined the effect of SNAP in the presence of BoNT/C. As shown in Fig. 7C and Table III, co-expression of BoNT/C with SNAP practically abolished the effect of SNAP on RCF and reduced the right shift of $V_{1/2}$. When the channel was expressed alone, BoNT/C had no significant effect on the RCF or on the $V_{1/2}$ (not shown). These results support the notion that the ability of SNAP to enhance RCF and to slow down the rate of onset of inactivation requires the presence of endogenous Syx, as predicted. The ability of SNAP to shift $V_{1/2}$ to the right is less affected by the reduced amounts of endogenous Syx, possibly because the amounts of Syx that remain after the knock down are sufficient to mediate this effect, or alternatively, this effect does not require Syx.
using Xenopus oocytes and in vitro binding analyses. Thus, using reciprocal co-immunoprecipitation and immunocytochemistry analyses in PC12 cells, we demonstrated the association of Kv2.1 with Syx and SNAP and the co-localization of the channel with Syx at plasma membranes. Furthermore, we determined a quantitative and saturable direct physical interaction of Syx with the proximal half of the Kv2.1 C terminus, whereas the direct interaction of SNAP-25 with the channel was rather weak and was not confined to a particular region. Taken together, these results led us to suggest that in neuroen-

**FIG. 5. Effects of Syx, SNAP, and a combination of Syx and SNAP on the activation of Kv2.1.** A–C, four groups of oocytes, Kv2.1, + Syx, + SNAP, and + Syx + SNAP (abbreviations as in Fig. 3), were tested. Normalized conductance ($G/G_{\text{max}}$)-voltage relationships were derived from current-voltage relationships obtained from peak currents elicited by 300-ms steps to the denoted potentials (with intervals of 1 s between episodes). Representative current traces in single oocytes are shown in the two right panels of A. $G$ values were obtained from the peak currents, assuming a reversal potential of $-98$ mV for K$^+$ ions. Mean data were fitted to one-component (B and C) or two-component (A) Boltzmann equations (see legend to Table II). $N = 5$, $n = 25$; $N = 2$, $n = 10$; and $N = 2$, $n = 10$ ($N$, number of experiments; $n$, total number of oocytes tested in all experiments) in A–C, respectively; in each panel the same batches of oocytes were used for the two groups tested. Calculated mean values are shown in Table II. Representative current traces (leak subtracted) from single oocytes of the same batch injected with Kv2.1 alone or with Syx mRNAs are shown in right panels of A.

| Effect of Syx | $V_{a1/2}$ | $\alpha$ | $V_{a2/2}$ | $\alpha^H$ |
|---------------|------------|----------|------------|----------|
| Kv2.1         | 9.36 ± 3.37| 16.87 ± 0.72| -46.81 ± 2.11| 0.92 ± 1.098|
| + Syx         | 7.73 ± 0.99 | 18.62 ± 0.91 |            |          |
| Effect of SNAP|            |          |            |          |
| Kv2.1         | 10.65 ± 0.86| 13.35 ± 0.69|            |          |
| + SNAP        | 8.91 ± 0.65 | 13.88 ± 0.65|            |          |
| Effect of the combination of Syx and SNAP | | | | |
| Kv2.1 + SNAP  | 12.88 ± 1.95 | 12.77 ± 0.77 | | |
| Syx + SNAP    | 13.40 ± 0.66 | 13.35 ± 0.69 | | |
doocrine cells the channel interacts directly with Syx and that the interaction with SNAP is possibly via Syx. This notion was further substantiated in oocytes by concomitant biochemical and electrophysiological analyses. We showed that in manually dissected plasma membranes the physical interaction of Syx with the channel is strong. However, the interaction of SNAP with the channel is weak, becoming substantial only in the presence of Syx. Correspondingly, Syx is required for the functional effect of SNAP. Furthermore, we characterized distinct gating (both activation and inactivation) modes of the channel in the presence of Syx and/or SNAP that are contingent on the assembly status of the t-SNARE complex (see below). On the basis of these conclusions we suggest (as elaborated below) the occurrence of dynamic coupling of a voltage-gated K⁺ channel to the exocytotic apparatus of neuroendocrine cells that is dependent on the assembly/disassembly of the t-SNARE complex (possibly within the context of the whole SNARE complex), and that this coupling serves to tune the exocytosis of these cells.

**Modes of Interaction of Kv2.1 with Syx and SNAP**—Under the "Results," we presented a working hypothesis to account for the results of the electrophysiological experiments and the biochemical experiments carried out in plasma membranes of oocytes expressing Syx, or SNAP, or the combination of SNAP with Syx (Figs. 3–6 and Tables I and II). To substantiate the hypothesis, we confirmed an emerging prediction by knocking down endogenous Syx (Fig. 7 and Table III). On the basis of the results, we propose a model that describes the interactions of Kv2.1 with Syx and SNAP (Fig. 8). In this model the channel has two potential binding sites, one for Syx (site I) and the
other for the Syx:SNAP complex (site II). Subject to the occupancy of these sites, the channel can exist in one of three gating modes. 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, Syx binds to site I (which is presumably located on the proximal half of the C terminus of Kv2.1; Fig. 2C). In mode C, in the presence of both Syx and SNAP, SNAP scavenges Syx to form the high affinity Syx:SNAP complex that binds to site II, and site I becomes practically vacant. In the following we present the experimental basis for the above postulations.

Our assumption that I and II are distinct binding sites was based on the different functional effects produced by Syx alone and by Syx and SNAP in combination (Fig. 3 and Fig. 5). However, one cannot exclude only one Syx-binding site that mediates different functional consequences depending on whether SNAP is also bound to Syx. That the Syx:SNAP complex, and not SNAP alone, is required for occupancy of site II in mode C was concluded from the observation that both the physical and the functional interactions of SNAP with the channel were dependent on Syx. First, the binding of SNAP alone to the channel, both in vitro (Fig. 2C) and in plasma membranes of oocytes (Fig. 6A), was weak, but was markedly strengthened in oocytes in the presence of Syx and occurred on the background of massive formation of Syx:SNAP complexes (Fig. 6A). Second, the effect of SNAP alone was reversed upon knock down of endogenous Syx (Fig. 7 and Table III). However, one cannot exclude the possibility that in mode C individual Syx and SNAP molecules bind to different sites of Kv2.1 simultaneously and modulate channel gating coordinately. The vacancy of site I in mode C due to scavenging of Syx by SNAP was postulated on the basis of two complementary findings showing that the binding of Syx was reduced in the presence of co-expressed SNAP (Fig. 6A) and, vice versa, was increased upon knock down of endogenous SNAP (Fig. 6B). Also, the reversal of the effects of Syx alone, both on inactivation and on activation of the channel, observed in the presence of SNAP (Fig. 3C and 5C) are consistent with dissociation of free Syx from the channel. The affinity of Syx for site I is probably higher than the

![Figure 7](image_url)

**Fig. 7. Effects of knock down of endogenous Syx. A** A, effect of an AS-ODN (ASy) on modulation of the inactivation by SNAP. Oocytes were injected, 2 days before the assay, with Kv2.1 mRNA, alone or together with SNAP mRNA or together with SNAP mRNA and ASy. A representative experiment (5 oocytes per group), performed as in Fig. 3 is shown. Analysis and mean values derived from two such experiments are shown in Table III. B, mean rate constants of onset of inactivation at −10 mV (inset) derived from one exponential decay fits to peak normalized currents elicited by 350-ms test pulses to +50 mV following −10 mV prepulses of increasing duration (two experiments with total of 10 oocytes per group). C, effect of BoNT/C on modulation of the inactivation by SNAP. Data obtained from 5 oocytes per group were analyzed as in Fig. 3 and mean values are shown in Table III.

| Table III |
|---|
| Effects of knock down of endogenous Syx on the voltage dependence of inactivation of Kv2.1 |
| Each panel (top to bottom) summarizes inactivation parameters derived from 2 experiments (with 5 oocytes per group in each experiments), corresponding to representative experiments shown in Fig 7. Experimental protocols and analyses are as in Table I. |
| | $V_{1/2}$ | $a$ | Normalized RCF |
| | mV | | % |
| Syx knock down by ASy | | | |
| Kv2.1 | −18.54 ± 1.23 | −6.55 ± 0.92 | 100 |
| + ASy | −19.82 ± 2.26 | −7.76 ± 0.67 | 107.18 ± 8.56 |
| + SNAP | −9.70 ± 0.92 | −6.81 ± 0.11 | 160.83 ± 4.71 |
| + SNAP + ASy | −10.67 ± 0.92 | −5.07 ± 0.80 | 103.94 ± 11.67 |
| Syx knock down by BoNT/C | | | |
| Kv2.1 | −18.26 ± 2.09 | −5.07 ± 0.30 | 100 |
| + Syx | −25.93 ± 2.99 | −4.93 ± 0.26 | 108.35 ± 8.47 |
| + Syx + BoNT/C | −16.81 ± 4.49 | −5.69 ± 0.75 | 112.27 ± 2.38 |
| + SNAP | −9.74 ± 2.10 | −14.86 ± 0.99 | 193.35 ± 2.02 |
| + SNAP + BoNT/C | −12.76 ± 2.58 | −7.45 ± 0.38 | 100.44 ± 11.74 |

* $p$ values were obtained by t test in two-group experiments and by one-way analysis of variance in multigroup experiments. $p < 0.001$.

* $p < 0.05$. 
affinity of Syx/SNAP for site II, as the amount of Syx that co-precipitated with Kv2.1 in the plasma membrane of oocytes expressing Syx was markedly decreased in the presence of co-expressed SNAP (Fig. 6A).

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 mean activation and inactivation curves at the specific mode (Fig. 8, upper panels). The overlap of activation and inactivation properties means that a considerable fraction of these channels are open at resting potential. The biophysical characteristics of mode B are derived from the curves determined in the experiments concerning expression of Syx alone (Figs. 3B and 5A and Tables I and II). The activation curve is presented assuming that Syx affects the whole population of channels and not just a fraction of the channels, as was the case in Fig. 5A. Thus, upon binding of Syx to site I, the channel shifts from mode A to mode B, and the conductivity window of the unoccupied channel (Fig. 8, yellow area, mode A) is extended to include more hyperpolarized potentials as well (red area, mode B). The biophysical characteristics of mode C are derived from the curves determined in experiments concerning the combined expression of Syx and SNAP (Figs. 3D and 5C and Tables I and II). Thus, in mode C the conductivity window is shifted back to depolarized potentials, similar to those in the unoccupied channel (Fig. 8, yellow area), and the conductance at these potentials is even increased (green area). These changes arise first from the depolarizing shift of inactivation (relative to mode A) and the enhancement of the RCFs which are induced by the binding of Syx/SNAP complex to site II. Second, the hyperpolarizing shifts of inactivation and activation that are prominent in mode B are eliminated due to the concomitant dissociation of Syx from site I site.

Physiological Significance of the Interaction of Kv2.1 with Syx and SNAP—In the following we would like to link the finding that the Kv2.1 channels interact physically with Syx and SNAP in neuroendocrine cells (Fig. 1) with the modal behavior of the channels presented in Fig. 8. Assuming that the interaction of the t-SNARE complex with the channels occurs within the context of the whole exocytotic SNARE complex, we would like to propose a mechanism that may contribute to the regulation of exocytosis in neuroendocrine cells for review (34). On the basis of the high affinity of Syx to the Kv2.1 channels and its abundance, it is conceivable to assume that at resting membrane potentials the channels are associated with Syx and reside in mode B. Due to the conductivity window of this mode that includes hyperpolarized membrane potentials close to the resting potential, small membrane depolarizations are filtered out, contributing to membrane potential stabilization. Upon strong membrane depolarizations, opening of L-type Ca$^{2+}$ channels and the consequent Ca$^{2+}$ ions influx is associated with the assembly of SNARE complexes at spots of docked vesicles available for release. At these spots, upon association with the complexes, the Kv2.1 channels will shift to mode C. The conductivity window in this mode that is shifted to depolarized potentials will enhance repolarization of membrane potential and consequently the cessation of Ca$^{2+}$ influx at the spots that have just undergone exocytic events. 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 in neuroendocrine cells.

Another possible physiological significance of our findings is consistent with the role of SNAREs, in particular syntaxin 1A, in regulation of ion channel trafficking demonstrated for the cystic fibrosis transmembrane regulator and endothelial sodium channels to regulate Cl$^-$ and Na$^+$ conductance properties of epithelial apical membranes, respectively (35), and also suggested by us for the Kv1.1 channel (6). In the case of Kv2.1 we demonstrate that in the presence of overexpressed Syx or SNAP the amount of channel protein detected in the plasma membrane is markedly increased (Fig. 6A). Markedly, this effect of up-regulation of Kv2.1 channel density in the plasma membrane by Syx is opposite to the down-regulation of the other channels.
Interaction of Kv2.1 with Syntaxin 1A and SNAP-25

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