Delayed-rectifier K⁺ channels (K_{DR}) are important regulators of membrane excitability in neurons and neuroendocrine cells. Opening of these voltage-dependent K⁺ channels results in membrane repolarization, leading to the closure of the Ca^{2+} channels and cessation of insulin secretion in neuroendocrine islet β cells. Using patch clamp techniques, we have demonstrated that the activity of the K_{DR} channel subtype, K_{V}1.1, identified by its specific blocker dendrotoxin-K, is inhibited by SNAP-25 in insulinoma HIT-T15 β cells. A co-localization study of rat brain confirmed that SNAP-25 interacts with the K_{V}1.1 protein. Cleavage of SNAP-25 by expression of botulinum neurotoxin A in HIT-T15 cells relieved this SNAP-25-mediated inhibition of K_{DR}. This inhibitory effect of SNAP-25 is mediated by the N terminus of K_{V}1.1, likely by direct interactions with K_{V}1.1 and/or K_{Ca}β subunits, as revealed by co-immunoprecipitation performed in the Xenopus oocyte expression system and in vitro binding. Taken together we have concluded that SNAP-25 mediates secretion not only through its participation in the exocytotic SNARE complex but also by regulating membrane potential and calcium entry through its interaction with K_{DR} channels.

In islet β cells, glucose-mediated Ca^{2+}-evoked insulin secretion is initiated by the closure of ATP-sensitive K⁺ channels, which in turn causes membrane depolarization and the resultant opening of L-type Ca^{2+} channels (1). The cessation of insulin secretion is brought about by the closure of these Ca^{2+} channels by membrane repolarization, which is primarily effected by the opening of a voltage-dependent K⁺ channels (K_{V}) of the delayed rectifier subtype (K_{DR}) (2). Regulation of K_{DR} activity therefore directly affects the duration and extent of Ca^{2+} channel opening-ensuing Ca^{2+} influx. In neurons, the short duration of action potentials regulates rapidly activating and inactivating N-type Ca^{2+} channels, resulting in ultrashort (μs) Ca^{2+} fluxes acting on docked synaptic vesicles (3). In contrast to neurons that have a higher proportion of readily releasable docked vesicles (~10%), less than 5% of insulin-containing secretory granules in islet β cells are morphologically docked, with the vast majority of insulin granules located more distantly from the membrane within a reserve pool (4). A more sustained Ca^{2+} influx effected by a much longer train of action potentials would be necessary to reach and mobilize this reserve pool of insulin granules to the plasma membrane and then to effect their exocytosis (3, 4). This contributes to the more sustained phase of secretion, also observed in other neuroendocrine cells (5). In the islet β cell, since this glucose-sensitive sustained phase of insulin secretion is regulated by the K_{DR} channel, it would be an ideal drug target for the treatment of non-insulin-dependent or type 2 diabetes. In particular, drugs that interfere selectively with it would be superior to the current treatment with sulfonfonylureas that act on β cell K_{ATP} channels in a glucose-independent manner, often resulting in deleterious side effects such as hypoglycemia (6). However, very little is known about the K_{DR} channel or the identities of the molecules interacting with this channel (2).

The target-SNAREs, SNAP-25 and syntaxin, and the vesicle-SNARE, vesicle-associated membrane protein, are thought to comprise the minimal machinery required for membrane fusion and exocytosis (7, 8). These proteins all possess α-helical domains that interact to form a stable coiled-coil complex, the formation of which likely drives membrane fusion (7, 8). Clos-tridial neurotoxins, which specifically cleave these SNAP proteins, have been valuable tools in revealing SNAP protein functions (9–11). t-SNARE proteins syntaxin 1A and SNAP-25 can also directly interact with membrane ion channels involved in regulating the secretory process. Syntaxin 1A and SNAP-25 bind to (12, 13) and modulate neuronal (12–14) and neuroendocrine (pancreatic islet β cells) (15, 16) Ca^{2+} channels. More recently, syntaxin has also been shown to modulate epithelial Na^{+} (17, 18), Cl⁻ (19, 20), and voltage-gated K⁺ (21) channels.

In this study, we have begun to explore the molecular interactions between K_{DR} channels and the critical t-SNARE, SNAP-25. Here we show that SNAP-25 interacts with insulinoma HIT-T15 cell K_{DR} specifically via the K_{V}1.1 subunit. Overexpression of SNAP-25 or exogenously applied recombinant SNAP-25 protein inhibits HIT cell K_{DR} activity. Cleavage of SNAP-25 by botulinum neurotoxin A (BoNT/A) light chain expression relieves the actions of endogenous and exogenous SNAP-25 on K_{DR}. Furthermore, the N-terminal domain but not
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Previously transfected GFP-positive HIT cells (passage 65–75) were studied with standard whole-cell and cell-attached patch clamp techniques (29) as we have previously described (30). The standard pipette solution contained the following (in mM) except where noted in the text: 140 KCl, 1 MgCl\(_2\), 10 HEPES, 4 Na\(_2\)ATP, 0.3 EGTA, and 8 mM CaCl\(_2\), pH 7.2. The external solution contained (in mM): 140 NaCl, 4 KCl, 1 MgCl\(_2\), 2 CaCl\(_2\), 10 HEPES, pH 7.4. Axopatch 1D and 200B patch clamp amplifiers were used together with pCLAMP software (v6.0, Axon Instruments, Union City, CA) to record channel currents. Currents were typically elicited from a holding potential of ~70 mV. Data were presented as mean ± S.E. and compared by Student’s t test for single comparisons and by analysis of variance for multiple comparisons. p < 0.05 was considered to be statistically significant. Single-channel recording and analyses were performed in the cell-attached configuration in which HIT-T15 cells were bathed in the internal solution (high K\textsuperscript{+}) and pipettes contained a normal external solution. Test pulses of 30 s from ~70 to ~10 mV were applied every 3 min. Analog signals were filtered at 2 kHz using a Bessel filter. Single channel data were analyzed using Fetchan and pSTAT software (pCLAMP 6.0, Axon Instruments). Recombinant proteins including GST-SNAP-25, GST\(_\text{a}TA\), GST\(_\text{T}1\text{B}\), GST-K\textsubscript{V1.1}\text{a}, and GST-K\textsubscript{V1.1}\text{b} were generated as previously described (27, 28) and dialyzed into a cell via the patch pipette. A single voltage step to +30 mV from a holding potential of ~70 mV was used to assess the effects of these test proteins on K\textsubscript{p}. RESULTS

Insulinoma HIT Cell K\textsubscript{DR} Is Similar to That of Islet \textit{β} Cells—To determine whether the K\textsubscript{DR} in HIT-T15 (HIT) cells are similar to those reported in islet β cells (2, 31, 32), we functionally and molecularly characterized this channel protein. Like islet β cell K\textsubscript{DR} (2, 32), HIT cell K\textsubscript{DR} possesses several similar functional properties (Fig. 1A). These properties include K\textsuperscript{+} ion selectivity, as demonstrated by an inhibitory effect of Ca\textsuperscript{2+} ions, when intracellular KCl was replaced with CsCl (Fig. 1A, CsCl), a low threshold of activation (~30 mV), and slow inactivation (~250 ms, shown in Fig. 1A, inset, and graphic summary). Characteristic of K\textsubscript{DR}, the HIT cell K\textsubscript{DR} was inhibited by high doses of the semi-selective K\textsuperscript{+} channel blocker tetraethylammonium (20 mM TEA in Fig. 1A, inset), which caused ~85% reduction (p < 0.05) in the outward current at +30 mV (31). Because large conductance Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} currents (K\textsubscript{Ca}) also contributes to the outward currents in islet β cells (32), we determined the contribution of this channel to total outward currents. Like others (31), we also found that K\textsubscript{Ca} contributed little to the HIT cell outward currents, as evidenced by (a) insensitivity to low intracellular Ca\textsuperscript{2+} concentrations (with 8 mM Ca\textsuperscript{2+}, <8% reduction in outward currents, data not shown) or the Ca\textsuperscript{2+} channel blocker nifedipine (Fig. 1A), (b) insensitivity to low doses of TEA (~10% reduction at 1 mM TEA, not shown), or (c) inhibition by 100 mM iiberiotoxin, a specific large conductance K\textsubscript{Ca} blocker (Fig. 1A, iiberiotoxin (IbTX)). Nonetheless, to negate even this small contribution of K\textsubscript{Ca} to the outward currents, all pipette solutions contained low Ca\textsuperscript{2+} concentration (<8 mM).

SNAP-25 Inhibits Whole-cell K\textsubscript{DR} Subtype, K\textsubscript{V1.1}\textsuperscript{a} Currents—We and others previously showed that in pancreatic islets and insulinoma cell lines including HIT cells, SNAP-25 expression is required for insulin exocytosis (22, 33, 34). Furthermore, t-SNARE proteins syntaxin 1A and SNAP-25 can also directly interact with membrane ion channels involved in regulating the secretory process (15, 16). To determine whether SNAP-25 can modulate K\textsubscript{DR} channels, we overexpressed SNAP-25 in HIT cells (Figs. 1B, 2B, and 3B). We already reported that overexpressed SNAP-25 proteins are appropri-
insulin secretion was altered by GFP transfection (22).

We therefore overexpressed BoNT/A light chain in HIT cells. BoNT/A expression reduced endogenous SNAP-25 levels of HIT cells by ~10% of control levels (Fig. 2A) (Fig. 1B). We reasoned that if SNAP-25 genuinely acts to inhibit K\textsubscript{DR}, then BoNT/A cleavage of SNAP-25 should enhance K\textsubscript{DR} currents. We therefore overexpressed BoNT/A-transfected cells (n = 6) (C, right panel). The asterisk indicates p < 0.05 versus basal. K\textsubscript{DR} current levels after the DTX-K addition in control or SNAP-25- or BoNT/A-overexpressing cells (see graph) were very close. The dotted lines are the zero current level. Currents are normalized to cell membrane capacitance.

Fig. 1. HIT-T15 cell K\textsubscript{DR} identification. Typical whole-cell K\textsubscript{DR} currents evoked with one-step voltage depolarization to +30 mV in a control cell before (○) and after it is inhibited by TEA (20 mM) application to the extracellular solution (■) (A, inset). A, effects of various K\textsuperscript{+} channel blockers:iberiotoxin (ibTX; 10 \textsuperscript{−5} M, ▲), n = 6), TEA (20 mM, ■, n = 6), CsCl (replaced KCl in the pipette solution, 140 mM, ●, n = 5), and a Ca\textsuperscript{2+} channel blocker, nifedipine (NIF; 10 \textsuperscript{−5} M, ○, n = 6) on the mean steady-state current-voltage relationship recorded from control HIT cells (○, n = 12). Whole-cell currents were evoked in response to a series of test pulses between −50 and +50 mV in 20-mV steps from a holding potential of −70 mV. K\textsubscript{DR} currents were remarkably reduced by TEA and CsCl. B, representative families of whole-cell K\textsubscript{DR} currents recorded from a control and a SNAP-25- and a BoNT/A-transfected cell. Currents were evoked in response to a series of test pulses from −50 to +50 mV in 10-mV steps from a holding potential of −70 mV. Steady-state current-voltage curves were obtained from control (C, n = 18), SNAP-25- (●, n = 18) or BoNT/A-transfected cells (■, n = 14) (B, right panel). Data are presented as the mean ± S.E. The asterisk indicates p < 0.05 against control by analysis of variance. C, effects of DTX-K, a specific K\textsubscript{+}1.1 blocker, on K\textsubscript{DR} currents. Representative outward currents recorded from a control and a SNAP-25- and a BoNT/A-transfected cell before (○, basal) and after 300 nM DTX-K addition (●). Current traces were caused by one-step depolarization to +30 mV from a holding potential of −70 mV (C, left 3 panels). The bar graph shows the effects of DTX-K on the steady-state K\textsubscript{DR} currents (mean ± S.E.) in the control (n = 6) and SNAP-25- (n = 6) or BoNT/A-transfected cells (n = 6) (C, right panel). The asterisk indicates p < 0.05 versus basal. K\textsubscript{DR} current levels after the DTX-K addition in control or SNAP-25- or BoNT/A-overexpressing cells (see graph) were very close. The dotted lines are the zero current level. Currents are normalized to cell membrane capacitance.

Fig. 1B shows representative whole-cell K\textsubscript{DR} current traces after transfection and a graphical summary of the peak currents, which have been normalized by cell membrane capacitance for minimizing variations in cell size. Transfection of HIT cells with SNAP-25 resulted in a 3-fold increase in SNAP-25 expression in total HIT lysates (Fig. 2A, lane 4). Transfection efficiency as determined by the frequency of GFP-positive cells is consistently ~40%. With this transfection efficiency of ~40%, the transfected cells studied by patch clamp would be estimated to contain about a 5–6-fold increase in SNAP-25 compared with endogenous levels. In cells overexpressing SNAP-25, the peak K\textsubscript{DR} current (86.9 ± 6.2 pA/picofarads, n = 18, p < 0.05) was inhibited at +30 mV by 22% compared with control cells (111.3 ± 7.9 pA/picofarads, n = 16) (Fig. 1B). As reasoned that if SNAP-25 genuinely acts to inhibit K\textsubscript{DR}, then BoNT/A cleavage of SNAP-25 should enhance K\textsubscript{DR} currents. Therefore, in cells expressing BoNT/A, it can be assumed that all endogenous SNAP-25 would be cleaved (22). K\textsubscript{DR} currents in these BoNT/A-expressing cells were augmented at +30 mV by 22% (135.4 ± 8.9 pA/picofarads, n = 14, p < 0.05) compared with control cells (Fig. 1B). Indeed, these effects of BoNT/A are opposite to the effects of SNAP-25 overexpression, which would therefore sug-
gest that BoNT/A cleavage of SNAP-25 removes the inhibitory actions of full-length SNAP-25. BoNT/A cleavage of SNAP-25 also revealed a smaller band (Fig. 2A, lower unfilled arrowhead of the arrowhead doublets to the right of lanes 4 and 7), which is likely the SNAP-25 cleavage product representing amino acids 1–197 (22, 34).

To identify the KDR subtype upon which SNAP-25 acted, we first applied dendrotoxin (DTX), which blocks Kv1.1 and Kv1.2 channels, and subsequently applied dendrotoxin-K (DTX-K), which more specifically blocks only Kv1.1 channels. We found that both toxins exhibited identical effects, and therefore, we show only the more specific DTX-K results in Fig. 1C. DTX-K (300 nM) application had no significant additional effects in SNAP-25-expressing cells compared with controls in which there was a 33% drop in KDR current (p < 0.05). Thus, the KDR subtype upon which SNAP-25 acted was Kv1.1. We confirmed the presence of the Kv1.1 channel protein in HIT cell lysates as shown in Fig. 2A in lane 2 (HIT Cell lysate, Control). This Kv1.1 antibody (Alomone Labs) has been depleted of antibodies that reacted with closely related Kv isoforms. Kv1.1 was previously identified in HIT cells (35) and also in human islets and human insulinoma β cells (2). Furthermore, HIT-T15 does not express

**FIG. 2.** Effects of SNAP-25 and BoNT/A transfection on KDR channel expression and channel kinetics. A, expression of SNAP-25 (lower panel) and the voltage-activated K⁺ channel (Kv) subtype, Kv1.1 (upper panel) in HIT cells. HIT cells transfected with an empty vector (Control, lanes 2 and 5), SNAP-25 (lanes 3 and 6) or BoNT/A (lanes 4 and 7) were lysed by sonication or subjected to subcellular fractionation to obtain a purified plasma membrane fraction. Western analysis using specific antibodies to SNAP-25 (22) and Kv1.1 (Alomone Labs) was performed on these HIT cell lysates (15 µg, lanes 2–4) and plasma membrane fractions (70 µg, lanes 5–7). Rat brain synaptosomal membrane fraction (LP1, 10 µg of protein) was used as the positive control. In BoNT/A-transfected HIT cells, we observed a slightly smaller band (by ~1 kDa), indicated by lower open arrowhead on lanes 4 and 7) SNAP-25 immunoactive band, which is likely the larger SNAP-25 cleavage product (amino acids 1–197). B (top left), representative whole-cell KDR activation current traces were evoked by depolarization to +10 mV for 300 ms from a holding potential of −70 mV and normalized to the same amplitude to compare their activation time course. Time constants of activation were obtained by fitting each current trace with a monoeponential equation over test voltages from +10 to +40 mV (B, bottom left). Each point was expressed as the mean ± S.E. The asterisk indicates p < 0.05 against control. SNAP-25 (○, n = 8) decelerated KDR activation, whereas SNAP-25 cleavage by BoNT/A expression (■, n = 8) accelerated KDR activation when compared with control cells (□, n = 12). B (top right), representative whole-cell KDR inactivation current traces were evoked by a 10-s pulse to +10 mV from a holding potential of −70 mV, and these traces were normalized to the same amplitude to compare their decay. Time constants of inactivation were fitted by a biexponential equation to calculate fast (not shown) and slow time constants, represented by the histogram (B, bottom right). Data were the mean ± S.E. from control (n = 8), SNAP-25 (n = 6), and BoNT/A-transfected cells (n = 6), respectively. The asterisk indicates p < 0.05 against control. SNAP-25 accelerated KDR inactivation, whereas SNAP-25 cleavage by BoNT/A expression decelerated KDR inactivation when compared with control cell KDR inactivation.
the closely related Kᵥ1.2 channel protein, as confirmed by us at the protein (by anti-Kᵥ1.2 antibody, Alomone Labs) and mRNA (by reverse transcription-PCR) levels (36).

Because the KᵥDR current in HIT cells is likely to be contributed by Kᵥ channels other than Kᵥ1.1 (36), we next examined whether the SNAP-25-sensitive component of the KᵥDR current is predominantly Kᵥ1.1. In Fig. 1C, we expressed BoNT/A to cleave endogenous SNAP-25, which predictably increased the KᵥDR currents (representative trace on the left, and summary on the right) over controls as was shown in Fig. 1B. More importantly, the application of DTX-K (300 nM) inhibited the KᵥDR current of the BoNT/A-transfected HIT cells to precisely the same extent as the control and SNAP-25-overexpressing cells (Fig. 1C), indicating that the SNAP-25-sensitive KᵥDR current in the HIT cells is indeed from Kᵥ1.1 channels.

SNAP-25 Decelerates Activation and Accelerates Inactivation of Whole-cell KᵥDR Currents—There are two possible explanations for the differences in whole-cell KᵥDR current magnitude observed in Fig. 1B. First, SNAP-25 or BoNT/A expression could affect KᵥDR synthesis and cell surface expression. In this way, changes in peak KᵥDR current would reflect a change in channel density rather than in KᵥDR activity. Fig. 2A (upper panel) shows that there is no obvious difference in the levels of Kᵥ1.1 protein (indicated by rat brain positive control, 86 kDa) expression in whole-cell lysates of HIT cells overexpressing SNAP-25 (lane 3) or BoNT/A (lane 4) when compared with control HIT cell lysates (lane 2), indicating that there was no gross effects on Kᵥ1.1 synthesis. To assess the effects on Kᵥ1.1 expression at the cell surface, which could be due to changes in endocytic or exocytic events, we determined Kᵥ1.1 levels in the plasma membrane fraction (Fig. 2A, upper panel, lanes 5–7). To our surprise, Kᵥ1.1 expression in the plasma membrane preparation was reduced to 84% of control in the SNAP-25-overexpressing HIT cells (lane 6, Fig. 2A). This reduction of Kᵥ1.1 expression at the cell surface would therefore at least contribute to the decreased current density observed in SNAP-25-transfected cells (Fig. 1B). However, if the primary action of SNAP-25 is to regulate Kᵥ1.1 transport to the cell surface, we would expect an augmented expression of Kᵥ1.1 in the plasma membrane after cleavage of endogenous SNAP-25 by overexpression of BoNT/A. This was not the case since BoNT/A overexpression, which reduced the membrane SNAP-25 levels to ~10% of controls, did not cause an increase in the plasma membrane levels of Kᵥ1.1. In fact, the Kᵥ1.1 levels in the plasma membrane (lane 7, Fig. 2A) were reduced to 49% of control, which we believe may in part be due to nonspecific proteolysis during the membrane preparation caused by the BoNT/A expression. Of note, the plasma membrane SNAP-25 levels of the SNAP-25-overexpressing HIT cells was greater than 5-fold normal levels, which is much greater in proportion to the reduction of membrane Kᵥ1.1 levels, suggesting a pharmacological effect of the overexpressed SNAP-25. In the SNAP-25- and BoNT/A-transfected HIT cell lysates and plasma membrane, syntaxin 1A levels did not change (data not shown).

The most plausible explanation is that endogenous SNAP-25 physiologic actions are to inhibit KᵥDR channel activity. We tested this possibility by examining the effects of SNAP-25 on the activation and inactivation kinetics of the whole-cell KᵥDR current. Fig. 2B demonstrates that the overexpressed SNAP-25 acts to decelerate KᵥDR activation and accelerate KᵥDR inactivation, with the time constants for activation and inactivation at +10 mV of 2.15 ± 1.0 ms (n = 8) and 3.2 ± 0.3 s (n = 6), respectively. These values were significantly different (p < 0.05) than those for controls, which had activation and inactivation time constants of 16.3 ± 1.0 ms (n = 12) and 4.3 ± 0.3 s (n = 8), respectively. In contrast, cleavage of SNAP-25 by BoNT/A had the opposite effect, resulting in acceleration of activation and deceleration of inactivation (shown in Fig. 2B), with the time constants for activation and inactivation of 11.6 ± 0.7 ms (n = 8) and 5.4 ± 0.5 s (n = 6), respectively. These values were also significantly different (p < 0.05) than those for controls (as above). These differences in time-dependent kinetics of the KᵥDR channel effected by SNAP-25 and SNAP-25 cleavage by BoNT/A would contribute to the reduction or augmentation, respectively, of the peak KᵥDR current observed in cells overexpressing SNAP-25 or BoNT/A (shown in Fig. 1B).

SNAP-25 Inhibits Single KᵥDR Channels—Single channel studies on cell-attached patches revealed single K⁺ channels in HIT cells that possess a unitary conductance of 10 picosiemens, as determined from the slope conductance (Fig. 3A), which is similar to that reported for KᵥDR in β cells (31). This conductance is like that reported for the Kᵥ1.1 voltage-activated K⁺ channel isoform expressed in oocytes (37). We next attempted to determine whether SNAP-25 affected the probability of channel opening or the conductance of single KᵥDR channels in cell-attached patches (Fig. 3, B–D). Sample traces are shown in Fig. 3B. We examined these single KᵥDR channel currents evoked by a voltage pulse to ~10 mV in all transfected cells. We found that neither SNAP-25 nor BoNT/A transfection had any effects on KᵥDR channel conductance, with all single channel slope conductances calculated to be 10 picosiemens. This is reflected by the observation that the mean unitary channel current amplitude at ~10 mV for each of the transfections was not significantly different from control cells, ranging from 0.47 to 0.51 pA (Fig. 3C). However, SNAP-25 overexpression greatly reduced the number of KᵥDR channel openings (Fig. 3C), and furthermore, the mean KᵥDR channel open time (7.6 ± 3.7 ms, n = 5, p < 0.05) shown in Fig. 3D was greatly shortened by 59% compared with control cells (18.2 ± 2.3 ms, n = 5). In contrast, cleavage of endogenous SNAP-25 by BoNT/A transfection resulted in a profound increase in the number of channel openings (Fig. 3C) and a prolongation in KᵥDR channel open time by 150% (45.6 ± 3.2 ms, n = 5, p < 0.05 versus control in Fig. 3D). These altered single channel activities collectively contribute to the observed changes in the macroscopic, whole-cell currents arising from this population of KᵥDR channels shown in Figs. 1B and 2B.

The Time-dependent Inhibitory Actions of Exogenous SNAP-25 on KᵥDR Are Relieved by BoNT/A Proteolysis—To more clearly distinguish the effects of the overexpressed SNAP-25 on the cell surface expression of Kᵥ1.1 (in Fig. 2A) and the direct effects of SNAP-25 on the Kᵥ1.1 channel, we examined the effects of acutely applied exogenous recombinant SNAP-25 protein (introduced via the patch pipette after membrane rupture) in control (Fig. 4, A–C) and BoNT/A-expressing cells (Fig. 4C). This strategy also allowed us to examine the time course of action of SNAP-25 on KᵥDR. Furthermore, SNAP-25 overexpression in other cell types leads to chronic effects such as alteration in axonal growth (38) and vesicular trafficking processes (39). It has been previously shown (40) that for a 10-megaohm access-resistance pipette, a protein with a molecular mass of 50 kDa can diffuse into the cytosol with a time constant of 5 min. Because the diffusion time constant varies with the third root of the molecular mass of the diffusing substance, we expected that the concentration of the smaller SNAP-25 (25 kDa) in cytosol would be greater than 72% of the pipette concentration within 5 min. Therefore, we expected to see exogenously applied SNAP-25-mediated effects within 5 min, which was indeed the case.

GST-SNAP-25 inhibited KᵥDR currents in a dose- and time-dependent manner. Fig. 4A shows a representative series of
K<sub>DR</sub> currents demonstrating a time-dependent reduction in K<sub>DR</sub> currents in a control HIT cell dialyzed with 10<sup>-9</sup> M GST-SNAP-25. Fig. 4B is a summary (n = 5–6 cells each) of the dose-dependent inhibition of K<sub>DR</sub> by SNAP-25. 10<sup>-10</sup> M GST-SNAP-25 had minimal effects on K<sub>DR</sub> even 5 min after membrane rupture when compared with GST (10<sup>-8</sup> M) alone, which had no effect (even after up to 30 min, as shown in Fig. 4C). In contrast, 10<sup>-9</sup> M GST-SNAP-25 caused a significant 25% reduction of K<sub>DR</sub> currents 4 min after membrane rupture (Fig. 4B). At a higher concentration of GST-SNAP-25 (10<sup>-8</sup> M), the inhibition reached a significant level just 2 min after membrane rupture, with a maximal level of 39% occurring at 5 min (Fig. 4B). In Fig. 4C, we recorded for up to 30 min and found that this maximal inhibition of K<sub>DR</sub> by 10<sup>-8</sup> M GST-SNAP-25 was maintained. These acute changes on K<sub>DR</sub> currents by exogenous application of SNAP-25 precisely mirrored those caused by SNAP-25 overexpression (Fig. 1B), which supports our thinking that a dominant action of the excess SNAP-25 is to inhibit K<sub>DR</sub> currents.

We next wanted to examine the effects of the active cleavage of SNAP-25 by BoNT/A on K<sub>DR</sub> activity. To demonstrate this, we dialyzed GST-SNAP-25 into BoNT/A-expressing HIT cells. In <i>in vivo</i> expression of BoNT/A light chain ensured continued cleavage of SNAP-25 from both endogenous synthesis and exogenous application. The initial K<sub>DR</sub> current recorded immediately after membrane rupture in BoNT/A-expressing cells was 23 ± 1% (n = 5, p < 0.05) higher than that recorded in control cells (Fig. 4C). The initial higher K<sub>DR</sub> values in the BoNT/A cells is due to cleavage of endogenous SNAP-25, which is expected to be the case before dialysis of exogenous GST-
SNAP-25 into the cytosol (Fig. 1B). As we dialyzed GST-SNAP-25 (10^{-8} M) into these BoNT/A-transfected cells, we observed a decline in K_{DR} currents that paralleled the decline seen with GST-SNAP-25 dialyzed into control cells over a similar time period of 12 min after membrane rupture (Fig. 4C). This reached a maximum level of 84 ± 1% (n = 5, p < 0.05) of the initial control cell value, which when compared with the initial value of BoNT/A-transfected cells (123%), was a reduction of 39% (Fig. 4C). This percentage reduction is identical to that seen with GST-SNAP-25 dialysis into control cells. Of note, after 12 min, the K_{DR} currents in the BoNT/A-transfected cells began to recover as a result of BoNT/A action (Fig. 4C).

The time course of this recovery of K_{DR} currents resulting from BoNT/A cleavage of the dialyzed SNAP-25 is consistent with the predicted enzymatic activity of BoNT/A, as reported by in vitro proteolysis of recombinant SNAP-25 by BoNT/A (41). By 30 min, K_{DR} activity completely recovered, reaching levels identical to those recorded immediately after initial membrane rupture (Fig. 4C). This indicates complete cleavage of SNAP-25, rendering it incompetent to modulate K_{DR}.

SNAP-25 Interacts with Kv1.1 and Kv1p1.1 Subunits—These functional data therefore suggested the possibility that SNAP-25 could interact with Kv1.1 channel proteins. To test this possibility, we examined whether SNAP-25 is in a complex with the α-subunit of the Kv1.1 channel (Fig. 5A). To demonstrate the general applicability of these findings to neurons and since these proteins are less abundant in HIT cells, we performed immunoprecipitation studies using rat brain LP1 synaptosomal fractions (s-LP1) shown in lane 1 to be enriched in SNAP-25 and Kv1.1 proteins (Fig. 5A). Of note, lane 2 shows that a rabbit anti-SNAP-25 antibody generated against the α-subunit of the Kv1.1 channel (Fig. 5A).

We showed earlier that Kv1.1 is in complex with Kv α auxiliary subunits in brain synaptosomes and that this channel exists in multimolecular complex consisting also of the t-SNARE, syntaxin 1A (21). Also, we showed that the Kv1.1/ Kv1p1.1 channel interacted physically with syntaxin 1A expressed in Xenopus oocytes. Thus, syntaxin 1A could mediate...
SNAP-25 Modulates $K_{DR}$

Fig. 5. SNAP-25 interacts with Kv1.1 proteins. **A**, co-precipitation of rat brain SNAP-25 and Kv1.1. Lane 1, solubilized LP1 fraction (α-LP1) of rat brain synaptosomes shows clear bands for Kv1.1 and SNAP-25 proteins. Lane 2 shows that the SNAP-25 antibody (SNAP-25) immunoprecipitated (IP) SNAP-25 from the solubilized LP1 fraction and co-precipitated Kv1.1 channel proteins. Control studies were performed in lanes 3 and 4. Lane 3, control preimmune IgG precipitated an ~50-kDa protein (the IgG heavy chain). Neither protein-G-Sepharose in lysis buffer (lane 4) or preimmune IgG (lane 3) precipitated SNAP-25 or Kv1.1 channel proteins. B and C, the Kv1.1 (α) and Kv1.1/Kv2.1 (αβ) channels interact physically with SNAP-25 in oocytes. B, top, digitized PhosphorImager scan of SDS–PAGE analysis of [35S]Met/Cys-labeled Kv1.1 SNAP-25 proteins co-immunoprecipitated by Kv1.1 antibody from homogenates of oocytes that were un.injected (control), injected with Kv1.1 (10 ng/oocyte; α mRNA only, co-injected with SNAP-25 (1.25 ng/oocyte; α + SNAP-25), or injected with SNAP-25 alone. B, bottom, homogenates of each group of oocytes were subjected to immunoprecipitation by SNAP-25 antibody. The protein samples were analyzed on a 12% PAGE. The results shown are from one of three independent experiments. C, top, reciprocal co-immunoprecipitation using SNAP-25 antibody, carried out in oocytes that were either un.injected (control), injected with Kv1.1 (30 ng/oocyte) together with Kv1.1 (30 ng/oocyte) without or with SNAP-25 (10 ng/oocyte) mRNAs (αβ and αβ + SNAP-25, correspondingly), or injected with SNAP-25 alone (SNAP-25). Bottom, homogenates of each group of oocytes were subjected to immunoprecipitation by Kv1.1 antibody. Oocytes used in this experiment had a low level of endogenous proteins migrating as Kv1.1 and precipitated by the Kv1.1 antibody, as we report previously (42). The protein samples were analyzed on an 8% PAGE. Arrows indicate the relevant proteins. The electrophoretic mobilities of molecular mass standards (size in kDa) are shown along the right (B) or left (C) of each autoradiogram.

The interaction between Kv1.1 and SNAP-25 in synaptosomes. To examine whether Kv1.1 could interact directly with SNAP-25 without the mediation of other SNARE proteins or neuronal presynaptic proteins, we employed the heterologous expression system of Xenopus oocytes. We showed by SDS–PAGE analysis of metabolically labeled proteins that SNAP-25 expressed in oocytes co-immunoprecipitated using Kv1.1 antibody with Kv1.1 (α) co-expressed alone (α; Fig. 5B) or together with Kv2.1 (β; not shown). The specificity of the channel interaction with SNAP-25 was verified by reciprocal co-immunoprecipitation of Kv1.1 and Kv2.1 with SNAP-25 using antibody against SNAP-25 (Fig. 5C). The faint immunoreactive bands corresponding to Kv1.1 and Kv2.1 in oocytes injected with αβ without SNAP-25 are probably the result of coprecipitation with the endogenous SNAP-25 homolog(s) (previously we also detected endogenous syntaxin homolog(s) (21)) of the channel proteins (which were better expressed in these oocytes than in oocytes coexpressing also SNAP-25; compare the expression of Kv1.1 in Fig. 5C, bottom panel, lanes αβ + SNAP-25 and αβ). As previously shown (42), the Kv1.1 protein is expressed in oocytes mainly in the form of a doublet of ~57 and ~54 kDa polypeptides that are N-glycosylated and represent functional channels.

We then proceeded to determine the domain within the Kv1.1/Kv2.1 protein with which SNAP-25 would directly interact. This study is particularly important since we had detected a small amount of endogenous syntaxin homolog(s) in oocytes (21), which raised the possibility that SNAP-25 binding to the Kv1.1 could be mediated by the binding to the endogenous syntaxin. We have measured the in vitro binding of [35S]labeled SNAP-25 synthesized in reticulocyte lysate to the following bacterially expressed recombinant proteins: GST αC, corresponding to amino acids 411–495, i.e. the whole-length C terminus of Kv1.1; GSTαT1A and GSTαT1B, corresponding to amino acids 1–71 (containing 30 amino acids that comprise T1A domain) and 72–143 of the N terminus of Kv1.1, respectively (both are involved in tetramerization of Kvα subunits (43–45), and the latter is involved also in Kvα subunit binding (46, 47); GST βC, corresponding to amino acids 75–397, a region in Kvα that is conserved among the different Kvα isoforms and is involved in binding to Kv1.1; and βfull, corresponding to the whole length of Kvα1.1 (amino acids 1–397). Fig. 6A shows that SNAP-25 bound to full-length Kvα1.1 and to its C terminus, to the N terminus, and preferentially to the T1A domain but not

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2 S. Tsuk, D. Chikvashvili, and I. Lotan, unpublished results.
to the C terminus of Kv1.1. Next we set out to test if there was a causative relationship between the direct interaction of the channel with SNAP-25 and the functional interaction that leads to inhibition of the current. To this end we tried to acutely rescue the channel in HIT cells overexpressing SNAP-25 from the functional effects of SNAP-25 by dialysis of a peptide corresponding to a domain of the channel that is involved in SNAP-25 binding. The N-terminal domain of Kvα1.1 was chosen for the following considerations. The oocyte results demonstrated that Kvα1.1 alone can bind SNAP-25, and the in vitro binding results pointed to its N terminus. Also, the in vitro binding results showed that the C-terminal domain of Kvβs, a region that is conserved among the different Kvβ isoforms, does bind SNAP-25. Because this domain binds the N-terminal domain of Kv1.1 (46, 47), we assumed that the impact of its possible physical interaction with SNAP-25 could be transferred to the N terminus of Kv1.1, conferring its functional effect. In any case, whether it interacts directly with SNAP-25 or indirectly via interaction with Kvβs, excess soluble N-terminal Kv1.1 is likely to interfere with the interaction of the endogenous membrane-bound Kv1.1 protein with SNAP-25. Induced dialysis of the recombinant αT1A or N termini (amino acids 1–168) but not the αT1B (data not shown), C-termini (amino acids 411–495), or GST resulted in the augmentation of the outward current (28–35% over control levels), presumably preventing the functional effect of SNAP-25 by disrupting its physical interaction with the channel proteins (Fig. 6B). We further showed that neither the N-or C-terminal domains had any effect on KDR currents of BoNT/A-transfected HIT cells (data not shown). Taken together, these binding and functional data point to a link between the functional effects of SNAP-25 on the Kv1.1 channel and its physical interaction with this channel protein.

**DISCUSSION**

Here we present the first evidence that SNAP-25 interacts with and inhibits the K_Dr (subtype Kv1.1) channel. Although the overexpressed SNAP-25 had a small effect in reducing the cell surface expression of Kv1.1 (Fig. 2A), dialysis of excess recombinant SNAP-25 protein acutely inhibited K_Dr currents in both control and BoNT/A-transfected HIT cells (Fig. 4), which supports a primary inhibitory role of SNAP-25 on the Kv1.1 channel. More importantly, the experiments on BoNT/A cleavage of endogenous SNAP-25 (Figs. 1–3) demonstrated the specific actions of the endogenous SNAP-25 in inhibiting K_Dr currents.

Our biophysical analysis showed that SNAP-25 inhibited K_Dr activity in part by altering channel kinetics so that the channel assumes a reluctant state by slowing channel activation and enhancing channel slow (C-type) inactivation (Figs. 1–3). Biochemical analysis in Xenopus oocytes and in vitro binding studies revealed that the K1.1 channel interacts physically with SNAP-25 both via the membrane pore-forming Kvα subunit and the peripheral auxiliary Kvβ subunits (Figs. 5 and 6A). Both the functional and the physical interactions indicate that the cytosolic N-terminal fragment of Kvα, particularly the αT1A domain, acutely reverses the inhibitory effects of SNAP-25 on K_Dr currents (Fig. 6B). The crystal structures of the Kvβ subunit and the T1 assembly domain of the N terminus of Kvα subunit (48, 49) reveals that submembrane modulating com-
plexes interact with the core domain of a channel responsible for the activation and C-type inactivation gating (50). Very recently it has been demonstrated that a cytosolic C-terminal complex conveys inhibition of HCN pacemaker channel gating (51). Cleavage of SNAP-25 by BoNT/A expression relieved inhibition of Kᵥ₁.1 by SNAP-25 by reversing the SNAP-25 effects on Kᵥ₁.1 kinetics. This indicates endogenous SNAP-25 has a clamping effect on Kᵥ₁.1.

Even though SNAP-25 can interact with the Kᵥ₁.1 channel directly, it is likely that the molecular complex also involves other proteins. In fact, we recently report direct interactions of syntaxin 1A with Kᵥ₁.1 channel enhancing its rapid inactivation (21). In that report, we showed that in brain synaptosomes, the Kᵥ₁.1 and Kᵥ₄ subunits exist in a macromolecular complex also comprising syntaxin 1A, SNAP-25, and synaptotagmin. Such an interaction between Kᵥ₁.1 and the exocytotic SNARE complex proteins is analogous to those described for the Ca²⁺ channels. For example, SNAP-25 inhibited P/Q-type Ca²⁺ channels, and this inhibition could be overcome by the formation of a SNARE complex of SNAP-25 with syntaxin 1A and synaptotagmin I (52). Similar modulation of N- and L-type Ca²⁺ channels, and this inhibition could be overcome by the formation of a SNARE complex of SNAP-25 with syntaxin 1A and synaptotagmin. Such an interaction between Kᵥ₁.1 and the exocytotic SNARE complex proteins is analogous to those described for the Ca²⁺ channels. For example, SNAP-25 inhibited P/Q-type Ca²⁺ channels, and this inhibition could be overcome by the formation of a SNARE complex of SNAP-25 with syntaxin 1A and synaptotagmin. Such an interaction between Kᵥ₁.1 and the exocytotic SNARE complex proteins is analogous to those described for the Ca²⁺ channels. For example, SNAP-25 inhibited P/Q-type Ca²⁺ channels, and this inhibition could be overcome by the formation of a SNARE complex of SNAP-25 with syntaxin 1A and synaptotagmin.