Cognate soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins are now known to associate with the secretory vesicle with both the target plasma membrane and Ca\(^{2+}\) channels in order to mediate the sequence of events leading to exocytosis in neurons and neuroendocrine cells. Neuroendocrine cells, particularly insulin-secreting islet \(\beta\)-cells, t-SNARE proteins, 25-kDa synaptosomal-associated protein (SNAP-25), and syntaxin 1A, independently inhibit the L-type Ca\(^{2+}\) channel (L_{Ca}). However, when both are present, they actually exhibit stimulatory actions on the L_{Ca}. This suggests that the positive regulation of the L_{Ca} is conferred by a multi-SNARE protein complex. We hypothesized an alternate explanation, which is that each of these SNARE proteins possess distinct inhibitory and stimulatory domains that act on the L_{Ca}. These SNARE proteins were recently shown to bind the Lc_{753–893} domain corresponding to the II and III intracellular loop of the α1C subunit of the L_{Ca}. In this study, using patch-clamp methods on primary pancreatic \(\beta\)-cells and insulinoma HIT-T15 cells, we examined the functional interactions of the botulinum neurotoxin A (BoNT/A) cleavage products of SNAP-25, including NH\(_2\)-terminal (1–197 amino acids) and COOH-terminal (amino acid 198–206) domains, on the L_{Ca}, particularly at the Lc_{753–893} domain. Intracellular application of SNAP-25_{1–206} in primary \(\beta\)-cells decreased L_{Ca} currents by ~15%. The reduction in L_{Ca} currents was counteracted by coapplication of Lc_{753–893}. Overexpression or injection of wild-type SNAP-25 in HIT cells reduced L_{Ca} currents by ~30%, and this inhibition was also blocked by the recombinant Lc_{753–893} peptide. Expression of BoNT/A surprisingly caused an even greater reduction of L_{Ca} currents (by 41%), suggesting that the BoNT/A cleavage products of SNAP-25 might possess distinct inhibitory and positive regulatory domains. Indeed, expression of SNAP-25_{1–197} increased L_{Ca} currents (by 19% at 10 mV), and these effects were blocked by the Lc_{753–893} peptide. In contrast, injection of SNAP-25_{198–206} peptide into untransfected cells inhibited L_{Ca} currents (by 47%), and more remarkably, these inhibitory effects dominated over the stimulatory effects of SNAP-25_{1–197} overexpression (by 34%). Therefore, the SNARE protein SNAP-25 possesses distinct inhibitory and stimulatory domains that act on the L_{Ca}. The COOH-terminal 197–206 domain of SNAP-25, whose inhibitory actions dominate over the opposing stimulatory NH\(_2\)-terminal domain, likely confers the inhibitory actions of SNAP-25 on the L_{Ca}. We postulate that the eventual accelerated proteolysis of SNAP-25 brought about by BoNT/A cleavage allows the relatively intact NH\(_2\)-terminal SNAP-25 domain to assert its stimulatory action on the L_{Ca}, to increase Ca\(^{2+}\) influx, and this could in part explain the observed weak or inconsistent inhibitory effects of BoNT/A on insulin secretion. The present study suggests that distinct domains within SNAP-25 modulate L_{Ca} subtype Ca\(^{2+}\) channel activity in both primary \(\beta\)-cells and insulinoma HIT-T15 cells. Diabetes 51:1425–1436, 2002

In pancreatic islet \(\beta\)-cells, insulin exocytosis involves an intimate and sequential relationship between the Ca\(^{2+}\) influx via plasma membrane Ca\(^{2+}\) channels and the exocytotic fusion machinery. However, dysregulation of Ca\(^{2+}\) influx across the Ca\(^{2+}\) channels has been postulated as a mechanism of islet \(\beta\)-cell death caused by an undefined serum factor in patients with type 1 diabetes (1) as well as during treatment with sulfonylureas (2) and exposure to cytokines (3). It therefore is important to identify the precise molecules and their domains that bind and regulate the islet \(\beta\)-cell Ca\(^{2+}\) channels.

Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins that mediate fusion of these synaptic and hormone-containing vesicles are also capable of directly binding and modulating the Ca\(^{2+}\) channels (4–6). In the pancreatic islet \(\beta\)-cells, an excellent neuroendocrine cell model for study of the SNAP/CA\(^{2+}\) channel interactions, the L-type Ca\(^{2+}\) channel (I_{CaL}) and Ca\(^{2+}\) influx, was found to be colocalized to the sites of insulin granule exocytosis (7). In this regard, Wiser et al. (8) reported that the t-SNAREs syntaxin 1A and 25-kDa synaptosomal-associated protein (SNAP-25); and the v-SNARE synaptotagmin bind the cytoplasmic domain (Lc_{753–893}) of the II–III L-loop of the α1C subunit of L-type C-class, which is common to the neuronal N-type Ca\(^{2+}\) channel. They further showed that injection of this Lc_{753–893} peptide disrupted depolarization-evoked insulin secretion but not I_{CaL} activity per se; and that syntaxin 1A inhibition of I_{CaL} activity could be reversed by coexpression of synaptotagmin. These functional results led these...
workers to postulate that in the basal state, syntaxin 1A binds and inhibits the LCa and that during stimulation, synaptotagmin associated with the approaching insulin granule binds syntaxin 1A, forming a complex of the vesicle with the LCa termed an excososome (8). With this binding, synaptotagmin would then prevent syntaxin 1A inhibition of the LCa thereby allowing Ca\(^{2+}\) entry to occur at the site of exocytosis (8).

Despite progress in examining the structure/function of SNARE proteins in membrane fusion (9), the precise molecular interactions between these SNARE proteins and Ca\(^{2+}\) channels are only now becoming clearer, particularly through the use of clostridial neurotoxins that specifically cleave SNARE proteins (10). Early studies have shown that tetanus toxin, which cleaves VAMP and botulinum toxins A and C1 (which cleave SNAP-25 and syntaxin 1A), inhibit neurotransmitter and insulin release (11–14), but the inhibition by botulinum neurotoxin A (BoNT/A) could be reversed by an increasing extracellular Ca\(^{2+}\) concentration (13,15). Intuitively, the increased Ca\(^{2+}\) influx observed in BoNT/A-treated chromaffin cells raises the possibility that cleavage products of the substrate by BoNT/A might directly affect Ca\(^{2+}\) influx, perhaps by actions on the Ca\(^{2+}\) channels.

In this work, we have demonstrated the modulation of LCa subtype Ca\(^{2+}\) channel activity by full-length SNAP-25 in primary pancreatic β-cells and then used the neuroendocrine model, insulinoma HIT-T15 cells, to further examine the effects of BoNT/A cleavage products of SNAP-25, including NH2-terminal 1–197 amino acid and COOH-terminal amino acid 198–206, on the LCa. HIT cells reliably and uniformly take up multiple plasmids, including one containing the green fluorescence proteins (GFPs) that identify the transfected cells for patch-clamp studies (16). This capability has allowed us to identify the cells expressing the NH2-terminal truncated SNAP-251–197 protein with or without injection of the SNAP-25198–206 peptide. Surprisingly, we found that NH2-terminal SNAP-251–197 possesses a positive regulatory action on the LCa, explaining the previously observed increased Ca\(^{2+}\) influx in BoNT/A-treated cells. The stimulatory action of SNAP-251–197 on the LCa opposes its inhibitory effects on exocytotic fusion (15). In contrast, the COOH-terminal SNAP-25198–206 potently inhibited LCa activity and overcame the stimulatory effects of the SNAP-251–197 domain. This COOH-terminal region therefore confers the inhibitory effect of full-length SNAP-25 on the LCa. Blockade of SNAP-251–197 effects on the LCa by the LCa753–893 domain of the channel further indicates the specific interactions of these protein domains.

**RESULTS**

**Effect of GST SNAP-251–206 on voltage-gated Ca\(^{2+}\) currents in primary pancreatic β-cells.** To test for possible modulation of voltage-gated Ca\(^{2+}\) currents by SNAP-25, pancreatic β-cells were dialyzed with GST (10–9 mol/l, control), GST SNAP-251–206 (10–9 mol/l), LCa753–893 (10–6 mol/l) alone, or LCa753–893 (10–6 mol/l) together with GST SNAP-251–206 (10–6 mol/l) through the recording pipette. Whole-cell voltage-gated Ca\(^{2+}\) currents from cells subjected to different treatments were evoked by step-depolarizing voltage pulses from a holding potential of

**RESEARCH DESIGN AND METHODS**

**Primary pancreatic β-cell culture.** Islets of Langerhans were isolated from mice and dispersed into single cells that were then grown in RPMI-1640 medium (Gibco, Middlesex, UK).

**Cell culture and transfections.** HIT-T15 cells were grown at 37°C in 5% CO\(_2/95\)% air in RPMI-1640 medium supplemented with 20 mmol/l glucose, 10% FCS (Gibco, Gaithersburg, MD), penicillin (100 units/ml), and streptomycin (100 mg/ml). When the cells were confluent, they were passaged and transiently transfected with 2 mg empty plasmid vector (control) or plasmid DNA encoding for SNAP-251–206, BoNT/A light chain, or SNAP-251–197 (16). The transfection of plasmid DNA was facilitated using FBS-1 lipid (Invitrogen, Carlsbad, CA) at a ratio of 1:6 (wt/wt). After a 4-h incubation at 37°C in 5% CO\(_2/95\)% air, normal RPMI-1640 medium was replaced. Transfection efficiency was determined by using pCNA/His/Lac Z (Invitrogen) as a control plasmid for transfections, followed by determination of β-galactosidase expression. In a previous study (16), HIT-T15 cells were found to uniformly intake the plasmids. Therefore, successfully transfected cells were confirmed by coexpression and visualization of the GFPs (Clontech, Palo, CA).

**Whole-cell patch-clamp studies.** Whole-cell Ca\(^{2+}\) currents were recorded in pancreatic β-cells from adult male and female mice. Pipettes were pulled from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) on a horizontal programmable puller (DMZ Universal Puller; Zeitz-Instrumente, Augsburg, Germany). Typical electrode resistance was 3–5 MΩ. Electrodes were filled with an internal solution containing glutathione-S-transferase (GST) (10–7 mol/l), GST SNAP-251–206 (10–7 mol/l), LCa753–893 (10–7 mol/l), or LCa753–893 (10–7 mol/l) combined with GST SNAP-251–206 (10–7 mol/l). The standard internal solution contained the following (in mmol/l): 150.0 N-methyl-D-glucamine, 1.0 MgCl\(_2\), 2.0 CaCl\(_2\), 5.0 HEPES, and 3.0 Mg ATP (pH 7.2 adjusted with HCl). The cells were bathed in a solution containing the following (in mmol/l): 138.0 NaCl, 5.6 KCl, 1.2 MgCl\(_2\), 10.0 CaCl\(_2\), 5.0 HEPES, and 5.0 HEPES (pH 7.2 (10)). After obtaining a seal, the holding potential was set at −70 mV during the experiment, and depolarizing voltage pulses (70 mV, 100 ms, 0.05 Hz) were applied. The resulting currents were recorded with an Axopatch 200 amplifier (Axon Instruments, Foster City, CA). All recordings were made at room temperature (22°C). The amplitude of whole-cell Ca\(^{2+}\) currents was normalized by the cell capacitance. Acquisition and analysis of data were done using the software program PCCLAMP (Axon Instruments).

On the third day after the transient transfection, the HIT-T15 cells were trypsinized and dispersed into single cells for patch-clamp study. Drops of medium containing these cells were placed in a 1-ml chamber mounted on the stage of an inverted phase-contrast microscope and allowed to stick to the bottom for 10 min. The RPMI-1640 medium was replaced by external solution composed of (in mmol/l): 138.0 NaCl, 5.6 KCl, 1.2 MgCl\(_2\), 1.0 CaCl\(_2\), 5.0 HEPES, and 5.0 HEPES (pH 7.4). After obtaining a seal, the holding potential was set at −70 mV during the experiment, and depolarizing voltage pulses (70 mV, 100 ms, 0.05 Hz) were applied. The resulting currents were recorded with an Axopatch 200 amplifier (Axon Instruments, Foster City, CA). All recordings were made at room temperature (22°C). The amplitude of whole-cell Ca\(^{2+}\) currents was normalized by the cell capacitance. Acquisition and analysis of data were done using the software program PCCLAMP (Axon Instruments).
−70 to 0 mV at 0.05 Hz. As shown in Fig. 1, whole-cell Ca\(^{2+}\) currents recorded from cells treated with GST SNAP-251–206 gradually and markedly decreased during recording in comparison with those from control or Lc753–893-treated cells. Interestingly, the gradual reduction in whole-cell Ca\(^{2+}\) currents induced by the intracellular application of GST SNAP-251–206 was counteracted by coapplication of Lc753–893. Furthermore, there was no significant difference in Ca\(^{2+}\) current density between control cells and cells treated with Lc753–893 (10\(^{-6}\) mol/l) alone or Lc753–893 (10\(^{-6}\) mol/l) together with GST SNAP-251–206.

Identification of L\(_{Ca}\) currents in HIT-T15 cells. To record L\(_{Ca}\) currents efficiently, Cs\(^{+}\) was included in the pipette solution, and Ba\(^{2+}\) and TEA were added to the bath solution to block voltage-gated outward K\(^{+}\) currents. Ba\(^{2+}\) also increased the L\(_{Ca}\) conductance. Among 140 cells (15.6 ± 0.1 pF) tested, 119 displayed inward currents in response to depolarizing pulses from a holding potential of −70 mV (Fig. 2). The peak current-voltage relationship from a control cell shows that the inward current appeared at a high voltage of 30 mV, reached the maximum at 10 mV, and reversed at 50 mV (Fig. 2).

**FIG. 1.** Effect of GST-SNAP-251–206 on voltage-gated Ca\(^{2+}\) currents in the pancreatic β-cell. A: Cells dialyzed with GST as control, Lc753–893 alone, or Lc753–893 together with GST SNAP-251–206 display slight run-down in whole-cell Ca\(^{2+}\) currents evoked by step-depolarizing voltage pulses from a holding potential of −70 to 0 mV. Cells dialyzed with GST SNAP-251–206 show a markedly gradual decrease in whole-cell Ca\(^{2+}\) currents with recording time. B: Compiled data illustrate that the intracellular application of GST SNAP-251–206 (n = 10) significantly inhibited whole-cell Ca\(^{2+}\) currents with recording time, as compared with the intracellular application of GST (control, n = 9), Lc753–893 (n = 10) alone, or Lc753–893 together with GST SNAP-251–206 (n = 10). Data are means ± SE. Statistical significance was evaluated by one-way ANOVA followed by least significant difference test. *P < 0.05 and **P < 0.01 vs. controls; *P < 0.05 and ***P < 0.01 vs. Lc753–893; +P < 0.05 vs. Lc753–893 plus GST SNAP-251–206.
relationship to the left by 10 mV (Fig. 2B). In the presence of nifedipine (10^-6 mol/l), the peak inward current amplitude was reduced to 13.7 ± 2.0% of control (n = 6). Thus, the predominant inward current (>85%) in HIT-T15 cells results from the activation of L_Ca.

**Inhibitory effect of the full-length SNAP-25 protein on L_Ca currents in HIT-T15 cells.** To examine the effect of wild-type SNAP-25 protein on L_Ca currents, we introduced the full-length recombinant GST SNAP-25 protein into HIT-T15 cells via the patch pipette. This exogenously applied recombinant SNAP-25 also allows us to examine the time course of action of SNAP-25 on L_Ca. It has been previously shown that a protein with a molecular weight of 50 kDa can diffuse through a pipette (access resistance of 10 MΩ) into the cytosol with a time constant of 5 min (20).

As shown in the lower panel of Fig. 3A, the inward current began to decrease from 2 min after membrane rupture in a HIT-T15 cell treated with 10^-9 mol/l GST SNAP-25, whereas such a decline was not seen with 10^-9 mol/l GST alone in the patch pipette (Fig. 3A, upper panel). The inhibition of the inward current became significant around 4 min. At 5 min, the peak inward current amplitude in GST SNAP-25–treated cells (n = 8) was reduced by 32% compared with that in GST-treated cells (n = 8, P < 0.05) (Fig. 3B). In a preliminary study, we found that GST alone (10^-9 mol/l, n = 4) did not alter the time course of the inward current seen in control HIT-T15 cells (n = 4). Thus, cells treated with GST were used as vehicle control cells. This result indicates that SNAP-25 protein can inhibit L_Ca currents in HIT-T15 cells, and this is consistent with the previous finding that coexpression of SNAP-25 and L_Ca suppressed the Ca^{2+} currents (4). We then examined whether the inhibitory effect caused by SNAP-25 was related to its direct interaction with the cytoplasmic domain separating repeats II and III I-loop of the α1C subunit of the L_Ca (8) by adding both synthetic II–III L-loop (1 μmol/l Lc753–893, a generous gift from Dr. D. Atlas, Jerusalem, Israel) and GST SNAP-25 (10^-9 mol/l) to the pipette solution. As shown in Fig. 3C, SNAP-25 failed to suppress the inward current in the concomitant presence of Lc753–893. Lc753–893 itself had no significant effect on the inward current (Fig. 3C), consistent with the previous report (8). Our results indicate that Lc753–893 blocks the binding site at SNAP-25 for the L_Ca and subsequently prevents the SNAP-25–induced inhibition. This was surprising for us because we previously thought that Lc753–893 would merely extend the distance between Ca^{2+} entry through L_Ca and the Ca^{2+} sensing site on the insulin granule, thereby disrupting exocytosis, but would not directly affect L_Ca activity (8). These results suggest that the interaction between SNAP-25 and the II–III I-loop of L_Ca is necessary for SNAP-25 to negatively modify L_Ca activity in HIT-T15 cells.

**Domains within SNAP-25 protein have distinct effects on L_Ca activities.** We then explored which domain within SNAP-25 protein is responsible for its modulation of L_Ca activities. Acute treatment with BoNT/A cleaves SNAP-25 at Gln197–Arg208 to generate a membrane-bound
NH₂-terminal SNAP-25₁₋₁⁹₇ fragment and a cytosolic COOH-terminal consisting of nine amino acids, SNAP-25₁⁹₈₋₂⁰₆ (11,21). These cleavage products could compete with each other and with the remaining intact SNAP-25, making it difficult to distinguish their independent actions. We therefore used the strategy of overexpressing SNAP-25 proteins and BoNT/A light chain. The overexpressed SNAP-25 proteins will be targeted to the plasma mem-

**FIG. 3.** Inhibitory effect of GST SNAP-25₁₋₂⁰₆ on I_{ca} currents in HIT-T15 cells. A: Representative inward currents obtained at various times after breakthrough from two HIT-T15 cells dialyzed with 10⁻⁹ mol/l GST (control) and 10⁻⁹ mol/l GST SNAP-25₁₋₂⁰₆, respectively. Inward currents were elicited by depolarization to 10 mV for 300 ms from a holding potential of −70 mV. B: Peak inward currents with time from HIT-T15 cells treated with 10⁻³ mol/l GST (control, ○; n = 8) and 10⁻⁹ mol/l GST SNAP-25₁₋₂⁰₆ (●, n = 8). Each point is means ± SE. *P < 0.05 against corresponding control data using Student’s t test. C: Lc₇₅₃₋₈₉₃ (1 μmol/l) reversed the inhibitory effect of GST SNAP-25₁₋₂⁰₆. Peak inward currents from HIT-T15 cells treated with 10⁻³ mol/l GST (control, ○; n = 6), 10⁻⁹ mol/l GST SNAP-25₁₋₂⁰₆ (●, n = 6), 1 μmol/l Lc₇₅₃₋₈₉₃ plus 10⁻⁹ mol/l GST SNAP-25₁₋₂⁰₆ (●, n = 6), and 1 μmol/l Lc₇₅₃₋₈₉₃ (▲, n = 6). Each point represents the means ± SE. *P < 0.05 against corresponding control data using ANOVA. Peak inward currents from each cell were normalized by cell membrane capacitance to avoid cell size variation. Dotted lines are the zero current level.
brane to effectively compete with endogenous SNAP-25, and the overexpressed BoNT/A will have access to all cellular SNAP-25 proteins, including those in complexes that would have cycled into uncomplexed forms. Furthermore, sufficient time for cytosolic proteolysis would deplete the cleavage products.

We first compared the effects of the overexpression of wild-type SNAP-25 (SNAP-251–206) and the mutant NH2-terminal SNAP-251–197. Mutant SNAP-251–197, like SNAP-251–206, is capable of binding membrane syntaxin 1a (22,23), and the regions within this portion of the truncated SNAP-25 protein have been shown to directly bind to neuronal N-type Ca2+ (24) and neuroendocrine LCa (8).

Our previous report demonstrated that transfection with wild-type SNAP-25 and mutant SNAP-251–197 resulted in targeting of these proteins specifically to the plasma membrane and that these SNAP-25 protein–transfected cells were identified by the coexpressed GFP (16). In a control study, the peak inward current-voltage relationship in HIT-T15 cells (n = 4) cotransfected with empty vectors and vectors with cDNA encoding for GFP did not show significant difference from that of untransfected cells (n = 4, data not shown). Therefore, those cells cotransfected with empty vectors and GFP vectors were used as control cells in all sets of experiments involving various transfectants, if not indicated otherwise. Figure 4A displayed the current traces evoked by voltage steps from −60 to 50 mV for 300 ms from a holding potential of −70 mV. Dotted lines are the zero current level. B: Current-voltage curves obtained by plotting the peak inward current amplitudes against test pulse voltages. Data were normalized by cell membrane capacitance and summarized from HIT-T15 cells transfected with empty vector (control, ○; n = 16), SNAP-251–206 (●; n = 16), BoNT/A (∆; n = 17), and SNAP-251–197 (■; n = 12). Data are the means ± SE. *P < 0.05 and **P < 0.01 vs. control (ANOVA).

FIG. 4. Effects of different transfectants on LCa currents in HIT-T15 cells. A: Representative inward current traces from four HIT-T15 cells transfected with empty vector (control), SNAP-251–206, BoNT/A, and SNAP-251–197. Inward currents were evoked by depolarization from −60 to 50 mV for 300 ms from a holding potential of −70 mV. Dotted lines are the zero current level. B: Current-voltage curves obtained by plotting the peak inward current amplitudes against test pulse voltages. Data were normalized by cell membrane capacitance and summarized from HIT-T15 cells transfected with empty vector (control, ○; n = 16), SNAP-251–206 (●; n = 16), BoNT/A (∆; n = 17), and SNAP-251–197 (■; n = 12). Data are the means ± SE. *P < 0.05 and **P < 0.01 vs. control (ANOVA).
LC loop of the α1C subunit. We used another set of experiments in which 1 mmol/l Lc753–893 peptide was injected via the patch pipette into HIT-T15 cells already expressing mutant SNAP-251–197. The data were collected 5 min after the membrane breakthrough to allow Lc753–893 peptides to diffuse into the cell. As shown in Fig. 5A, injection of the Lc753–893 peptide blocked the stimulatory effect of SNAP-251–197 at potentials from −20 to 10 mV. For example, the inward current density at 10 mV was −8.7 ± 0.5 pA/pF in SNAP-251–197–transfected cells (n = 8, P < 0.05 vs. control) and reversed to −7.3 ± 0.5 pA/pF after injecting Lc753–893 into SNAP-251–197–transfected cells (n = 7), which was close to −7.0 ± 0.3 pA/pF in control cells (n = 8) (Fig. 5B). Taken together, these results indicate that SNAP-251–197 has an opposite effect on LCa currents, as compared with SNAP-251–206, and that its excitatory action is also associated with direct interaction with the LC loop of the LCa.

We then examined the effects of depletion of endogenous SNAP-25 protein by transfection with BoNT/A light chain. We predicted that because wild-type SNAP-25 inhibits LCa, its depletion by BoNT/A should relieve this inhibition. Surprisingly, Fig. 4A shows that transfection with BoNT/A paradoxically decreased the LCa currents to an even greater extent than wild-type SNAP-251–206. The current-voltage relationship demonstrated that the BoNT/A-induced inhibition was also voltage dependent, with statistical significance between 0 and 20 mV (Fig. 4B). At a membrane potential of 10 mV, BoNT/A inhibited the peak inward current amplitude by 41%, whereas wild-type SNAP-251–206 inhibited it by only 27% (Fig. 4B).

The greater inhibition by BoNT/A expression is likely due to absence of a tonic-positive regulatory effect of the NH2-terminal SNAP-251–197 domain on LCa currents. Wild-type SNAP-25–mediated inhibition could therefore not be ascribed to this product. COOH-terminal SNAP-251–197–transfected cells treated with Lc753–893 (1 mmol/l) (▲, n = 6). We began to record current traces 10 min after membrane rupture to allow Lc753–893 peptide to dialyze the cytosol sufficiently. Data are means ± SE and are normalized to cell membrane capacitance. *P < 0.05 and **P < 0.01 vs. control (ANOVA).

**FIG. 5.** Lc753–893 reversed the stimulatory effect of SNAP-251–197. A: Representative inward current traces elicited by 300-ms depolarization to step voltages, as indicated in a control cell, a SNAP-251–197–transfected cell, and a SNAP-251–197–transfected cell dialyzed with Lc753–893 (1 μmol/l). The holding potential was −70 mV. Dotted lines indicate the zero current level. B: Current-voltage curves were determined by plotting peak inward currents against step voltages in control cells (○, n = 6), SNAP-251–197–transfected cells (▲, n = 6), and SNAP-251–197–transfected cells treated with Lc753–893 (1 mmol/l) (●, n = 6). We began to record current traces 10 min after membrane rupture to allow Lc753–893 peptide to dialyze the cytosol sufficiently. Data are means ± SE and are normalized to cell membrane capacitance. *P < 0.05 and **P < 0.01 vs. control (ANOVA).
and SNAP-25 1 affect LCa kinetics. Therefore, both voltage- and time-dependent activation and inactivation of the LCa were affected by different transfec
tions. Voltage-dependent activation curves were obtained by depolarization to 10 mV from a holding potential of −70 mV. Peak inward currents with time from control HIT-T15 cells (○, n = 6), control cells dialyzed with 10−9 mol/l SNAP-25197, SNAP-25198-transfected cells (●, n = 6), and SNAP-251,197-transfected cells dialyzed with 10−9 mol/l SNAP-
251,198 (▲, n = 6). Each point is the means ± SE. Current values were normalized to cell membrane capacitance.

47% at 10 min, as compared with control cells (n = 6). Furthermore, introduction of synthetic SNAP-25198 (10−9 mol/l) into HIT-T cells transfected with SNAP-25197 also resulted in the reduction of the inward current, with a maximum inhibition of 34% occurring at 10 min after membrane rupture, as compared with control cells (Fig. 6). These data suggest that SNAP-25198 is likely the putative domain within SNAP-25 that inhibits LCa currents and exerts a dominant effect over the NH2-terminal SNAP-25197 domain.

**Effects of different transfections on voltage-dependent activation and inactivation of the LCa.** Because LCa current amplitudes were affected by different transfections, we further explored how these transfections could affect LCa kinetics. Therefore, both voltage- and time-dependent activation and inactivation were examined in the following studies. Voltage-dependent activation curves were obtained by plotting normalized peak tail currents against test pulses. In this set of experiments, only expression of the truncated SNAP-251,197 in HIT-T15 cells shifted the activation curve 10 mV toward the left (Fig. 7), indicating that the LCa is easier to activate at lower voltages in the presence of SNAP-251,197; consistent with its stimulatory effect on this channel (Fig. 4B). However, transfection with SNAP-251,206 or BoNT/A did not affect activation kinetics (Table 1). Parameter values for voltage-dependent activation kinetics are summarized in Table 1.

Voltage-dependent inactivation of the LCa was assessed using a standard two-step protocol in which long duration prepulses (15 s) of varying test potentials were followed by a short step to 10 mV. The steady-state inward current, after the 10-mV test pulse from each prepulse, was measured as a fraction of the maximal inward current. The voltage-dependent inactivation curve was obtained by plotting these fractional values against prepulse voltages and best-fitted by the Boltzmann equation (Fig. 7). No transfections significantly altered the inactivation curve.

Only control and SNAP-251,197 data for voltage-dependent inactivation are shown in Fig. 7. The values for voltage-dependent inactivation parameters are also summarized in Table 1.

**Effects of different transfectants on time-dependent activation and inactivation of the LCa.** Time constants during activation were obtained by fitting depolarization-evoked inward current traces with a mono-exponential function. Figure 8A shows that the inward current was activated faster in the cell transfected with SNAP-251,197 but almost unaffected in the cell transfected with SNAP-251,206 or BoNT/A. By calculation, the activation time constant was reduced from 2.8 ± 0.2 ms in control cells (n = 11) to 2.0 ± 0.1 ms in SNAP-251,197 (n = 11, P < 0.05), suggesting that SNAP-251,197 accelerates the activation rate of the LCa, again consistent with its stimulatory effect on this channel. The values for activation time constants were given in Table 2.

Time-dependent inactivation of the LCa was studied using a long (9 s) depolarizing pulse to 10 mV (Fig. 8B). Time constants (τ1 and τ2) of the inward current decay were calculated by fitting current traces with a biexponential equation. As shown in Table 2, SNAP-251,206 accelerated the inactivation rate of the LCa with τ1 and τ2 increased by 1.5- and 1.6-fold, respectively, which was in agreement with the previous study performed in Xenopus oocyte (4). The absolute values for τ1 were larger in our experiment. The reason for this was probably the replacement of the bath solution Ca2+ with Ba2+, which could slow down the inactivation rate of the LCa. Transfection with BoNT/A also increased the inactivation rate, but transfection with SNAP-251,197 significantly decelerated it.
TABLE 1
Alteration of voltage-dependent kinetics of the $L_{Ca}$

<table>
<thead>
<tr>
<th></th>
<th>Activation</th>
<th>Inactivation</th>
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<tbody>
<tr>
<td></td>
<td>$V_{1/2}$ (mV)</td>
<td>$k$ (mV)</td>
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<tr>
<td>Control</td>
<td>$-5.0 \pm 0.9$ (6)</td>
<td>$13.4 \pm 0.9$ (6)</td>
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<tr>
<td>SNAP-25$_{1-206}$</td>
<td>$-3.2 \pm 0.6$ (5)</td>
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<tr>
<td>BoNT/A</td>
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<td>$12.7 \pm 1.5$ (5)</td>
</tr>
<tr>
<td>SNAP-25$_{1-197}$</td>
<td>$-15.1 \pm 1.8$ (6)$^*$</td>
<td>$10.2 \pm 1.9$ (6)</td>
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</tbody>
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Data are means $\pm$ SE. The number of cells used is shown in parentheses. Definitions for $V_{1/2}$ and $k$ are given in the legend for Fig. 7. $^*P < 0.05$ against control.

Effects of different transfectants on $Ca^{2+}$ influx through the $L_{Ca}$. In pancreatic $\beta$-cells, $Ca^{2+}$ entry through $L$-type channels triggers the exocytosis of insulin granules (25). Therefore, modulation of $L_{Ca}$ channel activity by SNAP-25 proteins could consequently affect $Ca^{2+}$ influx and cytoplasmic-free $Ca^{2+}$ concentration ([Ca$^{2+}$]$_i$). To test this, we measured [Ca$^{2+}$]$_i$ in single HIT-T15 cells using Fura 2 as Ca$^{2+}$ indicator. The HIT-T15 cells studied were transfected with the empty vector (control), SNAP-25$_{1-206}$, BoNT/A, or SNAP-25$_{1-197}$. Transfection was again determined by coexpression of GFP in each experiment. HIT-T15 cells were placed in a 1-ml chamber with Ca$^{2+}$-containing physiological solution (see RESEARCH DESIGN AND METHODS). After the basal [Ca$^{2+}$]$_i$ level was recorded, the cells were treated with 10 mmol/l KCl, which depolarizes the cell membrane and subsequently activates $L_{Ca}$. The difference between the peak transient rise in [Ca$^{2+}$]$_i$ and the basal [Ca$^{2+}$]$_i$ was considered as net Ca$^{2+}$ influx through $L_{Ca}$. To minimize the possible inter- and intra-assay variations, [Ca$^{2+}$]$_i$ values from cells (usually 3–4) with efficient transfection in each field were averaged, and only one field was counted from each chamber. Figure 9A shows that expression of SNAP-25$_{1-206}$ and BoNT/A in HIT-T15 cells decreased the peak transient rise in [Ca$^{2+}$]$_i$, whereas SNAP-25$_{1-197}$ had an opposite effect. Data for the peak transient rise in [Ca$^{2+}$]$_i$ were averaged in Fig. 9B. In control cells, the peak transient rise in [Ca$^{2+}$]$_i$ was 199.1 $\pm$ 10.4 nmol/l ($n = 6$). SNAP-25$_{1-206}$ and BoNT/A reduced it to 150.6 $\pm$ 5.4 nmol/l ($n = 6$, $P < 0.05$) and 134.1 $\pm$ 7.8 nmol/l ($n = 6$, $P < 0.05$), respectively, whereas SNAP-25$_{1-197}$ raised it to 248.1 $\pm$ 18.1 nmol/l ($n = 6$, $P < 0.05$). These data, therefore, are in agreement with our $L_{Ca}$ current recordings (Fig. 4) and provide further evidence that domains within SNAP-25 protein have distinct effects on the $L_{Ca}$.

DISCUSSION
In this study, we demonstrate for the first time that SNAP-25 specifically modulates $L_{Ca}$ subtype $Ca^{2+}$ channel activity in insulin-secreting cells. This indicates that SNAP-25 not only functions as a key component in a core complex to trigger insulin exocytosis but also interacts with the voltage-gated $L_{Ca}$ to control $Ca^{2+}$ influx, which plays an important role in the formation of the core complex (4,8). The degree of inhibition of full-length SNAP-25 on whole-cell $Ca^{2+}$ currents in the primary pancreatic $\beta$-cell was smaller than that observed in the HIT-T15 cell. This is probably due to the relative small contribution of the $L_{Ca}$ subtype $Ca^{2+}$ channel to the total L-type $Ca^{2+}$ currents in normal $\beta$-cells. The predominant
subtypes of the L_{Ca} in islet \(\beta\)-cells is the L_{D} subtype (26–28), whereas HIT-T15 cells predominantly contain the L_{C} subtype. These results also suggest that although the L_{C} and L_{D} subtypes of L_{Ca} share a 70% homology (29), the L_{C} subtype appears to be more sensitive to SNAP-25. In contrast, syntaxin 1A appears to similarly inhibit both the L_{C} and L_{D} subtypes of HIT-T15 L_{Ca} (4,8,28).

We also present the first evidence that domains within the SNAP-25 protein are capable of causing distinct changes in L_{Ca} kinetics. Wild-type SNAP-25 reduced the inward current amplitude and accelerated its inactivation rate, indicating its inhibitory regulatory role on the L_{Ca}, which is consistent with the previous report (4). It is likely that SNAP-25_{1-206} acts on the inactivation state of the channel and results in its faster decay. However, BoNT/A expression, which depleted all cellular SNAP-25 proteins, resulted in an even greater inhibition of L_{Ca} currents. Specifically, BoNT/A expression decreased the current

### TABLE 2
Alteration of time-dependent kinetics of the L_{Ca}

<table>
<thead>
<tr>
<th></th>
<th>Activation</th>
<th>Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\tau_1) (ms)</td>
<td>(\tau_2) (ms)</td>
</tr>
<tr>
<td>Control</td>
<td>2.8 ± 0.2 (11)</td>
<td>6,096.0 ± 505.2 (6)</td>
</tr>
<tr>
<td>SNAP-25_{1-206}</td>
<td>2.9 ± 0.3 (11)</td>
<td>4,127.6 ± 280.2 (6)*</td>
</tr>
<tr>
<td>BoNT/A</td>
<td>2.6 ± 0.4 (11)</td>
<td>4,245.7 ± 273.9 (6)*</td>
</tr>
<tr>
<td>SNAP-25_{1-197}</td>
<td>2.0 ± 0.1 (11)*</td>
<td>9,886.6 ± 1,028.2 (6)†</td>
</tr>
</tbody>
</table>

Data are means ± SE. The number of cells used is given in parentheses. *\(P < 0.05\) and †\(P < 0.01\) vs. control.

FIG. 9. Effects of different transfectants on \([Ca^{2+}]_i\) in HIT-T15 cells. A: Transient rise in \([Ca^{2+}]_i\), in response to 10 mmol/l KCl from HIT-T15 cells transfected with empty vector, SNAP-25_{1-206}, BoNT/A, and SNAP-25_{1-197}. B: Peak transient rise in \([Ca^{2+}]_i\), summarized from control (n = 6), SNAP-25_{1-206}-transfected (n = 6), BoNT/A-transfected (n = 6), and SNAP-25_{1-197}-transfected (n = 6) HIT-T15 cells, respectively. Each bar is the means ± SE. *\(P < 0.05\) against control (ANOVA); ††\(P < 0.01\).
amplitude, accelerated the inactivation rate, and reduced the Ca$^{2+}$ influx. BoNT/A expression has the advantage over the acute application of BoNT/A. The latter could acutely generate cleavage products that compete with each other and with the remaining intact SNAP-25, therefore preventing assessment of the independent effect of the functional domains within SNAP-25. The greater inhibition observed with BoNT/A expression suggests that SNAP-25 contains not only negative but also positive regulatory domains. Indeed, we found the COOH-terminal SNAP-25 contains not only negative but also positive functional domains within SNAP-25. The greater inhibition by overexpression of SNAP-25 and BoNT/A, whereas the membrane-bound NH$_2$-terminal SNAP-25$_{1-197}$ conferred a paradoxical stimulatory effect on the L$_{\text{Ca}}$. The fact that COOH-terminal SNAP-25$_{198-206}$ peptide to confer the inhibitory effect of SNAP-25 and of BoNT/A, whereas the membrane-bound NH$_2$-terminal SNAP-25$_{1-197}$ conferred a paradoxical stimulatory effect on the L$_{\text{Ca}}$. The fact that COOH-terminal SNAP-25$_{198-206}$ peptide is able to override the positive regulatory effect of SNAP-25$_{1-197}$ indicates that the negative regulatory role of SNAP-25 on the L$_{\text{Ca}}$ probably predominates in the islet β-cell. Nonetheless, this dominant-negative regulatory effect of SNAP-25 is balanced by the positive regulatory domain, as evidenced by its complete absence effected by BoNT/A transfection, which caused an even greater inhibition of the Ca$^{2+}$ channel than the SNAP-25 overexpression. It therefore seems likely that both negative and positive regulatory domains of the SNAP-25 protein are operative in modulating the Ca$^{2+}$ channel in the islet β-cell.

SNAP-25$_{1-197}$ increased the current amplitude (Fig. 4), activated L$_{\text{Ca}}$ at lower membrane potentials (Fig. 7), and decelerated its inactivation rate (Fig. 8B). These results indicate that SNAP-25$_{1-197}$ may interact with more than one site at the L$_{\text{Ca}}$. Injecting the Lc$_{753-893}$ peptide into HIT cells transfected with SNAP-25$_{1-197}$ or wild-type SNAP-25$_{1-206}$ restored the current-voltage curve to control levels, indicating that both SNAP-25 proteins modulated the L$_{\text{Ca}}$ by direct interaction with the channel. This is further supported by the previous study showing a direct binding between SNAP-25 and L$_{\text{Ca}}$ (8). Nonetheless, wild-type SNAP-25 and mutant SNAP-25$_{1-197}$ must be bound to the II-III L-loop of L$_{\text{Ca}}$ in distinct conformations to assert these apparent opposite effects on L$_{\text{Ca}}$ and the resulting Ca$^{2+}$ influx (Fig. 9). In our previous report, where data collected were from a field of both transfected and untransfected cells (transfection efficiency ~45%), we did not observe an obvious difference in KCl-evoked Ca$^{2+}$ influx between SNAP-25$_{1-197}$- and wild-type SNAP-25$_{1-206}$-transfected cells (16). However, in the current study, data were collected from cells with the highest protein expression, as determined by GFP coexpression, therefore permitting the effects to be seen. Despite its positive modification of the L$_{\text{Ca}}$ overexpression of SNAP-25$_{1-197}$ caused a net inhibition of insulin secretion in HIT-T15 cells (16), likely due to the formation of nonfunctional exocytotic SNARE complexes (22–23).

Injection of the COOH-terminal SNAP-25$_{108-206}$ inhibited L$_{\text{Ca}}$ currents to a greater extent than wild-type SNAP-25, and interestingly, these inhibitory effects dominated over the stimulatory effects of SNAP-25$_{1-197}$ expression. The greater inhibition by overexpression of BoNT/A over the wild-type SNAP-25 further supports our hypothesis that these two domains of SNAP-25, NH$_2$-terminal SNAP-25$_{1-197}$, and COOH-terminal SNAP-25$_{108-206}$ exert a “yin yang” effect on the L$_{\text{Ca}}$. This might in part explain why the presence of higher levels of extracellular Ca$^{2+}$ or higher membrane depolarization could abrogate the inhibitory effects of the acutely applied BoNT/A (13,15), wherein the COOH-terminal SNAP-25$_{198-206}$, which initially blocks the L$_{\text{Ca}}$ binds insulin granule VAMP-2 to form a nonfunctioning fusion complex (16). However, its subsequent accelerated proteolysis in the cytosol would leave the relatively intact membrane-bound SNAP-25$_{1-197}$ unopposed to act on the L$_{\text{Ca}}$ to increase Ca$^{2+}$ influx. Whereas SNAP-25$_{1-197}$ also forms a nonfunctional docking complex (16), the increased Ca$^{2+}$ influx might overcome this inhibition. In support, Coorssen et al. (30) demonstrated that increased Ca$^{2+}$ levels could effect exocytosis independent of the SNARE complex and that the latter may serve to modulate Ca$^{2+}$ sensitivity in driving fusion. Furthermore, in contrast to a neuron that has a higher proportion of secretory granules in a readily releasable pool of docked vesicles (~10%), <4% of insulin secretion granules in β-cells are in this pool (25,31). The vast majority of insulin granules are located more distantly from the membrane, within a reserve pool. The initial phase of [Ca$^{2+}$]$_i$ rise is important for exocytosis of the readily releasable pool (32,33), whereas the subsequent sustained elevation of [Ca$^{2+}$]$_i$ acts to prime and mobilize the reserve pool to the readily releasable pool (32,33). The SNAP-25$_{1-197}$ stimulatory effects on the L$_{\text{Ca}}$ may compensate for the reduced insulin exocytosis caused by the nonfunctional docking complex by mobilizing more secretion granules from the reserve pool to the readily releasable pool.

Taken together, we have now provided evidence that domains within SNAP-25 protein have distinct effects on the L$_{\text{Ca}}$. These results have the potential to elucidate how SNARE proteins may not only regulate physiological insulin exocytosis but also be of pathophysiological significance in the dysregulation of Ca$^{2+}$ influx-mediated islet β-cell injury in diabetes (1–3). In fact, SNARE protein expression levels were diminished in islets of type 2 diabetic models, including Zucker fa/fa and GK rats (34,35), which exhibit abnormal regulation of Ca$^{2+}$ influx and insulin exocytosis (36). Reconstitution of these SNARE proteins, particularly SNAP-25 and syntaxin, partially restored insulin secretion to normal levels (35), likely by restoring the diabetic dysregulation of the distal components of the insulin exocytotic machinery, in particular the L$_{\text{C}}$ excitosome. Finally, the positive regulatory domains within these SNARE proteins, particularly within the NH$_2$-terminal SNAP-25$_{1-197}$ protein, could serve as targets for novel drug design to treat diabetes.

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