Insulin release by pancreatic β-cells is regulated by diverse intracellular signals, including changes in Ca\(^{2+}\) concentration resulting from Ca\(^{2+}\) entry through voltage-gated (Ca\(_V\)) channels. It has been reported that the Rab3 effector RIM1 acts as a functional link between neuronal Ca\(_V\) channels and the machinery for exocytosis. Here, we investigated whether RIM1 regulates recombinant and native L-type Ca\(_V\) channels (that play a key role in hormone secretion) and whether this regulation affects insulin release. Whole-cell patch clamp currents were recorded from HEK-293 and insulinoma RIN-m5F cells. RIM1 and Ca\(_V\) channel expression was identified by RT-PCR and Western blot. RIM1-Ca\(_V\) channel interaction was determined by co-immunoprecipitation. Knockdown of endogenous RIM1 and Ca\(_V\) channel subunit expression were performed using small interference RNAs. Insulin release was assessed by ELISA. Co-expression of Ca\(_V\)1.2 and Ca\(_V\)1.3 L-type channels with RIM1 in HEK-293 cells revealed that RIM1 may not determine the availability of L-type Ca\(_V\) channels but decreases the rate of inactivation of the whole cell currents. Co-immunoprecipitation experiments showed association of the Ca\(_V\)_β auxiliary subunit with RIM1. The lack of Ca\(_V\)_β expression suppressed channel regulation by RIM1. Similar to the heterologous system, an increase of current inactivation was observed upon knockdown of endogenous RIM1. Co-immunoprecipitation showed association of Ca\(_V\)_β and RIM1 in insulin-secreting RIN-m5F cells. Knockdown of RIM1 notably impaired high K\(^+\)-stimulated insulin secretion in the RIN-m5F cells. These data unveil a novel functional coupling between RIM1 and the L-type Ca\(_V\) channels via the Ca\(_V\)_β auxiliary subunit that contribute to determine insulin secretion.

Release of insulin-containing vesicles by pancreatic β-cells is regulated by various intracellular signals, including Ca\(^{2+}\). Physiologically, glucose stimulation increases the [ATP]/[ADP] intracellular ratio that closes ATP-sensitive potassium (K\(_{ATP}\)) channels, thereby depolarizing β-cell plasma membrane. This process in turn activates plasma membrane voltage-gated (Ca\(_V\)) channels, allowing Ca\(^{2+}\) to enter the cell and trigger insulin exocytosis (1, 2).

Ca\(_V\) channels are classified according to their activation threshold as low voltage-activated or high voltage-activated. Based on pharmacological profiles, high voltage-activated channels can be divided into L-type and non-L-type channels, the latter including the N, P/Q, and R subtypes (3, 4). Neurotransmitter release is attributed to Ca\(^{2+}\) influx through P/Q-type (Ca\(_V\)2.1) and N-type (Ca\(_V\)2.2) channels, whereas L-type (Ca\(_V\)1.2 and Ca\(_V\)1.3) channels are considered to be responsible for hormone secretion (3). At the molecular level, Ca\(_V\) channels are oligomeric complexes of at least three proteins or subunits, the pore-forming (Ca\(_V\)_α) subunit and the auxiliary Ca\(_V\)_α\(_{\text{δ}}\) and Ca\(_V\)_β subunits (3, 4).

Electrophysiological and molecular studies indicate that pancreatic β-cells express several subtypes of Ca\(_V\) channels. In particular, dihydropyridine-sensitive, L-type Ca\(_V\) channels are responsible for a significant portion of the high voltage-activated current (5, 6), and given that dihydropyridines potently suppress insulin secretion, L-channels are considered crucial for β-cell function (7). Of the four genes that encode Ca\(_V\)_α\(_{1}\) subunits of L-channels, either Ca\(_V\)_1.2 (formerly known as α\(_{1C}\)), Ca\(_V\)_1.3 (α\(_{1D}\)), or both have been identified in rodent and human islets as well as in various β-cell lines, including the rat insulinoma RIN-m5F cells (6, 8). Although the relative expression levels of the two genes and their importance for insulin secretion remain uncertain, immunoprecipitation experiments suggest that Ca\(_V\)_1.2 may represent ~50% of the L-type channels in this cell line (8).

In vivo and in vitro studies have shown that pancreatic islets respond to increases in extracellular glucose with a biphasic pattern of insulin release. The first phase lasts a few minutes and reflects the release of a pool of granules in close proximity to L-type channels (9, 10). Two mechanisms possibly contribute to the second phase of insulin secretion: the replenishment of the immediately releasable pool from the reserve pool and exocytosis of granules located far from Ca\(_V\) channels due to widespread increases in cytosolic Ca\(^{2+}\) during depolarization.
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The latter mechanism also involves non-L-type channels. Last, studies in mice lacking Ca\textsubscript{V}1.2 and Ca\textsubscript{V}1.3 channels have corroborated that L-type channels are crucial for β-cell physiology (11, 12).

Interestingly, it has been found that different members of the RIM family (13–16), putative effectors of Rab3, and some associated proteins (17) may functionally link Ca\textsubscript{V} channels to the machinery for exocytosis. Moreover, it has been reported that RIM1 modulates neuronal Ca\textsubscript{V}2.1 channels through its interaction with the Ca\textsubscript{V}\textbeta subunit, modifying the inactivation rate for a sustained Ca\textsuperscript{2+} influx and anchoring neurotransmitter-containing vesicles in the vicinity of the channels (16). In contrast to these findings, no evidence has been reported for an N-type (Ca\textsubscript{V}2.2) channel/RIM interaction at the presynaptic terminals using a chick calyx synapse preparation as well as in the heterologously expressed proteins in HEK293T cells (18, 19). These results argue against the hypothesis that RIM proteins may be critical for neuronal channel localization at the active zone. On the other hand, recent studies have shown also that RIM1 or RIM2 and RIM3 could indeed interact with native and recombinant mammalian N-type channels (20). Although the reason for this discrepancy is presently unknown, a model has emerged that could reconcile the conflicting results regarding the N- and P/Q-type channel/RIM interaction. In this model, RIM is part of a complex that tethers the synaptic vesicle to the channel, acting as a switch for a link between the channel and the synaptic vesicles that changes from high to low affinity states (19, 21).

In the present report, by using a strategy that combines patch clamp recordings with biochemical and molecular biology techniques, we provide evidence that RIM1 regulates recombinant L-type Ca\textsubscript{V} channels (of the Ca\textsubscript{V}1.2 and Ca\textsubscript{V}1.3 class) heterologously expressed in HEK-293 cells as well as native L-channels expressed in rat insulinoma RIN-m5F cells and also show that this regulation results in a facilitation of insulin secretion. These data stress the importance of RIM1 as a regulatory constituent of the insulin secretory machinery.

**Experimental Procedures**

**Cell Culture**—HEK-293 cells (ATCC) were grown in DMEM-high glucose medium supplemented with 10% horse serum, 2 mM l-glutamine, 110 mg/liter sodium pyruvate, 100 units/liter penicillin, and 100 μg/liter streptomycin. The rat insulin-producing RIN-m5F cells (ATCC) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine, 110 mg/liter sodium pyruvate, 100 units/liter penicillin, and 100 μg/liter streptomycin. Cell cultures were maintained at 37 °C in 5% CO\textsubscript{2}, 95% air humidified atmosphere.

**Recombinant Ca\textsubscript{V} Channel Expression and Electrophysiology**—After splitting HEK-293 cells on the previous day and seeding at 60% confluence, cells were transfected using the Lipofectamine Plus reagent (Invitrogen) with 1.6 μg of each plasmid cDNA encoding L-type channel pore-forming subunit Ca\textsubscript{V}1.2 (GenBankTM accession number X15539) or Ca\textsubscript{V}1.3 (AF370009) with Ca\textsubscript{V}\textbeta\textsubscript{2} (M80545) or Ca\textsubscript{V}\textbeta\textsubscript{3} (M88751), and Ca\textsubscript{V}\textalpha\textsubscript{2-δ} (M86621) in the presence or absence of RIM1 (NM_053270). For electrophysiology, 0.6 μg of a plasmid cDNA encoding the green fluorescent protein (Green-Lantern; Invitrogen) was added to the transfection mixture to identify and select transfected cells.

Electrophysiological recordings were performed according to the whole cell configuration of the patch clamp technique (22) at room temperature (22–24 °C) in a bathing solution containing 10 and 5 mM Ba\textsubscript{2+} (for Ca\textsubscript{V}1.2 and Ca\textsubscript{V}1.3, respectively), 125 mM TEA-Cl, 10 mM HEPES, and 10 mM glucose (pH 7.3). Patch pipettes were filled with a solution containing 120 mM CsCl, 10 mM HEPES, 10 mM EGTA, 5 mM MgCl\textsubscript{2}, 4 mM ATP, and 0.1 mM GTP (pH 7.3). Ba\textsuperscript{2+} was used as the charge carrier instead of Ca\textsuperscript{2+} for the following reasons: (i) conductance for Ba\textsuperscript{2+} versus Ca\textsuperscript{2+} ions through high voltage-activated Ca\textsubscript{V} channels is larger, thereby increasing the signal/noise ratio; (ii) it reinforces blockade of K\textsuperscript{+} currents; and (iii) rundown of the current is sometimes prominent, and the use of Ba\textsuperscript{2+} ameliorates this problem. More importantly, when experiments were performed with external solutions containing Ca\textsuperscript{2+}, cells exhibited a prominent Ca\textsuperscript{2+}-activated component, which difficult a clear evaluation of the action of RIM1 on L-type Ca\textsubscript{V} currents. This component was suppressed in external Ba\textsuperscript{2+} conditions. It is worth noting, however, that the effect of RIM1 on L-type currents is qualitatively similar in external Ba\textsuperscript{2+} and Ca\textsuperscript{2+} conditions (supplemental Fig. 1).

Recordings were made using an Axopatch 200B amplifier (Molecular Devices). Data acquisition and analysis were performed using pClamp10 software (Axon CNS) and Sigma Plot 11.0 (Systat Software Inc.) as described elsewhere (23, 24). Linear leak and parasitic capacitance components were subtracted on-line using a P/4 protocol. Membrane capacitance (C\textsubscript{m}) was determined as described previously (25) and used to normalize currents.

**Western Blots**—Cells were detached from culture dishes, washed with phosphate-buffered saline (PBS; pH 7.4), and lysed in single-detergent lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and Complete 1×; Roche Applied Science). Protein concentration was determined using the bicinchoninic acid assay. Samples with 50 μg of protein were boiled for 5 min within protein loading buffer (1.7% SDS, 0.1 M 2-mercaptoethanol, 5% glycerol, 58 mM Tris-Cl, 0.002% bromophenol blue, pH 6.8). Proteins were resolved in 8–10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After blocking with 5% nonfat dry milk in Tris-buffered saline Tween 20 (TBST; 100 mM Tris-Cl, 0.9% w/v NaCl, 0.2% Tween 20, pH 7.5), membranes were incubated overnight with primary antibodies anti-Ca\textsubscript{V}1.2α and anti-Ca\textsubscript{V}1.3α (Alomone Laboratories) used at a 1:200 dilution in blocking solution: anti-Ca\textsubscript{V}\textbeta\textsubscript{2} (1:1000) (26), anti-Ca\textsubscript{V}\textbeta\textsubscript{2} (1:500; Santa Cruz Biotechnology), or anti-Ca\textsubscript{V}\textbeta\textsubscript{3} (1:1000) (27), anti-RIM (1:1000; BD Bioscience). Membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibodies diluted in TBST with 5% nonfat dry milk and developed with Western Lightning PLUS ECL (PerkinElmer Life Sciences). For semiquantitative analysis, membranes were stripped and incubated with a mouse monoclonal anti-actin antibody (1:200 in TBST). Densitometry analysis was carried out using the ImageJ version 1.43 program (National Institutes of Health).
Co-immunoprecipitation—Aliquots of protein (1–1.5 mg) from RIN-m5F or HEK-293 cells (transfected with the CaV channels and RIM1) were incubated for 4 h at 4°C with 5 μg of anti-CaV β2, anti-CaV β3, or an irrelevant IgG0 (anti-Sp1 antibody, Santa Cruz Biotechnology, Inc.). Next, the complexes were incubated overnight with 20 μl of rProtein G-agarose (Invitrogen) at 4°C. The immunoadsorbents were recovered by centrifugation (5 min at 13,000 rpm) and washed three times by resuspension and centrifugation (5 min at 13,000 rpm) with wash buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS 0.5 mM PMSF) and two times with PBS. Samples were eluted into 30 μl of protein loading buffer. Immunoprecipitated proteins were subjected to Western blotting using the anti-RIM antibody. The specificity of the antibodies was assessed by Western blot (supplemental Fig. 2).

RT-PCR—Total RNA was extracted from RIN-m5F cells by TRIzol reagent (Invitrogen). Reverse transcription was done with 5 μg of total RNA using the SuperScript III first strand system for RT-PCR (Invitrogen). The sequences of forward and reverse primers used for RIM1 amplification were 5′-GGTCCAGTGTATCTCAGG-3′ and 5′-TTACTATGACCCGGATGACAGG-3′ (sense and antisense, respectively) (28). As a PCR control, β-actin was amplified using as a sense primer 5′-AAGATGACCCAGATCTGTG-3′ and antisense primer 5′-GAGTACTTGCGCTCAGGAGG-3′. The PCR was carried out in a total volume of 50 μl containing 5 μl of cDNA solution, 1× PCR buffer, 0.2 mM each deoxynucleotide triphosphate, 1.5 mM MgCl2, 0.5 μM each primer, and 2.5 units of Taq DNA polymerase on a Thermal Cycler (Thermo Scientific) for 25 cycles. Denaturation was carried out at 94°C for 45 s, annealing at 55°C for 30 s, and elongation at 75°C for 1 min. PCRs were performed using Taq DNA polymerase (Invitrogen) with a 0.5 μM concentration of each primer.

RNA Interference in RIN-m5F Cells—The siRNA sequences 5′-CUCAGAUUAGAGGUGAAG (dT) and 5′-AUCACAGCUCUAAUGUGAG (dT) for RIM1 were transfected in RIN-m5F or HEK-293 cells (transfected with the CaV1.2 channel). A scramble sequence was used as a control.

Insulin Secretion Assays—RIN-m5F cells were washed twice with PBS and preincubated with Krebs-Ringer buffer (KRB; 25 mM HEPES, 115 mM NaCl, 24 mM NaHCO3, 5 mM KCl, 1 mM MgCl2, 2.5 mM CaCl2, 0.1% BSA) for 20 min at 37°C in 5% CO2, 95% air humidified atmosphere. The preincubation buffer was removed and replaced with KRB buffer containing 40 mM KCl for 30 min at 37°C. Insulin secretion was assayed by the enzyme-linked immunosorbent assay (ELISA) using the rat insulin high range ELISA kit (Alpco) according to the manufacturer’s instructions.

Data Analyses—Statistical analyses were carried out using the SigmaPlot 11 software (Systat Software Inc.). The significance of observed differences was evaluated by Student’s unpaired t test. A probability less than 5% was considered to be significant. All experimental values are given as means ± S.E. Curve fitting was performed as reported previously (29).

RESULTS

First, to investigate the coupling of the Rab3-interacting molecule 1 (RIM1) to L-type CaV channels and the functional consequences of this interaction, we examined the effects of RIM1 on whole cell currents through recombinant L-channels using the HEK-293 cell line, a heterologous expression system that does not express endogenous CaV channels (25, 30) ( supplemental Fig. 3). Hence, CaV1.2α1 or CaV1.3α1 channels together with the CaV β (β2 or β3) and the CaV αδ-1 auxiliary subunits were co-transfected in HEK-293 cells in the absence or presence of RIM1 48 h before electrophysiological recordings. Fig. 1 shows the average current density-voltage relationships (peak current amplitude normalized by Cm) in response to 2-s membrane depolarizations from a holding potential (Vh) of −80 mV and with 10-mV incremental steps from −50 to +60 mV. As observed, no apparent differences in current densities were detected in the absence or presence of RIM1 (Table 1). It is worth noting that RIM1 expression in HEK-293 cells was confirmed after cDNA transfection by Western blot analyses (Fig. 1, C and F). Although RIM1 had no effect on the density and voltage dependence of the expressed currents, its co-expression profoundly affected current inactivation kinetics, as we shall discuss below.

RIM1 Alters L-type CaV Channel Inactivation and Increases Charge Transfer into Cells—Fig. 2A compares representative whole cell current traces obtained in control conditions and in the presence of RIM1. As shown, HEK-293 cells co-transfected with CaV1.2α1, CaV αδ-1, and CaV β2 produced robust macroscopic Ba2+ current (Iba) through recombinant CaV channels. Likewise, as expected from previous results, peak current amplitude was increased, and inactivation kinetics fastened by expression of the CaV β2 auxiliary subunit (Fig. 2A, bottom). Notably, Iba through CaV1.2 channels decayed with a significantly slower time course in RIM1-expressing cells than in the controls. The L-channel inactivation rate was quantitatively compared between RIM1-expressing cells and control cells by fitting L-current traces with single exponential functions. The time constant for CaV1.2 currents was ~1.5–2-fold slower in the presence of RIM1 (Fig. 2B, top). The parameters from the best fits are given in Table 1. As a consequence, the percentage of current inactivated at the end of the pulse with respect to the peak current amplitude (I remaining) was significantly larger in the RIM1-transfected cells when compared with cells not expressing RIM1 (Fig. 2B, bottom). These effects of RIM1 were also observed in cells expressing CaV1.3 channels (Fig. 2, C and D, and Table 1).

To investigate the functional significance of the L-type CaV channels–RIM1 coupling in more detail, we next calculated the amount of charge mobilized (i.e. the number of ions that passed through the channels) during depolarization (Fig. 3). By integrating the whole cell current transients elicited by depolarizing commands to 0 from a Vh of −80 mV, a net entry of ~354 ± 42 picocoulombs of charge was estimated in control cells expressing CaV1.2 channels. In the RIM1-expressing cells, digital integration of the currents through CaV1.2α1/CaV αδ-1/
**RIM1 and L-type Ca\(^{2+}\) Channel Interaction**

**FIGURE 1. RIM1 does not modify current density through recombinant L-type Ca\(^{2+}\) channels.** Average current density-voltage relationships for \(I_{\text{Ba}}\) recorded from HEK-293 cells expressing Ca\(_{\text{v}}\)1.2/\(\alpha\_\text{Ca}\)/\(\beta\_2\) (A and B) and Ca\(_{\text{v}}\)1.3/\(\alpha\_\text{Ca}\)/\(\delta\_-1/\beta\_3\) (D and E) channels in the absence and presence of RIM1 as indicated. The number of recorded cells is shown in parenthesis. C and F, Western blotting with a RIM antibody on membranes from HEK-293 cells transfected with Ca\(_{\text{v}}\)1.2/\(\alpha\_\text{Ca}\)/\(\delta\_-1/\beta\_3\) and Ca\(_{\text{v}}\)1.3/\(\alpha\_\text{Ca}\)/\(\delta\_-1/\beta\_3\), respectively. The examples shown are representative of three separate experiments. pF, picofarads; Error bars, S.E.

**TABLE 1**

| Channel composition | \(I_{\text{max}}\) \(\mu A/\mu F\) | \(\tau_{\text{inact}}\) \(\mu\text{s}\) | 
|---------------------|---------------------------------|---------------------------------|
| Ca\(_{\text{v}}\)1.2/\(\alpha\_\text{Ca}\)/\(\delta\) | -6.4 ± 1.2 | 698.6 ± 96.0 |
| Ca\(_{\text{v}}\)1.2/\(\alpha\_\text{Ca}\)/\(\delta\) + RIM1 | -5.1 ± 0.9 | 582.3 ± 54.4 |
| Ca\(_{\text{v}}\)1.3/\(\alpha\_\text{Ca}\)/\(\delta\) | -6.0 ± 1.4 | 1085.2 ± 269.9 |
| Ca\(_{\text{v}}\)1.3/\(\alpha\_\text{Ca}\)/\(\delta\) + RIM1 | -8.0 ± 2.5 | 1093.8 ± 376.1 |
| Ca\(_{\text{v}}\)1.2/\(\alpha\_\text{Ca}\)/\(\delta\)/\(\beta\_2\) | -18.7 ± 1.5 | 800.5 ± 58.9 |
| Ca\(_{\text{v}}\)1.2/\(\alpha\_\text{Ca}\)/\(\delta\)/\(\beta\_3\) + RIM1 | -21.6 ± 2.9 | 1163.1 ± 131.8 |
| Ca\(_{\text{v}}\)1.3/\(\alpha\_\text{Ca}\)/\(\delta\)/\(\beta\_2\) | -27.4 ± 4.7 | 788.4 ± 185.0 |
| Ca\(_{\text{v}}\)1.3/\(\alpha\_\text{Ca}\)/\(\delta\)/\(\beta\_2\) + RIM1 | -25.3 ± 3.1 | 2351.2 ± 614.7 |
| Ca\(_{\text{v}}\)1.3/\(\alpha\_\text{Ca}\)/\(\delta\)/\(\beta\_3\) + RIM1 | -36.1 ± 8.4 | 240.2 ± 20.0 |
| Ca\(_{\text{v}}\)1.2/\(\alpha\_\text{Ca}\)/\(\delta\)/\(\beta\_3\) | -49.8 ± 5.2 | 425.6 ± 59.3 |
| Ca\(_{\text{v}}\)1.3/\(\alpha\_\text{Ca}\)/\(\delta\)/\(\beta\_3\) | -158.7 ± 16.5 | 475.3 ± 43.1 |
| Ca\(_{\text{v}}\)1.3/\(\alpha\_\text{Ca}\)/\(\delta\)/\(\beta\_3\) + RIM1 | -129.3 ± 11.4 | 643.9 ± 57.4 |

\(p < 0.05\).

**Ca\(_{\text{v}}\)\(_{\beta_2}\) channels during the imposed depolarization yielded a value of \(-603 \pm 101\) picocoulombs, corresponding to a 1.7-fold increase in charge entry (Fig. 3A). In a similar manner, the average charge transfer was significantly increased by RIM1 from a value of \(-458 \pm 107\) to 791 ± 95 picocoulombs in cells expressing Ca\(_{\text{v}}\)1.3/\(\alpha\_\text{Ca}\)/\(\delta\_-1/\beta\_3\) channels (Fig. 3B). When Ca\(_{\text{v}}\)\(_{\beta_2}\) was co-transfected, qualitatively similar results were obtained for both Ca\(_{\text{v}}\)1.2- and Ca\(_{\text{v}}\)1.3-containing channels after RIM1 expression (Fig. 3, A and B, and Table 1). To study whether RIM1 had an effect on L-type channel availability, the voltage dependence of inactivation was evaluated using 10-s prepulse depolarizations from \(-90\) to \(+40\) mV, preceding a 140-ms test potential to 0 mV or \(-30\) mV (for the Ca\(_{\text{v}}\)1.2/\(\alpha\_\text{Ca}\) and Ca\(_{\text{v}}\)1.3/\(\alpha\_\text{Ca}\) channels, respectively). Normalized current amplitudes were compiled for 4–7 cells and plotted against the prepulse voltage, and mean data points were well described by a sigmoid equation. Fits to the mean inactivation data points and the parameters from the best fits are given in Fig. 4 and Table 1. Interestingly, co-expression with RIM1 shifted \(V_{1/2}\) to the right about 10 mV for Ca\(_{\text{v}}\)1.3/\(\alpha\_\text{Ca}\)/\(\delta\_-1/\beta\_3\) channels. In contrast, the \(V_{1/2}\) value was not significantly altered by RIM1 expression in Ca\(_{\text{v}}\)1.3/\(\alpha\_\text{Ca}\)/\(\delta\_-1/\beta\_3\) channels (Table 1). These results suggest that RIM1 affects mainly the rate of channel inactivation and has a minor impact on the inactivation of the channels at steady state. Therefore, we speculate that RIM1 has an important role in the transition rates between inactivation states while having less impact in the availability of the channel.
RIM1 and L-type Ca\(^{2+}\) Channel Interaction

**FIGURE 2. RIM1 changes the inactivation kinetics of recombinant L-type Ca\(_v\) channels.** A, representative whole cell currents recorded in HEK-293 cells expressing Ca\(_{v1.2}\)/Ca\(_{\alpha_2\delta}\) channels with Ca\(_{\beta_2}\) (upper traces) or Ca\(_{\beta_2}\) (lower traces) in the absence and presence (black and gray traces, respectively) of RIM1. Peak amplitude of the currents before and after RIM1 co-expression was normalized for comparison. Currents were elicited by 2-s depolarizing pulses from a V\(_m\) of −80 to 0 mV. B, comparison of time constant of inactivation (top) and percentage of current remaining at the end of the 2-s voltage step (I\(_{\text{rem}}\)) in cells expressing Ca\(_{v1.2}\)/Ca\(_{\alpha_2\delta}\)/Ca\(_{\beta_2}\) channels in the absence (solid bars) or presence (open bars) of RIM1. C, representative current traces recorded in HEK-293 cells expressing Ca\(_{v1.3}\)/Ca\(_{\alpha_2\delta}\) channels together with Ca\(_{\beta_2}\) or Ca\(_{\beta_2}\) in the absence and presence of RIM1 (as in A). Currents were elicited by 2-s depolarizing pulses from a V\(_m\) of −80 to −30 mV. D, comparison of time constant of inactivation (top) and percentage of current remaining at the end of the voltage step in cells expressing Ca\(_{v1.3}\)/Ca\(_{\alpha_2\delta}\)/Ca\(_{\beta_2}\) channels in the absence or presence of RIM1 (as in B). *, significant differences (p < 0.05) compared with control without RIM1. Error bars, S.E.

**FIGURE 3. RIM1 increases charge transfer into HEK-293 cells.** A, comparison of total charge transfer (maximum current integrated over time) in HEK-293 cells expressing Ca\(_{v1.2}\)/Ca\(_{\alpha_2\delta}\) channels with Ca\(_{\beta_2}\) or Ca\(_{\beta_2}\) in the absence (solid bars) and presence (open bars) of RIM1. Currents were elicited by 2-s depolarizing pulses from a V\(_m\) of −80 to 0 mV. B, comparison of total charge movement in HEK-293 cells expressing Ca\(_{v1.3}\)/Ca\(_{\alpha_2\delta}\) channels with Ca\(_{\beta_2}\) or Ca\(_{\beta_2}\) in the absence and presence of RIM1 (as in A). Currents were elicited by 2-s depolarizing pulses from a V\(_m\) of −80 mV to −30 mV. *, significant differences (p < 0.05) compared with control without RIM1. Error bars, S.E.

nals. However, given that RIM1 expression induces a shift in the inactivation curve of the currents through Ca\(_{v1.3}\)/Ca\(_{\alpha_2\delta}\)/Ca\(_{\beta_2}\), the possibility exists that the RIM1 effects may depend on channel subunit composition.

The Lack of Ca\(_{v}\)β Prevents the Effects of RIM1 on Recombinant L-type Channels—Seminal work by Kiyonaka et al. (16) established that RIM1 associates with different neuronal voltage-gated Ca\(^{2+}\) channels (Ca\(_{v2.1}\) and Ca\(_{v2.2}\)) via interactions with the Ca\(_{v}\)β subunit. In order to determine whether this mechanism is also valid for recombinant L-type channels (Ca\(_{v1.2}\) and Ca\(_{v1.3}\)), a series of experiments using Ca\(_{v}\)β2 and Ca\(_{v}\)β3 antibodies were performed to study whether RIM1 could be immunoprecipitated in samples from transfected HEK-293 cells (Fig. 5). In these experiments, negative controls were obtained with anti-Sp1 antibodies. As shown in Fig. 5, A and B, immunoprecipitation with Ca\(_{v}\)β2 antibodies results in a band between the size markers of 150 and 250 kDa (expected size 178 kDa) in both Ca\(_{v1.2}\) and Ca\(_{v1.3}\) channels. Similarly, using Ca\(_{v}\)β3 antibodies, RIM1 could be immunoprecipitated in HEK-293 cells expressing Ca\(_{v1.2}\) and Ca\(_{v1.3}\) channels (Fig. 5, C and D). In all cases, the negative control (IgG<sub>c</sub>) did not co-precipitate with RIM1. These data provide direct evidence that expression of Ca\(_{v1.2}\)α1 or Ca\(_{v1.3}\)α1 does not hinder the formation of a complex between the Ca\(_{v}\)β subunits and RIM1.

In order to confirm that the functional effects of RIM1 on L-type channels occur through its interaction with the Ca\(_{v}\)β subunit, we characterized its effects on whole cell Ba\(^{2+}\) currents through recombinant Ca\(_{v1.2}\) and Ca\(_{v1.3}\) channels expressed in HEK-293 cells in the absence of Ca\(_{v}\)β. Fig. 6 shows that, as expected, RIM1 expression did not result in appreciable changes in current density recorded by applying depolarizing pulses to 0 and −30 mV for Ca\(_{v1.2}\)α1- and Ca\(_{v1.3}\)α1-containing channels, respectively (Fig. 6A and Table 1). More importantly, co-expression of RIM1 did not affect inactivation kinetics of the L-type currents arising from recombinant Ca\(_{v1.2}\) and Ca\(_{v1.3}\) channels in the absence of the Ca\(_{v}\)β subunit (Fig. 6, B and C, and Table 1). In these experiments, RIM1 expression was confirmed after cDNA transfection by Western blot analysis (Fig. 6D). Taken together, these results confirm the role of the Ca\(_{v}\)β auxiliary subunit in structurally and functionally coupling RIM1 to L-type Ca\(_{v}\) channel complexes.

**RIM1 Regulates Native L-type Ca\(_{v}\) Channel Activity**—Once we established the regulation of recombinant L-type Ca\(_{v}\) channels by RIM1, we checked whether native channels were also regulated by RIM1 using the rat insulinoma RIN-m5F cell line as a model. To this end, RT-PCR and immunoblotting were first used as methods to analyze the expression of RIM1 and different subunits that compose the L-type channel complex. By using antibodies, conspicuous signals were consistently observed. The lack of Ca\(_{v}\)β prevents the effects of RIM1 on recombinant L-type channels, suggesting that RIM1 regulates native L-type Ca\(_{v}\) channels.

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observed in the RIN-m5F cells for CaV1.2/1, CaV1.3/1, CaV2.2, and CaV2.3 subunits in Western blot experiments (Fig. 7A).

Likewise, using specific primers, an expected cDNA fragment of 512 bp for RIM1 was amplified from RIN-m5F cells and from mouse brain used as control tissue (Fig. 7B). The expression of RIM1 was confirmed by Western blot analyses (Fig. 7C). The possible interaction between RIM1 and the L-type channels was then studied by both co-immunoprecipitation and knockdown of RIM1 using specific siRNAs.

First, to evaluate the siRNA silencing efficiency, RIM1 expression was determined by semiquantitative analysis of Western blots. Fig. 7D shows that the RIM1 expression level in RIN-m5F cells transfected with the RIM1 siRNA was significantly lower than those with scrambled siRNA control, whereas the actin expression levels remained essentially unchanged.

Semiquantitative analysis indicates that siRNA decreased the levels of RIM1 to 70% of the levels observed in cells transfected with the control siRNA (Fig. 7E). Altogether, these data indicate that RIN-m5F cells express RIM1 and that RIM1 levels can be successfully down-regulated by RNA silencing.

In agreement with the results obtained with the heterologous expression system, we found that silencing endogenous RIM1 expression with siRNAs increases the extent of inactivation of whole cell native currents. Examples of normalized $I_{\text{Ba}}$ traces elicited in RIN-m5F cells by 2-s depolarizing pulses from a $V_h$ of $-80$ mV in the control condition and after knocking down RIM1 are shown in Fig. 7F. As noted, comparison of normalized superimposed records shows that currents from RIM1 knockdown cells inactivate more rapidly than those from control cells. The extent of inactivation defined by the $I$ remaining decreased ~25% in the RIM1 knockdown cells (Fig. 7G).

RIM1 Associates with L-type Channels in RIN-m5F Cells and Regulates Insulin Secretion—Using antibodies against CaV2.2 and CaV2.3, RIM1 could be co-immunoprecipitated in samples from RIN-m5F cells. Negative controls were obtained with Sp1 antibodies. Hence, probing of RIM1 immunoprecipitated with anti-CaV2.2 (Fig. 8A) and CaV2.3 (Fig. 8B) antibodies revealed a band of ~180 kDa in the co-immunoprecipitated sample lane but not in the IgG0 control lane, indicative of the specificity of the immunoprecipitation.

Last, we investigated the physiological relevance of the RIM1 coupling to the L-type Ca$^{2+}$ channels by assessing insulin release from RIN-m5F cells in control conditions and after transfection with RIM1 siRNA. Basal insulin release to the culture medium remained unchanged in non-stimulated cells (Fig. 8C). In contrast, insulin release triggered by Ca$^{2+}$ influx in response to high K$^+$-induced membrane depolarization (extracellular K$^+$ concentration was increased from 5 to 40 mM for 30 min) was significantly decreased (~25%) in RIM1 knockdown cultures (Fig. 8C). Likewise, insulin release triggered by depolarization with high K$^+$ was also significantly decreased by siRNAs specific to the CaV2.2 and CaV2.3 subunits (Fig. 8, D and E). Altogether, these data demonstrate the importance of L-type channel regulation by RIM1 for fine tuning insulin release.
DISCUSSION

The present study reveals that the coupling between RIM1 and the Ca$_v$ auxiliary subunits is also operational in L-type Ca$_v$ channels. This interaction decelerates L-type current inactivation, producing a sustained depolarization-induced Ca$^{2+}$ influx in insulin-secreting cells that favors hormone release. RIM1 is a putative effector of Rab3 that associates selectively with the active form of the GTPase (31). RIM1 contains an N-terminal domain that interacts with Rab3 and two C2 domains located at the C terminus. Although mainly expressed in the brain, RIM1 is also expressed in pancreatic $\beta$-cells, where it is involved in insulin release (28). In line with this, we found that RIM1 expression significantly increased charge transfer into HEK-293 cells by slowing down the inactivation kinetics of L-type Ca$_v$1.2 and Ca$_v$1.3 channels. Likewise, our results show that siRNA-mediated RIM1 knockdown in RIN-m5F cells significantly affected L-type current inactivation and reduced insulin release triggered by depolarization with high K$^+$. Together, these results suggest that RIM1 might play a role in docking the Rab3-bound vesicles near Ca$_v$ channels, functionally coupling channel activity to the exocytotic machinery in insulin-secreting cells.

Likewise, by searching for possible binding partners of the RIM proteins, initial studies found that the C2A domain mediates the interaction of RIM1 with some synaptic proteins as well as with the pore-forming subunit of neuronal Ca$_v$1.2 channel (13). It has also been reported that the mouse RIM1 arginine-to-histidine substitution (R655H), which corresponds to the human autosomal dominant cone-rod dystrophy mutation, modifies RIM1 function in regulating L-type Ca$_v$1.4 channels of the retina (32) and that the II-III loop of the Ca$_v$1.2 channel modulates RIM1 function in regulating L-type Ca$_v$1.3 channel function (33). Although this identifies RIM proteins as scaffolding proteins with a role in maintaining a high Ca$_v$ channel density at active zones, they have not yet attained general acceptance as critical tethering molecules. Wong and Stanley (19) found that co-immunostaining with RIM1 and Ca$_v$2,2 antibodies neither co-localized nor co-varied at the transmitter release face and that the two proteins did not co-immunoprecipitate.

It should be noted, however, that parallel studies by Han et al. (34) and Kaeser et al. (35) have reported more recently an important role for RIM proteins in localizing Ca$_v$ channels to active zones. Based on protein/protein interaction studies, generation of conditional KO mice, electrophysiological recordings, Ca$^{2+}$ imaging, and quantitative immunofluorescence, these authors propose that the PDZ domains of RIM proteins stoichiometrically interact with Ca$_v$2,2 channels in vitro and that RIM proteins, by interacting directly through their PDZ domains with the Ca$_v$$\alpha_2\delta$ subunits, are essential for tethering Ca$_v$ channels to presynaptic terminals in vivo.

Figure 5: RIM1 interacts with Ca$_v$$\alpha_2\beta$ subunits in HEK-293 cells. Proteins from HEK-293 cells co-transfected with Ca$_v$1.2$\alpha$/$\alpha_2\delta$/Ca$_v$$\alpha_2\beta$ together with the Ca$_v$$\alpha_2\beta$ or Ca$_v$$\alpha_2\beta$ subunits were immunoprecipitated with anti-Ca$_v$$\beta_1$ (A), anti-Ca$_v$$\beta_3$ (B), or control (IgG$_\alpha$) antibodies and subjected to Western blot analysis with anti-RIM antibody. The ~180-kDa RIM1 band is visualized in the immunoprecipitation (IP) lane. Likewise, proteins from HEK-293 cells co-transfected with Ca$_v$1.3$\alpha$/$\alpha_2\delta$/Ca$_v$$\alpha_2\beta$-1 together with the Ca$_v$$\alpha_2\beta$ or Ca$_v$$\alpha_2\beta$ subunits were immunoprecipitated with the anti-Ca$_v$$\beta_1$ (C), anti-Ca$_v$$\beta_3$ (D), or control antibodies and applied to Western blots. Staining the immunoprecipitates with the RIM1 antibody identified the ~180 kDa RIM1 band. In all cases, control experiments with the irrelevant antibody as a substitute for the anti-Ca$_v$$\beta$ antibodies failed to co-immunoprecipitate RIM1. The examples shown are representative of three separate experiments. In all cases, data were collected from the same experiment, and the images are shown separately because they were acquired with different time exposures.

Figure 6: The lack of Ca$_v$$\alpha_2\beta$ prevents the effects of RIM1 on recombinant L-type Ca$_v$ channels. A, averaged peak L-type current density recorded from HEK-293 cells co-transfected with Ca$_v$1.2$\alpha$/$\alpha_2\delta$/Ca$_v$$\alpha_2\beta$-1 (without Ca$_v$$\alpha_2\beta$) in the absence (solid bars) or presence of RIM1 (open bars). The peak amplitudes before and after co-expression of RIM1 were normalized for C$_{m}$, Ba$^{2+}$ currents were elicited by 2-s pulses to 0 mV from a V$_0$ of ~80 mV for the Ca$_v$1.2 channel currents and to ~30 mV in the case of the Ca$_v$1.3 channel currents. B and C, comparison of time constants of inactivation (left) and percentages of $I_{\text{rem}}$ (right) in cells expressing Ca$_v$1.2$\alpha$/$\alpha_2\delta$/Ca$_v$$\alpha_2\beta$-1 channels and Ca$_v$1.3$\alpha$/$\alpha_2\delta$/Ca$_v$$\alpha_2\beta$-1, respectively, in the absence (solid bars) or presence (open bars) of RIM1 (n = 16–25 cells). Shown is Western blot analysis of proteins from HEK-293 cells co-transfected with Ca$_v$1.2$\alpha$/$\alpha_2\delta$/Ca$_v$$\alpha_2\beta$-1 with (+) or without (−) RIM1 using an anti-RIM1 antibody. The examples shown are representative of three separate experiments. Error bars, S.E.
speeds the rate of transmitter release by increasing the intrinsic Ca\textsuperscript{2+} sensitivity of release as well as by contributing to the tight co-localization of readily releasable vesicles with neuronal Ca\textsubscript{V} channels (34, 35).

Similarly, recent studies by Mori and colleagues (16, 20) have documented a functional coupling of RIM1 with neuronal Ca\textsubscript{V} channels mediated by its physical association with the Ca\textsubscript{V} auxiliary subunit via the C2B domain at the C terminus region. This interaction significantly suppressed voltage-dependent channel inactivation (16, 20, 36), enhancing membrane docking of vesicles and potentiating neurotransmitter release (16, 20). Likewise, different RIMs have been shown to physically associ-
RIM1 and L-type Ca$^{2+}$ Channel Interaction

ate with Ca$_{\alpha}\beta$ decelerating current inactivation, increasing depolarization-induced Ca$^{2+}$ entry and enhancing neurotransmitter release (20). Interestingly, the results of our co-immuno-precipitation experiments have identified a RIM1-Ca$_{\alpha}\beta$ channel complex formed by direct interaction of the Ca$_{\alpha}\beta_2$ and Ca$_{\alpha}\beta_3$ subunits with RIM1 heterologously expressed in HEK-293 cells. The identification of native RIM1-Ca$_{\alpha}$ channel complexes in RIN-m5F cells and the effect of the RIM1 knockdown on insulin release support a physiological role for the RIM1-Ca$_{\beta}$ subunit interaction. In this context, it is well known that the Ca$_{\alpha}\beta$ subunit interacts with the pore-forming Ca$_{\alpha}\alpha_1$ subunit from the cytoplasmic side to enhance functional channel trafficking to the plasma membrane and to modify multiple kinetic properties (34, 37). In particular, functional studies have shown that the Ca$_{\alpha}\beta$ subunit is a key determinant in Ca$_{\alpha}$ channel inactivation (38). For many types of high voltage-activated Ca$_{\alpha}$ channels, co-expression with Ca$_{\alpha}\beta$ tends to increase the rate of inactivation (30, 39, 40). Therefore, the possibility exists that RIM1 may act on the Ca$_{\alpha}\beta$ subunits to suppress the regulatory function of this auxiliary subunit on L-type Ca$_{\alpha}$ inactivation. As a consequence, association with Ca$_{\alpha}\beta$ may enable RIM1 to play an important physiological role in hormone release. Decreased L-channel inactivation by RIM1 interaction implies that a substantially larger Ca$^{2+}$ current would be maintained during depolarization facilitating insulin release.

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