Voltage-dependent calcium (Ca²⁺) channels are involved in many specialized cellular functions and are controlled by a diversity of intracellular signals. Recently, members of the RGK family of small GTPases (Rem, Rem2, Rad, Gem/Kir) have been identified as novel contributors to the regulation of L-type calcium channel activity. In this study, microarray analysis of the mouse insulinoma MIN6 cell line revealed that the transcription of Rem2 gene is strongly induced by exposure to high glucose, which was confirmed by real-time reverse transcriptase-PCR and RNase protection analysis. Because elevation of intracellular Ca²⁺ in pancreatic β-cells is essential for insulin secretion, we tested the hypothesis that Rem2 attenuates Ca²⁺ currents to regulate insulin secretion. Co-expression of Rem2 with Ca₁,1.2 or Ca₁,1.3 L-type Ca²⁺ channels in a heterologous expression system completely inhibits de novo Ca²⁺ current expression. In addition, ectopic overexpression of Rem2 both inhibited L-type Ca²⁺ channel activity and prevented glucose-stimulated insulin secretion in pancreatic β-cell lines. Co-immunoprecipitation studies demonstrate that Rem2 associates with a variety of Ca₉β subunits. Importantly, surface biotinylation studies demonstrate that the membrane distribution of Ca²⁺ channels was not reduced at a time when channel activity was potently inhibited by Rem2 expression, indicating that Rem2 modulates channel function without interfering with membrane trafficking. Taken together, these data suggest that inhibition of L-type Ca²⁺ channels by Rem2 signaling may represent a new and potentially important mechanism for regulating Ca²⁺-triggered exocytosis in hormone-secreting cells, including insulin secretion in pancreatic β-cells.

Rem, Rem2, Rad, and Gem/Kir (RGK) are members of a Ras-related GTPase subfamily, with many unique characteristics that distinguish them from other members of the Ras superfamily (1–5). All RGK GTPases share a common structure consisting of a conserved Ras-related guanine nucleotide binding core, a series of nonconservative amino acid substitutions within regions known to be involved in nucleotide binding and hydrolysis, a non-CAAAX-containing C-terminal extension, and large N-terminal extensions relative to other Ras family proteins. These extensions contain multiple phosphorylation sites, a C-terminal calmodulin binding domain in Rad and Gem, and 14-3-3 binding sites (6), and each has been proposed to play a role in RGK regulation (7–10). The conservation of structural features within the RGK proteins suggests shared mechanisms of regulation and control of common cellular functions. However, RGK GTPases differ in their putative effector (Gₓ) domains, suggesting that they may associate with distinct regulatory and effector proteins. In addition, each exhibits a unique, tissue-restricted, and non-overlapping expression pattern. Another distinctive characteristic is their regulation at the transcriptional level. For example, Gem is an early response gene and Rem expression is repressed by lipopolysaccharide stimulation (1, 4, 5, 11).

Although the cellular functions of the RGK family remain largely unknown, recent evidence suggests a role for these proteins in the regulation of both Ca²⁺ channel activity and cytoskeletal reorganization (7, 8, 12). Rem, Rad, and Gem have all been shown to interact with the Ca²⁺ channel β subunit, resulting in the down-regulation of Ca²⁺ channel function and either termination of Ca²⁺-dependent secretion (8, 12) or modulation of cardiac electrical conduction and contractile function (8, 14). While direct association with Ca₂⁺β subunits appears crucial to RGK-mediated inhibition of Ca²⁺ channel function, the nature of the regulatory mechanism remains to be determined. A model has suggested that Gem association sequesters Ca₂⁺β subunits, resulting in inhibition of Ca₁-α expression at the plasma membrane (12).

Intracellular Ca²⁺ is involved in a variety of cellular processes such as signal transduction, gene expression, and hormone release, and disruption of intracellular Ca²⁺ homeostasis readily induces cellular dysfunction (13). Insulin release by pancreatic β-cells is a Ca²⁺-dependent process, which follows the sequence of closure of the ATP-dependent K⁺ channels, membrane depolarization, and opening of voltage-dependent Ca²⁺ channels. A tight coupling is believed to exist between the exocytosis of insulin-containing secretory granules and the increase in the intracellular free Ca²⁺ concentration (15, 16). An uncontrolled, enhanced Ca²⁺ signal, however, may be detrimental to the β-cell (17) and there appears to be multiple safeguards to regulate Ca²⁺ levels within these cells. Indeed, it has been reported that pancreatic islets are severely impaired in their ability to secrete insulin following chronic exposure to high glucose concentrations, and that this dysfunction contributes to the development of diabetes (18, 19). In this context, persistent hyperglycemia might well cause sustained elevated [Ca²⁺] and abnormalities in glucose-induced secretion and suggest that regulation of basal Ca²⁺ plays an important role in glucose-evoked insulin release.

In this study we have demonstrated that exposure of pancreatic β-cells to glucose is associated with a significant increase in Rem2 expression as determined by quantitative real-time RT3-PCR and RNase
Rem2 Regulates Glucose-induced Ca\(^{2+}\) Responses in Pancreatic β-Cells

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An example of text from the journal article:

Protection analysis. Exogenous Rem2 inhibits L-type Ca\(^{2+}\) channel function when expressed in HEK293 cells, and importantly, blocks endogenous Ca\(^{2+}\) channel activity and glucose-stimulated insulin secretion in pancreatic β-cells. In addition, Rem2-mediated inhibition of L-type channel activity occurs without altering Ca\(^{2+}\) channel trafficking, indicating that Rem2 utilizes a regulatory mechanism distinct from that described for Gem to acutely regulate channel function. These data clearly identify Rem2 as a novel and potentially critical modulator of Ca\(^{2+}\)-dependent secretion in pancreatic islets. Furthermore, these data suggest that Rem2 signaling may control a previously unappreciated negative feedback regulatory cascade operating to protect pancreatic β-cells from uncontrolled Ca\(^{2+}\) signaling in the presence of persistent hyperglycemia.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**MIN6 cells of passage 24 to 30 were cultured and maintained in Dulbecco’s modified Eagle’s medium containing 5 mM glucose, 10% (v/v) fetal bovine serum, 1% penicillin/streptomycin, 2 mM glutamine, and 100 μg/ml β-mercaptoethanol (20). For glucose regulation experiments, cells were washed three times with 1× phosphate-buffered saline and grown overnight, unless otherwise indicated, in Dulbecco’s modified Eagle’s medium without fetal bovine serum containing the indicated glucose concentration(s). HIT-T15 cells were maintained in Ham’s F-12 containing 10% dialyzed horse serum and 2.5% fetal bovine serum, whereas HEK293 cells were cultured as described (1).

**Plasmids and Adenoviruses—**The original cloning of rat Rem2ΔN69 has been described previously (2). Subsequent sequence analysis suggested that this original clone may represent a shorter N-terminal Rem2 splice variant. To isolate the longer Rem2WT cDNA, we performed PCR on mouse expressed sequence tag EST4400995 (GenBankTM accession number AW909633) with primers that introduced a 5′ BamHI site and 3′ XhoI site to the entire putative open reading frame. In addition, PCR was performed to generate the shorter version of mouse Rem2 (Rem2ΔN69). These clones were introduced into 3xHA-pcDNA3.1 to allow expression of hemagglutinin (HA) epitope-tagged mRem2WT and mRem2ΔN69. Replication-deficient adenoviruses expressing GFP as a marker and HA-tagged rat Rem2ΔN69 were constructed and purified using the pAdTrack/AdEasy system as described (8).

**Microarray Analysis of Glucose-regulated Genes in MIN6 Insulinoma Cells—**Microarray analysis was performed using RNA isolated from MIN6 cells incubated on 1 or 25 mM glucose for 16 h using a Qiagen RNeasy® Mini kit according to the manufacturer’s instructions. First-strand synthesis was performed using the Brilliant Q-PCR RT-PCR kit (Stratagene) with 10 μg of isolated MIN6 RNA, after treatment with DNase I (Sigma). Real-time amplification of the cDNA was performed using the TaqMan Universal Master Mix (ABI) with TaqMan probes. The β-actin primers used were 5′-AGGTCTCATCATTGGCAACAGCA-3′ and 5′-CCTCTCATGAGAATGTAGTTT-3′; and were used in combination with the β-actin probe 5′-(Cy5)-TGCACACAGAGTCCTACACCAAGAAGG-(BHQ)-3′. Rem2 was amplified using the TaqMan Assays on Demand 20× Mix kit (ABI) according to the manufacturer’s instructions.

**Insulin Secretion Assays—**MIN6 cells were infected with 1×10⁷ plaque-forming units/ml of the indicated adenoviruses for 16 h in serum-free Dulbecco’s modified Eagle’s medium. Insulin secretion from the infected MIN6 cells was measured by equilibrating the cells for 2 h in 1× KRBB buffer (119 mM NaCl, 4.74 mM KCl, 2.54 mM CaCl\(_2\), 1.19 mM MgSO\(_4\), 1.19 mM KH\(_2\)PO\(_4\), 25 mM NaHCO\(_3\), 10 mM HEPES (pH 7.4), 0.1 g of bovine serum albumin). Cells were then washed twice with 1× PBS and incubated for 1 h in 1× KRBB buffer containing 3 or 30 mM glucose. The insulin concentration in the media was determined using the mouse ultrasensitive insulin enzyme-linked immunosorbent assay kit (ALPCO) according to the manufacturer’s recommendations. Insulin content in the cells was determined by acid-ethanol extraction of the cells.

**Electrophysiological Studies—**HEK293 cells were transiently transfected with plasmids 12–48 h before recordings as described (8). Transfected cells were identified by the expression of GFP. HIT-T15 cells were plated on polylysine-coated coverslips in 24-well tissue culture dishes at 20,000 cells per well. The next day, the cells were infected with the indicated adenovirus at 1×10⁷ adenovirus/ml. Adenovirus-infected cells were identified by GFP expression and recordings made after 22 h post-infection. The whole cell configuration of the patch clamp technique was used to measure ionic current. Patch electrodes with resistances of 1–2 megohm contained (in mM): 110 K-glucuronate, 40 CsCl, 3 EGTA, 1 MgCl\(_2\), 5 MgATP, and 5 Heps, pH 7.36. The bath solution consisted of (in mM): 102.5 CsCl, 40 BaCl\(_2\), 1 MgCl\(_2\), 10 tetraethylammonium chloride, and 5 Heps, pH 7.4. Signals were amplified with an Axopatch 200B amplifier and 333 kHz A/D system (Axon Instruments, Union City, CA). Data were analyzed with Clampfit 9 (Axon Instruments) and Origin 7.0 statistical software (OriginLab Corp., Northampton, MA). All recordings were performed at room temperature (20–22 °C). Adenoviral infected HIT-T15 cells were analyzed as above using a bath solution consisting of (in mM): 102.5 (or 140) CsCl, 40 (or 2.5) BaCl\(_2\), CaCl\(_2\), 1 MgCl\(_2\), 10 triethanolamine chloride, and 5 Heps, pH 7.4. In addition to their well characterized electrical properties (22–24), viral infected HIT-T15 cells were more amenable to patch clamp analysis that viral infected MIN6 cells.

**Rem2-Ca\(_{\beta2a}\) Subunit Interactions—**HA-tagged rat Rem2ΔN69 pcDNA and FLAG-Ca\(_{\beta2a}\), pCMV-T7F2, FLAG-Ca\(_{\beta1b}\), FLAG-Ca\(_{\beta2a}\), or pCMV-T7F2 were co-transfected into HEK293 cells by the calcium phosphate method (25). Forty-eight h post-transfection, the cells were washed with PBS, placed into 1 ml of Verseen (Invitrogen), harvested, pelleted, and then suspended in ice-cold immunoprecipitation buffer (20 mM Tris, pH 7.5, 250 mM NaCl, 1% Triton X-100, 0.5 mM dithiothreitol, 1× protease inhibitor mixture (Calbiochem), 10 mM MgCl\(_2\), 10 μM GTPγS). The cells were lysed, subjected to centrifugation, and 1 mg of the supernatant incubated in a 500-μl reaction containing 10 μl of packed Protein G-Sepharose (Amersham Biosciences) and 4 μg of anti-FLAG M2 monoclonal antibody (Sigma) for 3 h with gentle agitation.
Rem2 Regulates Glucose-induced Ca\(^{2+}\) Responses in Pancreatic β-Cells

### RESULTS

**Expression of Rem2 Is Induced by Glucose in MIN6 Cells**—The MIN6 insulinoma cell line is one of the few pancreatic β-cell lines that retain insulin secretory response to physiological concentrations of glucose and other secretagogues (20, 26), and has been used extensively in studies of the mechanisms of insulin secretion. A major challenge in diabetes research is to understand the pleiotropic effects of glucose on pancreatic β-cells in molecular terms. To identify glucose-responsive genes, mRNA expression in MIN6 cells incubated in high or low glucose was compared using oligonucleotide arrays. Four independent sets of cells were harvested following 16 h incubation in medium containing either 1 (low) or 25 mM (high) glucose, RNA was extracted, labeled, and hybridized onto microarrays. These studies identified a subset of genes that differed in their expression levels in response to elevated extracellular glucose levels by 1.5-fold or greater, in all four experiments. Many of the genes identified in this approach were not previously known to be expressed differentially in MIN6 cells in response to glucose, including the Rem2 GTPase. The microarray data shown in TABLE ONE indicate that exposure of MIN6 cells to high concentrations of glucose (25 mM) result in a ∼2.4-fold increase in Rem2 gene expression.

### Validation of Glucose-regulated Expression of Rem2 but Not Other RKG Proteins—To verify the observed increase in Rem2 mRNA in response to high glucose seen with microarray analysis, we quantified the Rem2 mRNA level in MIN6 cells grown on low or high glucose media using real-time RT-PCR and RNase protection analysis. RT-PCR analysis performed using cDNA from low or high glucose-induced MIN6 cells indicate a 4.1-fold increase in Rem2 mRNA levels in response to high glucose (30 mM) compared with β-actin levels used as control (Fig. 1). As a control for contamination of the cDNA with genomic DNA, we employed actin primers that give an additional larger product as a control.

| Treatment           | Sample | Average (S.D.) | p Value | -Fold induction |
|---------------------|--------|----------------|---------|-----------------|
| 1 mM Glucose        | 1      | 43.1           |         |                |
|                     | 2      | 72.8           |         |                |
|                     | 3      | 43.9           |         |                |
|                     | 4      | 72.9           |         |                |
| 25 mM Glucose       | 1      | 162.3          |         |                |
|                     | 2      | 128.1          |         |                |
|                     | 3      | 144            |         |                |
|                     | 4      | 128.8          |         |                |

| p | Value |
|---|-------|
| 2 \times 10^{-4} | 2.42  |

4 S. Özcan, unpublished data.
Rem2 regulates glucose-induced Ca\(^{2+}\) responses in pancreatic β-cells.

**Fig. 1.** Regulation of Rem2 transcription in the MIN6 insulinoma cell line. Real-time RT-PCR analysis of Rem2 and Gem expression in MIN6 cells treated with 3 or 30 mM glucose for 16 h. A, quantification of the real-time RT-PCR data, as fold increases in mRNA levels of Rem2 or Gem on 30 mM glucose over 3 mM glucose. The mRNA levels from 3 or 30 mM glucose-incubated MIN6 cells were normalized to β-actin levels. B, the RT-PCR products obtained from 3 or 30 mM incubated MIN6 cells after amplification with Rem2 or β-actin-specific primers were separated on a native polyacrylamide gel and stained with ethidium bromide.

were because of differences in osmotic pressure between cells cultured in the presence and absence of glucose, MIN6 cells were treated with 30 mM l-glucose, a nonmetabolized glucose analog that is not transported into cells. As seen in Fig. 2A, l-glucose did not affect Rem2 gene expression. Hence, the glucose-induced increase of Rem2 gene expression was not because of an osmotic change of the culture medium. Finally, to further validate the significance of the quantitative RT-PCR analysis, ribonuclease protection analysis was used to confirm that Rem2 expression is elevated in response to high glucose concentrations (Fig. 2B).

Thus, Rem2 is a glucose-responsive gene in pancreatic β-cell lines whose expression increases in response to high levels of glucose.

RGK GTPases exhibit distinct tissue-specific expression patterns and are transcriptionally regulated in response to a variety of cellular stimuli (1–5, 8). Thus, we next examined the regulated expression of the remaining members of the RGK GTPases in MIN6 cells following exposure to high glucose (Fig. 2B). RNase protection analysis indicates that Gem, but not Rem or Rad, is expressed in MIN6 cells. However, both RNase protection and real-time RT-PCR analysis indicate that Gem mRNA levels are modestly decreased following exposure to high glucose for 16 h (Figs. 1A and 2B). Thus, both Rem2 and Gem RT-PCR products are expressed in MIN6 cells, but only Rem2 mRNA appears to be up-regulated following glucose exposure.

**Rem2 Inhibits L-Type Ca\(^{2+}\) Channel Function—Ca\(_{\alpha,1.2}\) and Ca\(_{\alpha,1.3}\) L-type Ca\(^{2+}\) channels are believed to underlie Ca\(^{2+}\) currents in pancreatic β-cells (15, 16), and other members of the RGK GTase family have been found to regulate L-type Ca\(^{2+}\) channel function in cardiac muscle, neuronal, and endocrine cells (7, 8, 12, 14). Therefore, we next tested the ability of Rem2 to regulate current expression of heterologously expressed L-type Ca\(^{2+}\) channels. Sequence analysis of Rem2 orthologs (rat, mouse, and human) indicated that our original rat Rem2 cDNA clone may represent a splice variant that initiated from an internal methionine and therefore lacked 69 amino acids found in the majority of cloned cDNAs (2). We therefore examined the ability of both our original Rem2 clone (Rem2ΔN69) and full-length Rem2 (Rem2WT) clones to regulate Ca\(^{2+}\) channel function. As seen in Fig. 3A, HEK293 cells transiently co-transfected with Ca\(_{\alpha,1.2}\) and Ca\(_{\alpha,\beta2a}\) express greater than 25 pA/pF of peak inward current. In contrast, co-expression of wild-type Rem2 with Ca\(_{\alpha,1.2}\) and Ca\(_{\alpha,\beta2a}\) resulted in 95% reduction of detectable ionic current expression (from 25.8 ± 2.6 pA/pF, n = 18 to 1.4 ± 0.6 pA/pF, n = 6 of peak inward current; p = 0.0003) (Fig. 3A, open circles). I\(_{\text{Ca,L}}\) was dramatically reduced for all potentials when co-expressed with wild-type Rem2. Similar results were seen using Rem2ΔN69 (Fig. 3A, open triangles). These results are consistent with the ability of Rem, and other RGK GTases, to prevent L-type Ca\(^{2+}\) current expression (7, 8, 12, 14).

Next, we tested the effect of Rem2 co-expression on Ca\(_{\alpha,1.3}\) function, the second major L-type channel in pancreatic β-cells (15, 16). As seen in Fig. 3B, Rem2 expression inhibited expression of current through the Ca\(_{\alpha,1.3}\) and Ca\(_{\alpha,\beta2a}\) channel, but did not result in a complete reduction of current (~9 pA/pF of peak inward current remained). Rem2 co-expression significantly decreased peak inward current density by 78% compared with Ca\(_{\alpha,1.3}\) and Ca\(_{\alpha,\beta2a}\) alone (from 41.6 ± 6.5 pA/pF, n = 22, to 9.2 ± 3.5 pA/pF, n = 8, of peak inward current; p = 0.00001). The current voltage relationship shows no shift of peak current or obvious voltage dependence for either L-type channel (Fig. 3B). Taken together, these data strongly support the notion that Rem2 serves as a negative regulator of L-type Ca\(^{2+}\) channel function in pancreatic β-cells.

Recent studies have established members of the RGK GTases as Ca\(_{\beta}\) subunit binding partners (8, 12). To determine whether Rem2 also interacts with Ca\(_{\beta}\) channel β-subunits in vivo, HEK293 cells were transiently transfected with expression vectors encoding FLAG-tagged Ca\(_{\alpha,\beta}2a\) alone or co-transfected with HA-tagged Rem2. FLAG-tagged Ca\(_{\alpha,\beta}2a\) was then isolated by immunoprecipitation and bound HA-tagged Rem2 was visualized by immunoblotting. As seen in Fig. 4, HA-tagged Rem2 was found in the pelleted fraction in a FLAG-Ca\(_{\alpha,\beta}2a\)-dependent manner. HA-tagged Rem2 was also found to interact with Ca\(_{\alpha,\beta1}b\) and Ca\(_{\alpha,\beta4a}\), demonstrating a direct interaction of Rem2 with a variety of Ca\(_{\beta}\) subunit isoforms.

**Rem2 Inhibits L-type Ca\(^{2+}\) Channel Function in HIT-T15 Cells—Voltage-gated Ca\(^{2+}\) channels play essential roles in many cellular functions, including stimulus-secretion coupling in pancreatic β-cells (13, 15). To test the effect of wild-type Rem2 on pancreatic β-cell voltage-gated Ca\(^{2+}\) channels, we next infected HIT-T15 cells with recombinant adenovirus co-expressing HA-tagged Rem2ΔN69 and GFP or GFP expressing control adenovirus. HIT-T15 cells are insulin-secreting cells whose electrophysiological properties have been extensively characterized, and were amenable to patch clamp analysis following adenoviral infection. As seen in Fig. 5, Rem2 expression inhibited the peak inward current by 90% when compared with control adenovirus infected, or uninfected, HIT-T15 cells (29.5 ± 6.8 pA/pF, n = 7 to 3.1 ± 1.0 pA/pF, n = 10; p = 0.003). Taken together, these data strongly support the notion that Rem2 serves as a regulator of L-type Ca\(^{2+}\) channel activity in β-islet cells, and that elevated Rem2 expression acts to inhibit depolarization-induced calcium influx in insulin-secreting cells.
Rem2 Regulates Glucose-induced Ca\textsuperscript{2+} Responses in Pancreatic \( \beta \)-Cells

![Diagram](image1)

**Fig. 2.** Expression of RGK GTPases in the MIN6 insulinoma cell line. A, quantification of Rem2 mRNA levels by real-time RT-PCR in response to 3 and 30 mM o- or \( \alpha \)-glucose from two independent experiments. B, RNase protection analysis was carried out by preparing total RNA (20 \( \mu \)g) from MIN6 cells treated with low (3 mM) or high (30 mM) glucose for 16 h, hybridized with radiolabeled antisense RGK and L32 riboprobes, and then subjected to RNase protection analysis as described under "Experimental Procedures." The protected probes were resolved by polyacrylamide gel electrophoresis and visualized by autoradiography (16 h). The data are representative of three separate ribonuclease protection assays.

![Diagram](image2)

**Fig. 3.** Rem2 prevents de novo expression of Ca\textsubscript{\( \alpha \)} current. A, current voltage relationships for HEK293 cells transfected with Ca\textsubscript{\( \alpha \)1.2 + \( \beta \)\textsubscript{\( \alpha \)} + GFP \((n = 6)\) or Ca\textsubscript{\( \alpha \)1.2 + \( \beta \)\textsubscript{\( \alpha \)} + GFP-Rem2 \((n = 11)\). Representative Ba\textsuperscript{2+} current elicited by a 5-mV voltage step from \(-80\) to \(+80\) mV. Inset, exemplar 40 mV Ba\textsuperscript{2+} current from cells co-expressing Ca\textsubscript{\( \alpha \)1.2 + \( \beta \)\textsubscript{\( \alpha \)} + GFP (dark line) or Ca\textsubscript{\( \alpha \)1.2 + \( \beta \)\textsubscript{\( \alpha \)} + Rem2 (light line). B, current voltage relationships for HEK293 cells transfected with Ca\textsubscript{\( \alpha \)1.3 + \( \beta \)\textsubscript{\( \alpha \)} + GFP \((n = 22)\) or Ca\textsubscript{\( \alpha \)1.3 + \( \beta \)\textsubscript{\( \alpha \)} + GFP-Rem2 \((n = 10)\). The inset is described in panel A.

![Diagram](image3)

**Fig. 4.** In vivo interaction of Rem2 with VDCC \( \beta \) subunit isoforms. HEK293 cells were co-transfected with expression vectors encoding HA-tagged Rem2 and a vector encoding the indicated FLAG epitope-tagged \( \beta \) subunit. Cell extracts were prepared, incubated with anti-FLAG antibody, and recovered with protein G beads and analyzed by SDS-PAGE and immunoblotting. The presence of Rem2 in the pellet and soluble fractions was detected with anti-HA antibody, whereas the distribution of \( \beta \)\textsubscript{\( \alpha \)} was determined using anti-FLAG monoclonal antibody. These data are representative of three separate experiments.

Recent analysis of Gem-mediated channel regulation has suggested that Gem-\( \beta \)-subunit association inhibits the trafficking of newly synthesized \( \alpha \)1 subunits to the plasma membrane, resulting in a chronic down-regulation of functional channel expression (12, 14). To explore whether Rem2 utilizes a similar mechanism, we examined whether the number of surface-exposed Ca\textsuperscript{2+} channels in HIT-T15 cells was altered following adenoviral-mediated Rem2 expression. Surface proteins were biotinylated with membrane-impermanent sulfo-NHS-LC-biotin 24 h after adenoviral infection, the same time that patch clamp analysis demonstrated Rem2-mediated inhibition of Ca\textsuperscript{2+} channel function (Fig. 5A). Biotinylated surface proteins were isolated by incubation with streptavidin resin, and subjected to Western blot analysis. We found that the expression of surface Ca\textsubscript{\( \alpha \)}\( \alpha \)1 subunit (represented by the \( \sim 220\)-kDa band) was unchanged in Rem2-infected cells compared with either uninfected or Ad-GFP-infected HIT-T15 controls (Fig. 5, C and D). The expression of Ca\textsubscript{\( \alpha \)}\( \alpha \)1 proteins in whole cell lysates remained the same between Rem2 expressing cells and the controls indicating that Rem2 expression does not markedly reduce channel stability. Finally, Western blot analysis for GAPDH, a well characterized cytoplasmic protein, verified that cytoplasmic proteins were not present in the biotinylation-eluted surface protein preparation. Taken together, these studies indicate that Rem2 regulates channel activity in HIT-T15 cells without a detectable reduction in the density of membrane L-type Ca\textsuperscript{2+} channels.

**Rem2 Modulates Insulin Secretion in Pancreatic \( \beta \)-Cells—**To confirm that Rem2 might function as a regulator of secretagogue-stimulated insulin release, we next investigated the effects on insulin secretion of wild-type Rem2 protein expression. To achieve this efficiently, we infected MIN6 cells with adenoviral constructs engineered to express Rem2. MIN6 cells were either uninfected or infected with control "empty" adenovirus or the constructs expressing Rem2 or Rem, another RGK GTPase know to potentily inhibit L-type Ca\textsuperscript{2+} channel function.
Under basal conditions (3 mM glucose) MIN6 cells exhibited low levels of insulin secretion and this rate was not affected by Rem2 or Rem expression (data not shown). When treated with 30 mM glucose, insulin secretion was 3-fold higher in both uninfected and control adenovirus-infected MIN6 cells (Fig. 6). However, glucose-stimulated MIN6 cells expressing Rem2 or Rem exhibited reduced insulin secretion, resulting in insulin release equivalent to that of unstimulated cells (Fig. 6). Similar data were obtained when MIN6 cells were treated with nifedipine, which blocks insulin secretion by inhibiting L-type Ca\(^{2+}\) channel activity (data not shown) (15, 28). These data reinforce the importance of Ca\(^{2+}\) signaling in the regulation of insulin secretion from pancreatic \(\beta\)-cells in response to glucose. Taken together, these results suggest that changes in Rem2 expression represent a previously unrecognized regulatory mechanism controlling Ca\(^{2+}\) signaling and insulin secretion in pancreatic islet cells.

**DISCUSSION**

DNA microarray technology has allowed the global analysis of pancreatic \(\beta\)-cell responses to high glucose levels and offers a comprehensive analysis of the compensatory gene expression involved in islet cell response to chronic glucose stimulation. Using DNA microarray analysis, we found that expression of the Rem2 GTPase, a member of the Ras superfamily, is significantly increased in the MIN6 \(\beta\)-cell insulinoma cell line following exposure to high glucose, which was confirmed by quantitative RT-PCR and RNase protection analysis. Thus, Rem2 is a glucose-regulated gene in pancreatic \(\beta\)-islet cells. Because the physiological function of the Rem2 GTPase is unknown, we looked for a possible biological relationship between Rem2 expression and pancreatic islet cell function. We show here that Rem2 inhibits L-type Ca\(^{2+}\) channel current expression in both a heterologous system, and importantly in the HIT-T15 pancreatic \(\beta\)-cell line. Thus, Rem2 joins the rest of the RGK GTPase family, in a shared ability to regulate voltage-gated Ca\(^{2+}\) channel activity and its regulation at the level of gene expression. We hypothesize that this transcriptional regulation underlies a novel negative feedback mechanism in pancreatic \(\beta\)-islet cells in which chronic...
**Rem2 Regulates Glucose-induced Ca^{2+} Responses in Pancreatic \( \beta \)-Cells**

Hyperglycemia results in elevated Rem2 expression, which provides a selective reduction in voltage-gated Ca\(^{2+} \) channel activation resulting in a decrease in glucose-stimulated insulin secretion.

Pancreatic islet \( \beta \)-cells can be regulated by multiple stimuli, including nutrients and growth factors. Islet \( \beta \)-cell growth and function are regulated by both glucose concentration and growth factors acting through a complex network of intracellular signaling cascades (15, 16, 29). Changes in gene expression that result from activation of these signaling pathways are likely responsible for the adaptation of \( \beta \)-cells to physiological and pathological states and recent work has begun to characterize the changes in gene expression and the molecular mechanisms mediating \( \beta \)-cell responses to environmental conditions. Indeed, the founding member of the RGK family, Rad (Ras-related protein associated with diabetes), was first isolated based on its overexpression in muscle from some type II diabetic patients and was thought to be a candidate gene involved in the pathogenesis of non-insulin-dependent diabetes mellitus and insulin resistance (5), although subsequent studies have largely failed to support a correlation between Rad expression and diabetes (30). In this study, microarray analysis of the highly characterized MIN6 insulinoma cell line following high glucose exposure (TABLE ONE) demonstrates that Rem2 expression is regulated by glucose-mediated transcriptional control (Fig. 1). Thus, Rem2 is the latest of the RGK GTPases shown to be under transcriptional regulation (1, 3–5). Rem2 mRNA is highly expressed in brain and kidney, and found to have a complementary distribution to those of other RGK family members, being the only RGK expressed at high levels in the brain (2). Together with the current data, these results suggest that Rem2 may be widely expressed in neuroendocrine tissues. Rem2 also appears to be the only glucose-regulated member of the RGK family found in \( \beta \)-islets, although Gem/Kir is also expressed in MIN6 cells (Figs. 1 and 2). Interestingly, Ohsumi and colleagues (31) identified Gem as a glucose-regulated gene in MIN6 cells by microarray, but failed to identify Rem2. These differences may reflect divergence in the genetic response to acute versus chronic high glucose exposure because this study examined gene expression following an acute (45 min) high glucose challenge while we examined MIN6 cells after a prolonged exposure (16 h) (TABLE ONE). Indeed, Gem gene expression is up-regulated in response to a variety of stimuli, including serum (32). Thus, it is possible that both Rem2 and Gem/Kir function to regulate both \( \beta \)-cell Ca\(^{2+} \) channel activity and insulin secretion.

Insulin release by pancreatic \( \beta \)-cells is a Ca\(^{2+} \)-dependent process and a tight coupling is believed to exist between the exocytosis of insulin-containing secretory granules and the increase in the intracellular free Ca\(^{2+} \) concentration. An uncontrolled, enhanced Ca\(^{2+} \) signal, however, may be detrimental to the \( \beta \)-cell and there appears to be multiple safeguards to regulate Ca\(^{2+} \) levels within these cells (18, 19). To examine whether Rem2 might contribute to regulated insulin secretion, we first explored the ability of Rem2 to regulate L-type Ca\(^{2+} \) channel function. Co-immunoprecipitation studies suggest that Rem2 can associate with Ca\(_{\alpha,1}\) subunits when overexpressed in HEK293 cells (Fig. 4), and potently inhibits both endogenous L-type Ca\(^{2+} \) channel activity in HIT-T15 insulinoma cells (Fig. 5) and heterologously expressed Ca\(_{\alpha,1,2}/\beta_{2\alpha} \) and Ca\(_{\alpha,1,3}/\beta_{2\alpha} \) channels in HEK293 cells (Fig. 3). Thus, Rem2 acts to negatively control voltage-gated Ca\(^{2+} \) channel activity in pancreatic \( \beta \)-cells. Indeed, recent data indicate that secretagogue-induced insulin secretion is dependent on Ca\(_{\alpha,1,2} \) function (33), supporting a potential regulatory role for Rem2 in Ca\(^{2+} \)-mediated insulin secretion.

Recent studies characterizing Gem-mediated L-type Ca\(^{2+} \) channel regulation using either immunocytochemical methods (12) or by measuring intramembrane charge movement in ventricular myocytes (14) have suggested that Gem expression results in a significant reduction in the density of membrane L-type Ca\(^{2+} \) channels. These data have led to the hypothesis that Gem association with Ca\(_{\alpha,1,2} \) blocks its association with Ca\(_{\alpha,1,2} \) inhibiting the exit of newly synthesized Ca\(_{\alpha,1,2} \) subunits from the endoplasmic reticulum (34). However, we see potent Ca\(^{2+} \) channel inhibition in HIT-T15 cells within 24 h of adenosinergic-mediated Rem2 expression (Fig. 5A) without a detectable reduction in the density of membrane localized L-type Ca\(^{2+} \) channels (Fig. 5C). These data indicate that the mechanism of Rem2-dependent channel regulation is distinct from Gem, resulting in a significant reduction in L-type Ca\(^{2+} \) channel activity without interfering with channel trafficking. Importantly, these results suggest that Rem2 may allow acute channel regulation, on a time scale potentially much faster than mediated through effects on intracellular trafficking. However, the mechanism by which Rem2 reduces I\(_{Ca,L} \) remains unclear. Studies are underway to examine alternative regulatory models including the direct modulation of channel gating in \( \beta \)-islets.

Surprisingly, these data also demonstrate significant differences in Rem2-mediated Ca\(^{2+} \) channel control versus that of the Rem GTPase. Thus, Rem2 expression inhibited, but did not completely attenuate, activity of the Ca\(_{\alpha,1,3}/\beta_{2\alpha} \) and Ca\(_{\alpha,1,2}/\beta_{2\alpha} \) channels (Fig. 3). Similar analysis of Rem-, Rad-, and Gem/Kir-mediated channel regulation have uniformly demonstrated a complete absence of functional channel activity although relatively few Ca\(_{\alpha,1,3}/\beta_{2\alpha} \) channel combinations have been thoroughly examined (8, 12). These results suggest that members of the RGK GTPase family might display subtle differences in their ability to regulate Ca\(^{2+} \) channel function based upon subunit composition. These studies also suggest that Rem2 regulation is more subtle than simple competition for a limiting intracellular pool of Ca\(_{\alpha,1,3}/\beta_{2} \) subunits as previously suggested (12), because Rem2, Rem, and Rad each display equivalent Ca\(_{\alpha,1,3}/\beta_{2} \) binding avidity, but Rem2 does not result in a complete blockade of Ca\(^{2+} \) channel activity (Fig. 4 and Ref. 8). The existence of multiple splice variants within both Ca\(_{\alpha,1,3} \) and \( \beta \)-subunit families stresses the need to explore this issue more thoroughly and may provide additional rational for the large number of splice variants within L-type Ca\(^{2+} \) channel subunits (13, 35).

Desensitization of glucose-induced insulin secretion in human pancreatic islets is induced in parallel with major glucose-specific Ca\(^{2+} \) abnormalities (19). Sustained exposure to high concentrations of glucose selectively impairs the ability of pancreatic islets to secrete insulin in acute glucose stimulation (18) and abnormal handling of Ca\(^{2+} \) by islet cells is one of the primary defects initiating impairments in insulin action, as well as initiating diabetic complications (29, 36, 37). Our data suggest that the elevation of basal Ca\(^{2+} \) following chronic glucose stimulation in \( \beta \)-cells increases Rem2, thus reducing the capacity of Ca\(^{2+} \) influx in response to membrane depolarization. Thus, elevated Rem2 expression would provide a critical negative feedback mechanism to ensure controlled insulin release following high glucose exposure. However, chronic stimulation, resulting in habitual Rem2 elevation, may also contribute to prolonged and pathologic suppression of insulin release. Indeed, it has been suggested that a decreased Ca\(^{2+} \)-ATPase activity may contribute to the desensitization of \( \beta \)-cells to glucose in NIDDM (38, 39). We suggest a novel mechanism in which glucose-mediated Ca\(^{2+} \) increases result in prolonged elevation of Rem2 that in turn results in reduced voltage-gated Ca\(^{2+} \) channel activity in \( \beta \)-islets. It will be important to investigate the contribution, if any, of elevated Rem2 levels to these phenomena.

In summary, we have identified Rem2 as a glucose-regulated gene in pancreatic \( \beta \)-islet cells whose increased expression is expected to inhibit
both voltage-gated Ca\(^{2+}\) channel activity and insulin secretion. We hypothesize that modulation of this previously unrecognized regulatory factor underlies a novel negative feedback mechanism in which elevated glucose levels would provide a selective reduction in L-type Ca\(^{2+}\) channel activity and thus decrease glucose-stimulated insulin secretion. Clearly additional studies will be needed to clarify the upstream signals that regulate Rem2 activity, the nature of Rem2-mediated regulation of voltage-gated Ca\(^{2+}\) channel activity, and the importance of Rem2-mediated changes in calcium-signaling pathways to the control of insulin release from pancreatic \(\beta\)-islets in both normal and diabetic states.

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