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Ca\textsubscript{V2.3} calcium channels control second-phase insulin release

Xingjun Jing,\textsuperscript{1} Dai-Qing Li,\textsuperscript{1} Charlotte S. Olofsson,\textsuperscript{1} Albert Salehi,\textsuperscript{1} Vikas V. Surve,\textsuperscript{1} José Caballero,\textsuperscript{1} Rosita Ivarsson,\textsuperscript{1} Ingmar Lundquist,\textsuperscript{1} Alexey Pereverzev,\textsuperscript{2} Toni Schneider,\textsuperscript{2} Patrik Rorsman,\textsuperscript{3} and Erik Renström\textsuperscript{1}

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Concerted activation of different voltage-gated Ca\textsuperscript{2+} channel isoforms may determine the kinetics of insulin release from pancreatic islets. Here we have elucidated the role of R-type Ca\textsubscript{V2.3} channels in that process. A 20% reduction in glucose-evoked insulin secretion was observed in Ca\textsubscript{V2.3}-knockout (Ca\textsubscript{V2.3–/–}) islets, close to the 17% inhibition by the R-type blocker SNX482 but much less than the 77% inhibition produced by the L-type Ca\textsuperscript{2+} channel antagonist isradipine. Dynamic insulin-release measurements revealed that genetic or pharmacological Ca\textsubscript{V2.3} ablation strongly suppressed second-phase secretion, whereas first-phase secretion was unaffected, a result also observed in vivo. Suppression of the second phase coincided with an 18% reduction in oscillatory Ca\textsuperscript{2+} signaling and a 25% reduction in granule recruitment after completion of the initial exocytotic burst in single Ca\textsubscript{V2.3–/–} β cells. Ca\textsubscript{V2.3} ablation also impaired glucose-mediated suppression of glucagon secretion in isolated islets (27% versus 58% in WT), an effect associated with coexpression of insulin and glucagon in a fraction of the islet cells in the Ca\textsubscript{V2.3–/–} mouse. We propose a specific role for Ca\textsubscript{V2.3} Ca\textsuperscript{2+} channels in second-phase insulin release, that of mediating the Ca\textsuperscript{2+} entry needed for replenishment of the releasable pool of granules as well as islet cell differentiation.

Introduction

Systemic glucose tolerance is orchestrated by the regulated release of insulin and glucagon from the β and α cells of the pancreatic islets of Langerhans. The α and β cells are electrically excitable and use electrical signals to couple changes in blood glucose concentration to stimulation or inhibition of hormone release. In both cell types, influx of extracellular Ca\textsuperscript{2+} through voltage-gated Ca\textsuperscript{2+} channels with resultant elevation of intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) triggers exocytosis of the hormone-containing secretory granules. Like other electrically excitable cells, both α and β cells contain several types of voltage-gated Ca\textsuperscript{2+} channel (1, 2). Assigning physiological functions to the respective Ca\textsuperscript{2+} channels is central to the understanding of electrical and secretory activities in these cells.

Voltage-gated Ca\textsuperscript{2+} channels are divided into 3 subfamilies: (a) L-type high voltage–activated (HVA) Ca\textsuperscript{2+} channel family that comprises the Ca\textsubscript{V1.1}, 1.2, 1.3, and 1.4 channels and is inhibited by dihydropyridines (DHPs) (1, 3, 4); (b) non–L-type HVA channels Ca\textsubscript{V2.1} (P/Q-type), 2.2 (N-type), and 2.3 (R-type) that are sensitive to ω-agatoxin IVA and ω-conotoxin GVIA and SNX482, respectively (1, 4, 5); and (c) the low voltage–activated (LVA) T-type Ca\textsuperscript{2+} channel family (Ca\textsubscript{V3.1}, 3.2, and 3.3). The latter subtype differs electrophysiologically from the HVA Ca\textsuperscript{2+} channels in opening transiently already upon modest depolarization (6, 7) and fulfilling important roles in pacemaker cells (8).

The chain of events that couples an elevation in blood glucose to initiation of β cell electrical activity is well established and involves facilitated transport of the sugar into the β cell and its subsequent metabolic degradation by glycolysis and mitochondrial oxidation, resulting in closure of the ATP-sensitive K\textsuperscript{+} channels (K\textsubscript{ATP} channels) and β cell depolarization, with resultant activation of voltage-gated Ca\textsuperscript{2+} channels and regulated insulin exocytosis (9). Glucose-stimulated insulin secretion consists of a rapid first-phase of insulin secretion that lasts for approximately 10 minutes before declining to near-basal levels, followed by less prominent but sustained second-phase insulin secretion that can last for several hours (10, 11). The cellular mechanisms underlying biphasic insulin release remain unclear, but consensus exists that an elevation in [Ca\textsuperscript{2+}]\textsubscript{i} is required for both first- and second-phase insulin secretion (12). Type 2 diabetes is associated with a shift from biphasic to monophasic insulin release (10), and it is therefore important to establish the cell biology of insulin release kinetics.

In mouse pancreatic β cells, 50% of the whole cell Ca\textsuperscript{2+} current exhibits properties typical for L-type channels, is inhibited by DHP channel blockers such as isradipine or nifedipine, and is activated by BayK8644. The molecular identity of the β cell L-type Ca\textsuperscript{2+} channel involved in insulin secretion has been debated, but recent studies have established that Ca\textsubscript{V1.2} or α\textsubscript{1C} channels play a decisive role (13, 14).

In mouse β cells, there is evidence suggesting that the secretory granules and the L-type Ca\textsuperscript{2+} channels assemble into a tight functional complex (13, 15–17). Thanks to this organization, the β cells are capable of exocytosis at rates comparable to those encountered in chromaffin cells, although the Ca\textsuperscript{2+} channel density is only 5–10% of that in the latter cell type. This arrangement bears strong resemblance to the tight coupling of P/Q-type Cav1.2 as well as N-type Cav2.2 channels to rapid synaptic transmission (18, 19). No such interaction has been demonstrated for the R-type Cav2.3 channels.

Nonstandard abbreviations used: [Ca\textsuperscript{2+}]\textsubscript{i}, intracellular Ca\textsuperscript{2+} concentration; Ca\textsubscript{V2.3–/–}, Ca\textsubscript{V2.3}-knockout; DHP, dihydropyridine; P, femtofarad(s); HVA, high voltage–activated; K\textsubscript{ATP}, ATP-sensitive K\textsuperscript{+}; IVA, low voltage–activated; RRP, readily releasable pool.

Conflict of interest: The authors have declared that no conflict of interest exists.

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R-type Ca2+ channels play distinct functional roles.

Whole-cell Ca2+ currents in islet cells from WT and CaV2.3–/– mice.

(A) Whole-cell Ca2+ currents (i) evoked by a 300-ms voltage-clamp depolarization (V) in WT CaV2.3+/+ (black) and CaV2.3–/– (gray) β cells. β cells were identified by exhibiting half-maximal Na+ channel inactivation at membrane potentials (V) lower than –100 mV (half-maximal inactivation at –102 mV; inset). (B) Average integrated current-voltage (Q-V) relationships. Data are mean values ± SEM in 10 WT (filled circles) and 10 CaV2.3–/– (shaded circles) β cells. *P < 0.05. (C) Whole-cell Ca2+ currents were recorded as in A, but using CaV2.3–/– β cells. The recordings were made under control conditions (lower gray line) in the presence of R-type Ca2+ channel blocker SNX482 (100 nM; black line) and after addition of L-type Ca2+ channel blocker isradipine (isr) (2 μM; upper gray line). (D) Average Q-V relationships representing mean values ± SEM in 4 CaV2.3–/– β cells under control conditions (shaded circles), in the presence of SNX482 (filled circles), and after addition of isradipine (open circles). *P < 0.05, **P < 0.01, control versus SNX482 plus isradipine. (E) Whole-cell Ca2+ currents were recorded as in A, but in α cells identified by Na+ channel inactivation at membrane potentials greater than –100 mV (half-maximal inactivation at –49 mV; inset). (F) Q-V relationships in α cells. Data represent average values ± SEM in 8 WT (filled circles) and 4 CaV2.3–/– (shaded circles) α cells. pC, picocoulombs.

By contrast, recent evidence in neurons suggests that R-type channels are physically detached from the exocytotic machinery (20) and are not involved in rapid neurotransmission in mossy fibers (21).

About one-quarter of the β cell whole-cell Ca2+ current is sensitive to the R-type Ca2+ channel blocker SNX482, but the role of R-type Ca2+ channels in insulin secretion remains elusive. A general CaV2.3-knockout (CaV2.3–/–) mouse has been established. It exhibits a relatively modest neurological phenotype, including altered pain responses (22), impaired spatial memory (23), and enhanced fear reaction (24). Pancreatic islets express the endocrine splice variant of CaV2.3 (25), and previous investigations of glucose homeostasis in CaV2.3–/– mice have demonstrated a slight glucose intolerance and reduced glucose-induced insulin secretion (26). Here we have extended these initial observations by performing in vivo glucose tolerance tests, dynamic measurements of phasic insulin secretion in situ, static pancreatic hormone–release experiments in isolated islets, as well as single cell recordings of whole-cell Ca2+ currents and exocytosis and ratiometric measurements of the cytoplasmic Ca2+ concentration in WT and CaV2.3–/– islets. We demonstrate that whereas R-type Ca2+ channels play a minor role in rapid insulin release, their significance becomes more apparent during second-phase secretion. These data point to an emerging picture where Ca2+ influx through different β cell Ca2+ channels play distinct functional roles.

Results

Whole cell Ca2+ currents in α and β cells from CaV2.3+/+ and CaV2.3–/– mice. Integrated Ca2+ current versus voltage (Q-V) relations were measured in dissociated single pancreatic islet cells using the perforated-patch whole cell configuration. Insulin-releasing β cells were identified by the absence of voltage-gated Na+ currents at membrane potentials relevant for β cell electrical activity, that is, between the β cell resting membrane potential (~70 mV) and the peak of the β cell action potential (~10 mV; Figure 1A, inset). In β cells from CaV2.3–/– mice, the integrated Ca2+ currents observed at membrane potentials less than or equal to ~10 mV were reduced compared with WT cells. At ~10 mV, the reduction averaged approximately 23% (P < 0.05; Figure 1B). The CaV2.3–/– mice exhibited a selective loss of a high-voltage Ca2+ current component, and current amplitudes at voltages below ~10 mV were not affected. The R-type Ca2+ channel blocker SNX482 (100 nM) had no effect on voltage-gated Ca2+ currents in CaV2.3–/– mice, whereas the L-type inhibitor isradipine (2 μM) significantly reduced Ca2+ influx approximately 60% at potentials greater than or equal to ~30 mV (P < 0.05; Figure 1, C and D).

Cells exhibiting Na+ currents when holding at ~70 mV (Figure 1E, inset) were classified as non-β cells and likely represent glucagon-releasing α cells (27). The frequency of α cells was much lower in dispersed islet cells from CaV2.3–/– mice than cells made from WT islets. Counting all cells that could be functionally defined as belonging to either group, only approximately 7% of the CaV2.3–/– cells could be classified as α cells versus approximately 27% of the WT cells. In 4 cells from CaV2.3–/– mice with the electrophysiological properties expected for α cells, the Ca2+ current amplitude was not different from that observed in WT cells (Figure 1, E and F).

Single cell exocytosis in CaV2.3–/– and CaV2.3+/+ mice. Ca2+-elicited exocytosis in β cells was monitored as increases in whole-cell membrane capacitance and was elicited by trains of 10 500-ms voltage
Effects of Ca\textsubscript{v.2.3} ablation on single-cell exocytosis in islet cells. (A) Exocytosis evoked by trains of 10 depolarizations (V) and monitored as increases in cell capacitance (ΔC) in WT Ca\textsubscript{v.2.3}/+ (black) and Ca\textsubscript{v.2.3}/− (gray) β cells. (B) Average total increase in capacitance evoked by the trains (ΔC\textsubscript{TOT}). Data are mean values ± SEM in 6 WT (black bars) and 7 Ca\textsubscript{v.2.3}/− (gray bars) β cells. *P < 0.05. (C) ΔC evoked by intracellular dialysis of a Ca\textsuperscript{2+}-containing patch electrode solution (free [Ca\textsuperscript{2+}], approximately 1.5 μM) in WT (black) and Ca\textsubscript{v.2.3}/− (gray) β cells. (D) Average rates of exocytosis (ΔC/Δt) ± SEM evoked by Ca\textsuperscript{2+} dialysis in 10 WT (black bars) and 10 Ca\textsubscript{v.2.3}/− (gray bars) β cells. (E and F) Exocytosis and average ΔC were recorded as in A and B, but results are from α cells, and averages represent 5 WT (black) and 3 Ca\textsubscript{v.2.3}/− (gray) α cells. (G and H) ΔC and average rates of exocytosis were recorded as in C and D, but the data are from α cells, and mean responses are from 6 WT (black) and 3 Ca\textsubscript{v.2.3}/− (gray) α cells. pF, picofarads.

Figure 3
Ca\textsuperscript{2+} homeostasis in WT and Ca\textsubscript{v.2.3}/− islets. (A) [Ca\textsuperscript{2+}i] in an intact WT Ca\textsubscript{v.2.3}/− islet assayed by ratiometric fura-2 measurements. The islets were stimulated at the time points indicated by the arrows in the continued presence of previously added stimuli. (B) [Ca\textsuperscript{2+}i] was determined as in A, but the experiment was performed in an intact Ca\textsubscript{v.2.3}/− islet. Recordings selected for display are representative of 7 and 9 separate experiments in WT and Ca\textsubscript{v.2.3}/− islets, respectively. Statistical significances are provided in the text.
in neuronal tissue, their ablation might affect pancreatic hormone release in vivo by indirect mechanisms and not by directly affecting α and β cell function. Insulin and glucagon secretion was therefore also investigated in isolated islets. The first set of experiments investigated the effects of L-type and R-type channel blockers isradipine and SNX482, respectively, in WT Ca_{2.3}^{\text{+/+}} islets (Table 2). Basal insulin release (1 mM glucose) was low and was unaffected by either channel blocker. Elevation of extracellular glucose to 20 mM stimulated insulin release more than 15-fold. Isradipine (2 μM) suppressed glucose-stimulated insulin release 77%, whereas SNX482 (100 nM) reduced insulin secretion 17% (Table 2). The latter value agrees favorably with the inhibition observed in capacitance measurements (see Figure 2, A and B). Glucagon release was measured under the same conditions. At 1 mM glucose glucagon release was high and remained unchanged by either isradipine or SNX482. Elevating the glucose concentration (20 mM) reduced glucagon secretion 70% (P < 0.001; low versus high glucose). At high glucose, blockade of R-type channels by SNX482 failed to affect glucagon release, whereas isradipine stimulated glucagon secretion under the same condition.

We next compared the effects of the Ca_{2.3}^{\text{+}} channel blockers with the consequences of Ca_{2.3}^{\text{+}} gene ablation on pancreatic hormone release (Table 3). In WT Ca_{2.3}^{\text{+/-}} islets, elevating glucose from 1 to 20 mM again stimulated insulin release more than 15-fold. In Ca_{2.3}^{\text{+/-}} islets, glucose-stimulated insulin release was reduced approximately 25% compared with WT. The effects of isradipine on glucose-stimulated insulin release were comparable in WT (66% reduction) and in Ca_{2.3}^{\text{+/-}} islets (68% suppression). Insulin secretion elicited by stimulation with high extracellular K⁺ (50 mM) resulted in a 75% enhancement of release relative to that observed in the presence of glucose alone in WT islets. A similar relative stimulation was observed in Ca_{2.3}^{\text{+/-}} islets (96%), but in absolute terms, the response in the latter type of islets was reduced 13% compared with WT islets. Surprisingly, whereas elevating glucose from 1 to 20 mM suppressed glucagon release 58% in WT Ca_{2.3}^{\text{+/-}} islets (P < 0.001; low versus high glucose), the inhibitory action of the sugar was severely impaired in the Ca_{2.3}^{\text{+/-}} islets and amounted to a mere 27% (P < 0.01 versus WT). Isradipine exerted divergent actions in WT and Ca_{2.3}^{\text{+/-}} islets. As discussed above, in WT islets the

**Table 1**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Insulin secretion (ng/islet/h)</th>
<th>Glucagon secretion (pg/islet/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM glucose</td>
<td>0.2 ± 0.03</td>
<td>38.4 ± 3.0</td>
</tr>
<tr>
<td>1 mM glucose + 2 μM isradipine</td>
<td>0.2 ± 0.05</td>
<td>36.7 ± 6.1</td>
</tr>
<tr>
<td>1 mM glucose + 100 nM SNX482</td>
<td>0.3 ± 0.03</td>
<td>38.5 ± 4.5</td>
</tr>
<tr>
<td>20 mM glucose + 20 mM glucose</td>
<td>2.6 ± 0.2A</td>
<td>12.8 ± 4.7A</td>
</tr>
<tr>
<td>20 mM glucose + 0.7 ± 0.08A</td>
<td>22.8 ± 2.1A</td>
<td></td>
</tr>
<tr>
<td>2 μM isradipine</td>
<td>2.3 ± 0.2A</td>
<td>16.2 ± 4.1A</td>
</tr>
</tbody>
</table>

Average values for insulin and glucagon secretion ± SEM measured in 60-minute batch incubations of 10 islets from WT Ca_{2.3}^{\text{+/-}} mice under conditions as indicated. Data are from 10 independent experiments. *P < 0.001, #P < 0.05 versus the same condition in 1 mM glucose.

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**Table 2**

<table>
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<tr>
<td>1 mM glucose + 100 nM SNX482</td>
<td>0.3 ± 0.03</td>
<td>38.5 ± 4.5</td>
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<td>20 mM glucose + 20 mM glucose</td>
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</tr>
</tbody>
</table>

Average values for insulin and glucagon release ± SEM measured in 60-minute batch incubations of 10 islets from WT Ca_{2.3}^{\text{+/-}} mice under conditions as indicated. Data are from 10 independent experiments. *P < 0.001, #P < 0.05 versus the same condition in 1 mM glucose.
L-type Ca\(^{2+}\) channel antagonist stimulated glucagon release 52% when applied in the presence of 20 mM glucose. By contrast, isradipine reduced glucagon secretion in \(\text{Ca}V2.3^{-/-}\) islets 32% under high-glucose conditions. Increasing extracellular K\(^{+}\) enhanced glucagon secretion massively, approximately 320% in WT and 220% in \(\text{Ca}V2.3^{-/-}\) islets.

Phasic insulin release measured by in situ pancreatic perfusion. To assess the role of Ca\(^{2+}\)V2.3 in dynamic insulin release, we performed in situ pancreatic perfusions with fractionated sampling. When WT \(\text{Ca}V2.3^{+/+}\) pancreata were perfused (Figure 4A) with a low-glucose solution (3.3 mM), insulin release was barely detectable. After increasing the glucose concentration to 16.7 mM (at \(t = 11\) minutes), first-phase insulin release was initiated with a 2-minute delay. The peak in first-phase insulin secretion was attained 2 minutes after onset of release (\(t = 15\) minutes) and measured 8.8 ± 1.7 ng/ml (\(n = 4\)). Insulin release then exhibited a transient nadir phase (\(t = 18–21\) minutes) during which release rates averaged 4–5 ng/ml, before accelerating again during second-phase insulin secretion to approximately 18 ng/ml at \(t = 36\) minutes and later. When the same experiment was repeated in \(\text{Ca}V2.3^{-/-}\) pancreata (Figure 4B), the peak in first-phase insulin secretion was only slightly reduced (19%) and measured 7.1 ± 2.1 ng/ml (NS; \(n = 4\)). More importantly, second-phase insulin release was markedly suppressed and averaged 9.4 ± 2.7 ng/ml at \(t = 40\) minutes, representing a 46% reduction compared with WT (\(P < 0.05\)).

Genetic ablation of Cav2.3 may result in compensatory mechanisms resulting in rearrangements of the Ca\(^{2+}\) channels in the β cell itself or neighboring α and δ cells. In addition, we wanted to verify that the observed effects of Ca\(^{2+}\)V2.3 on insulin secretion are not limited to the background C57B mouse strain. Similar experiments were therefore made in pancreata from standard inbred NMRI mice, but instead Ca\(^{2+}\)V2.3 channel function was inhibited by SNX482. In the absence of the channel inhibitor, first-phase insulin secretion was initiated with a 2-minute delay and peaked 1 minute, 30 seconds later when it measured 15.3 ± 2.5 ng/ml (\(n = 6\)).

### Table 3

<table>
<thead>
<tr>
<th>Insulin release</th>
<th>Glucagon release</th>
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</thead>
<tbody>
<tr>
<td>(ng/islet/h)</td>
<td>(pg/islet/h)</td>
</tr>
<tr>
<td><strong>WT</strong></td>
<td><strong>CaV2.3^{-/-}</strong></td>
</tr>
<tr>
<td>1 mM glucose</td>
<td>0.2 ± 0.02</td>
</tr>
<tr>
<td>20 mM glucose</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>20 mM glucose +</td>
<td>0.8 ± 0.09(^b)</td>
</tr>
<tr>
<td>2 μM isradipine</td>
<td>5.4 ± 0.2(^b)</td>
</tr>
<tr>
<td>20 mM glucose +</td>
<td>4.7 ± 0.3(^b)</td>
</tr>
</tbody>
</table>

Average values for insulin and glucagon release measured in 60-minute batch incubations of 10 WT \(\text{Ca}V2.3^{+/+}\) and \(\text{Ca}V2.3^{-/-}\) islets under conditions as indicated. Data represent means ± SEM of 18 experiments in each group. \(^aP < 0.01\), \(^bP < 0.05\) for comparisons between WT and \(\text{Ca}V2.3^{-/-}\); \(^cP < 0.001\), \(^dP < 0.01\), \(^eP < 0.05\) versus results obtained in 20 mM glucose alone.

Figure 4

**Dynamics of insulin release.** (A) Insulin release measured in WT \(\text{Ca}V2.3^{+/+}\) pancreata before and after increasing the glucose concentration in the perfusate from 3.3 mM to 16.7 mM at \(t = 11\) minutes. Samples were taken at 60-second intervals, except during the first 10 minutes after increasing the glucose concentration (\(t = 11\) to 21 minutes, as indicated by the gray bar) when the sample interval was 30 seconds. (B) Insulin release was measured as in A, but the experiments were performed in \(\text{Ca}V2.3^{-/-}\) mice. To facilitate comparison with WT, mean values measured under that condition are indicated by the dotted line. Data in A and B represent means ± SEM from 4 and 5 experiments in WT and \(\text{Ca}V2.3^{-/-}\) mice, respectively. (C) Insulin release was measured as in A, but the experiments were performed in NMRI mice. (D) Insulin release was measured as in C, but SNX482 (100 nM) was included in the high-glucose solution. To facilitate comparison with mean values measured in the absence of SNX482, these values are denoted by the dotted line. Data in C and D represent means ± SEM from 6 experiments performed with and without SNX482, respectively. Statistical significance is provided in Results.
Insulin release then decayed toward a lower plateau level of approximately 8 ng/ml and increased slightly to approximately 10 ng/ml during the second phase (Figure 4C). When SNX482 (100 nM) was included in the perfusate (Figure 4D), first-phase insulin release was largely unaffected, and peak values averaged 13.0 ± 3.7 ng/ml (n = 6). By contrast, second-phase insulin secretion was reduced more than 80% (e.g., 1.8 ± 0.6 ng/ml versus 10.0 ± 0.6 ng/ml at t = 40 minutes in the presence or absence of SNX482, respectively; P < 0.01). It was verified that the glucose concentration in the effluent medium was identical in all experiments.

**Discussion**

Insulin-producing β cells contain multiple types of Ca\(^{2+}\) channel. Whereas the role of L-type Ca\(^{2+}\) channels in insulin secretion is amply documented, that of the non-L-type Ca\(^{2+}\) channels is less well understood. The advent of subtype-specific Ca\(^{2+}\) channel blockers in combination with the generation of transgenic knockout mice provides a unique opportunity for an in-depth analysis of the function(s) fulfilled by the different Ca\(^{2+}\) channel subtypes. Here we have used SNX482 and Cav2.3-null mice to study the significance of R-type Cav2.3 Ca\(^{2+}\) channels in islet insulin and glucagon secretion in vivo and in vitro.

**The role of Cav2.3 channels for β cell [Ca\(^{2+}\)]\(_i\) homeostasis and insulin secretion.** Here we demonstrate that pharmacological inhibition of R-type Cav2.3 Ca\(^{2+}\) channels using SNX482 does not affect first-phase insulin secretion, but reduces second-phase release a dramatic 80%. A similar preferential effect on late-phase insulin secretion is observed in Cav2.3\(^{-/-}\) mice (Figure 4). At the single β cell level, close inspection of the capacitance recordings (Figure 2, A and B) reveals that the initial component of exocytosis is not much affected in Cav2.3\(^{-/-}\) β cells. In fact, the overall reduction results exclusively from suppression of the late component of exocytosis (elicited by the third and subsequent depolarizations). We have previously demonstrated that opening of L-type Cav1.2 Ca\(^{2+}\) channels is tightly associated with rapid exocytosis and first-phase secretion. Thus, it appears that Ca\(^{2+}\) entry via T-type Cav1.2 and R-type Cav2.3 channels have distinct intracellular effects.

Cav2.3 ablation is associated with a 23% decrease in β cell Ca\(^{2+}\) current in Cav2.3\(^{-/-}\) mice (Figure 1). This is in good agreement with the response to acute application of SNX482 in WT mice (13), suggesting that there is little compensatory upregulation of other Ca\(^{2+}\) channels in the knockout mice. We acknowledge that Cav2.3 channel activity may represent only part of the R-type current component (24, 32). In addition, other reports suggest that all R-type current components are not blocked by SNX482 with the same efficacy (33). The observation that SNX482 fails to affect whole cell Ca\(^{2+}\) currents in Cav2.3\(^{-/-}\) β cells (Figure 1, C and D), however, indicates at least that SNX482, at the concentration used here (100 nM), does not affect any Ca\(^{2+}\) channels other than Cav2.3, but we cannot exclude the contribution of an SNX482-insensitive R-type Ca\(^{2+}\) current component. Indeed, approximately 40% of the whole cell Ca\(^{2+}\) current is unaffected by either SNX482 or the L-type blocker isradipine in Cav2.3\(^{-/-}\) β cells. Although most of this current probably represents P/Q-type Ca\(^{2+}\) currents as previously shown for WT β cells (13), it is possible that part of it reflects a SNX482-resistant portion of the R-type Ca\(^{2+}\) current.

**How do Cav2.3 Ca\(^{2+}\) channels regulate second-phase insulin secretion?** Secretory granules in pancreatic β cells can be classified according to their release competence (34). A limited (1–5%) pool is immediately available for rapid exocytosis upon stimulation (readily releasable pool, RRP). Once this pool of granules has been...
released, exocytosis proceeds at a slower rate determined by the supply of granules newly mobilized from a much larger reserve pool (35). The observation that only a late component of exocytosis contributes to glucose-stimulated (53) or paracrine (27, 29) glucagon release at a significant concentration (53) indicates that glucagon secretion is controlled by different mechanisms. High glucose results in strong enhancement of the exocytotic capacity (36). It is of interest that this effect is mediated by a global rather than a localized increase in [Ca\(^{2+}\)], and is operational already at concentrations as low as 200–300 nM, that is, 10-fold lower than those required to elicit fast exocytosis. Thus, even moderate reduction of Ca\(^{2+}\) entry and [Ca\(^{2+}\)], following inhibition of Ca\(^{2+}\)Ca\(^{2+}\) channels can have strong effects on insulin secretion in the longer term by inhibition of granule recruitment. Presumably, L-type Ca\(^{2+}\) channels still trigger granule exocytosis, but once the RRP is depleted, the supply of new granules for release becomes rate limiting. This would explain how ablation of the approximately 20% Ca\(^{2+}\)Ca\(^{2+}\) channel component can result in approximately 50% (knockout) to 80% (SNX482) reduction of second-phase insulin secretion. Thus, it seems justifiable to conclude that Ca\(^{2+}\) entry via R-type Ca\(^{2+}\) Ca\(^{2+}\) channels is functionally (and perhaps spatially) linked to granule mobilization and priming of insulin granules for release.

Ca\(^{2+}\) ablation and glucagon secretion. Ca\(^{2+}\) ablation is associated with disturbances of glucagon secretion. In isolated islets, the ability of glucose to suppress glucagon secretion is severely impaired (Table 3). These abnormalities are also detectable at the systemic level. In the in vivo glucose challenge test, 3-minute measurements (Figure 2, E–H) provide no support for this notion. Another explanation is that the α/β cells exhibit poorly regulated exocytosis and constitutively release both glucagon and insulin. This idea is reinforced by the observation that basal insulin release in Ca\(^{2+}\)–/– islets is higher than in WT islets (0.4 ± 0.05 versus 0.2 ± 0.02 ng/islet/hour in Ca\(^{2+}\)–/– and WT, respectively; Table 3). Yet another possibility is that glucagon coreleased with insulin from the α/β cells potentiates further secretion by a cAMP-dependent mechanism. We have reported elsewhere that glucagon increases β cell exocytosis 5-fold (38). The significance of paracrine mechanisms within the islet is apparent from the strong stimulatory action of isradipine on glucagon secretion in WT islets (Tables 2 and 3). The latter effect may reflect the relief from paracrine inhibition of glucagon secretion exerted by Zn\(^{2+}\) and GABA cosecreted with insulin from the β cells (39, 40).

Preliminary data from our laboratory indicate that R-type Ca\(^{2+}\) Ca\(^{2+}\) channels play a decisive role in somatostatin secretion from the β cells (Q. Zhang, A. Salehi, E. Renström, and P. Rorsman, unpublished observations). We can exclude that the loss of physiological glucose inhibition of glucagon secretion is secondary to relief from paracrine inhibition by somatostatin, however, since the dysregulation of glucagon secretion in Ca\(^{2+}\)–/– islets is not mimicked by SNX482.

Role of Ca\(^{2+}\) in islet cell differentiation. A surprising finding in the present study is that the majority of the glucagon immunoreactive cells in Ca\(^{2+}\)–/– islets coexpress insulin, indicating that Ca\(^{2+}\) channels play a role in the development of mature α cells. A transient peak in Ca\(^{2+}\) channel expression in glial cells along specific CNS pathways has been demonstrated to coincide with postnatal myelination of the white matter in the rat (41). It can be speculated that Ca\(^{2+}\) channel expression exerts a similar action in defining the differentiated mature islet cell lineages, but the exact underlying mechanism remains to be established.

Pathophysiological implications. Although R-type Ca\(^{2+}\) channels appear to be of little importance for first-phase insulin secretion and their contribution becomes apparent only during late exocytosis/second-phase insulin secretion, this does not mean that they are unimportant for systemic glucose homeostasis. Indeed, the Ca\(^{2+}\)–/– mice exhibited basal hyperglycemia (Table 1). In humans, Ca\(^{2+}\) is encoded by the CACNA1E gene, which is located on chromosome 1q25–31. Interestingly, a chromosomal region around 1q25 reveals linkage to type 2 diabetes in several independent studies in different populations. These include early onset type 2 diabetes in Pima Indians (42), Utah Caucasians (43), and English sib pairs (44). In addition, the 1q25–32 region demonstrates linkage with elevated blood glucose levels in the Framingham Heart Study (45). It is also pertinent that the above region confers deficient insulin secretion in type 2 diabetes in Pima Indians (46). Type 2 diabetes is a multifactorial polygenic disorder. Clearly, polymorphisms in the CACNA1E gene alone are not sufficient to trigger disease. Given the present functional data, however, it is tempting to speculate that dysfunctional Ca\(^{2+}\) Ca\(^{2+}\) channels may frequently be involved in creating the disturbed β cell phenotype in type 2 diabetes.

Methods

Experimental animals. As previously reported (26), the CACNA1E gene encoding Ca\(^{2+}\) was disrupted in vivo by deleting a region containing exon 2 on mating Ca\(^{2+}\)–/– and deleter mice on a C57Bl/6 background.
that expresses Cre recombinase constitutively under the control of the CMV promoter. Ca.2.3+/– mice containing 1 cre transgene were inbred, and pups with the Ca.2.3+/– genotype were selected and transferred by embryo transfer into a SPF facility. The transfer included breeding with C57Bl/6 mice and resulted in heterozygous Ca.2.3+/– mice. Only cre-negative pups were selected and inbred, yielding either Ca.2.3+/– or Ca.2.3+/– mice. Thus, the Ca.2.3+/– and Ca.2.3+/– mice used in this study have an identical genetic background. In Figure 4, inbred NMRI mice purchased from Charles River Wiga GmbH were used.

The mice were housed at a constant temperature (22–23°C) and 12-hour light cycles (6:00 a.m.–6:00 p.m.), with access to standard pellet food and water ad libitum. All experiments were evaluated and approved by the local ethical committee Malmö/Lund djurförsöksetiska nämnd, Lund District Court, Sweden.

Islet isolation and islet cell preparation. The mice were sacrificed by cervical dislocation, and collagenase was administered into the pancreas by retrograde injection via the pancreatic duct. After 15–20 minutes' incubation at 37°C, the islet suspension was washed 4 times with HBSS (4°C) before being manually collected. The islets were cultured in RPMI-1640 medium (Invitrogen Corp.) supplemented with 10% FCS, penicillin, and streptomycin. For the preparation of single cells, islets obtained by collagenase digestion were dissociated by incubation and gentle trituration in Ca²⁺-free medium. The resulting cell suspension was centrifuged, the supernatant discarded, and the pellet (containing the cells) was resuspended in RPMI-1640 medium, plated on plastic Nunc 35-mm Petri dishes, and maintained in tissue culture for up to 2 days.

Electrophysiology. The measurements were conducted using an EPC-10 patch-clamp amplifier in conjunction with the PULSE software suite (version 8.53; HEKA Elektronik). Whole-cell Ca²⁺ currents were measured in intact cells using the perforated-patch whole-cell channel aperture (Figure 1) using a pipette solution consisting of 76 mM Cs₂SO₄, 10 mM NaCl, 10 mM CsCl, 1 mM MgCl₂, 5 mM HEPES (pH 7.35 with KOH), and 0.24 mg/ml amphotericin B (47). Exocytosis was monitored as increases in cell capacitance using the sine + DC mode of the lock-in amplifier included in the PULSE software and the standard whole cell configuration. When eliciting exocytosis by trains of 10-50 ms voltage clamp depolarizations (Figure 2, A and E), the pipette solution consisted of 125 mM Cs-glutamate, 10 mM CsCl, 10 mM NaCl, 1 mM MgCl₂, 5 mM HEPES, 3 mM Mg-ATP, 0.1 mM cAMP, and 0.05 mM EGTA (pH 7.2 with CsOH). In Figure 2, C and G, this pipette solution was slightly modified to include 10 mM EGTA and 9 mM CaCl₂, and all Cs⁺ salts were replaced by corresponding K⁺ salts. The resulting free intracellular Ca²⁺ concentration of this Ca²⁺/EGTA buffer was estimated to 1.5 μM using the binding constants of Martell and Smith (48, 49). The extracellular bath solution consisted of 138 mM NaCl, 5.6 mM KCl, 2.6 mM CaCl₂, 1.2 mM MgCl₂, 5 mM glucose, and 5 mM HEPES (pH 7.4 with NaOH). In the recordings of whole-cell Ca²⁺ currents (Figure 1) and depolarization-evoked exocytosis (Figure 2, A and E), 20 mM of NaCl was equimolarly replaced by the K⁺ channel blocker TEA-Cl to facilitate the separation of the small voltage-gated Ca²⁺ currents from the large outward K⁺ current. The DHP isradipine (Pfizer Inc.) was prepared as stock solution in DMSO (final concentration less than or equal to 0.1%). The R-type blocker SNX482 (Peptide Institute Inc.) was dissolved directly in the perfusate or, when comparisons involved more than 2 groups, ANOVA.

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