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RESEARCH ARTICLE

$Ca_V1.2$ and $Ca_V1.3$ channel hyperactivation in mouse islet β cells exposed to type 1 diabetic serum

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Abstract The voltage-gated Ca^{2+} (Ca_V) channel acts as a key player in β cell physiology and pathophysiology. β cell Ca_V channels undergo hyperactivation subsequent to exposure to type 1 diabetic (T1D) serum resulting in increased cytosolic free Ca^{2+} concentration and thereby Ca^{2+}-triggered β cell apoptosis. The present study was aimed at revealing the subtypes of Ca_V1 channels hyperactivated by T1D serum as well as the biophysical mechanisms responsible for T1D serum-induced hyperactivation of β cell Ca_V1 channels. Patch-clamp recordings and single-cell RT-PCR analysis were performed in pancreatic β cells from Ca_V1 channel knockout and corresponding control mice. We now show that functional $Ca_V1.3$ channels are expressed in a subgroup of islet β cells from $Ca_V1.2$ knockout mice ($Ca_V1.2^{-/-}$). T1D serum enhanced whole-cell Ca_V currents in islet β cells from $Ca_V1.3$ knockout mice ($Ca_V1.3^{-/-}$). T1D serum increased the open probability and number of functional unitary Ca_V1 channels in $Ca_V1.2^{-/-}$ and $Ca_V1.3^{-/-}$ β cells. These data demonstrate that T1D serum hyperactivates both $Ca_V1.2$ and $Ca_V1.3$ channels by increasing their conductivity and number. These findings suggest $Ca_V1.2$ and $Ca_V1.3$ channels as potential targets for anti-diabetes therapy.

Keywords Apolipoprotein · Calcium channel · Genetic ablation · Patch-clamp recording · Single-cell RT-PCR · Type 1 diabetes

GY and YS contributed equally to this study.

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Abbreviations

Ca_V	Voltage-gated calcium
$Ca_V1.2^{-/-}$	$Ca_V1.2$ subunit knockout
$Ca_V1.3^{-/-}$	$Ca_V1.3$ subunit knockout
DHP	Dihydropyridine
T1D	Type 1 diabetic

Introduction

The voltage-gated calcium (Ca_V) channel Ca_V1 is critical to β cell physiology and pathophysiology [1–5]. The two Ca_V1 channel subtypes $Ca_V1.2$ and $Ca_V1.3$ are identified in β cells including human and rat islet β cells [1, 2]. The level of $Ca_V1.3$ subunit mRNA is 13.5 and 2.5 times higher than that of $Ca_V1.2$ subunit mRNA in human and rat islet β

cells, respectively [6, 7]. Ca_V1 channel subtypes in mouse islet β cells are still a debated topic. Some studies claim that the mouse islet β cell only possesses $Ca_V1.2$ channels, since commonly used concentrations of dihydropyridines (DHPs) can no longer alter Ca_V currents when $Ca_V1.2$ subunits or their DHP sensitivity are genetically ablated [8, 9]. However, these studies ignore the fact that the DHP sensitivity of $Ca_V1.3$ channels is lower than that of $Ca_V1.2$ channels. For example, nifedipine at 10 μM only blocks about 40 % of the $Ca_V1.3$ channel-mediated Ca^{2+} currents, whereas nifedipine at 100 nM can completely ablate $Ca_V1.2$-mediated Ca^{2+} currents in the mouse hair cell [10]. Furthermore, $Ca_V1.3$ subunit mRNA and protein are convincingly detected in mouse islet β cells [11–13]. Genetic ablation of mouse $Ca_V1.3$ subunits abrogates basal insulin secretion [12]. The presence of functional $Ca_V1.3$ channels in mouse islet β cells is therefore still a matter of uncertainty [1, 2].

Dysregulation of β cell Ca_V1 channels impairs β cell function and even kills β cells resulting in diabetes [2, 8, 14–21]. The reduced expression of $Ca_V1.2$ and $Ca_V1.3$ subunits and the consequent decrease in Ca_V1 channel activity blunt stimulus–secretion coupling in Zucker diabetic fatty rat β cells [15]. β cell-specific $Ca_V1.2^{-/-}$ selectively abrogates the initial rapid component of insulin exocytosis and thereby first phase insulin secretion [8]. Trinucleotide expansion in the human $Ca_V1.3$ gene has been revealed in a subgroup of patients with type 2 diabetes [16, 17]. Point mutation of the human $Ca_V1.2$ subunit results in hyperactivation of β cell Ca_V1 channels, thereby causing excessive insulin secretion, episodic hypoglycemia and even death of some affected individuals [18]. Type 1 diabetic (T1D) serum hyperactivates β cell Ca_V1 channels, leading to increased cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and thereby β cell apoptosis [2, 19, 20]. However, for a long time the lack of β cell-specific Ca_V1 knockout mouse models prevented us from understanding if T1D serum affects either Cav1.2 or Cav1.3 channels or both, and the underlying biophysical mechanisms.

In the present study, combined application of Ca_V1 knockout mouse models, patch-clamp techniques and single-cell RT-PCR analysis leads to satisfactory circumvention of the aforementioned issues and results in the following novel observations. First, a subgroup of mouse islet $Ca_V1.2^{-/-}$ β cells accommodate functional $Ca_V1.3$ channels. This solves the uncertainty about the presence and function of $Ca_V1.3$ channels in the mouse islet β cell and provides a convenient small animal model for further investigations of physiology and pathophysiology of β cell $Ca_V1.3$ channels. Second, T1D serum hyperactivates both $Ca_V1.2$ and $Ca_V1.3$ channels in the β cell. This adds a new dimension to the molecular pathogenesis of type 1 diabetes. Finally, T1D serum-induced hyperactivation of β cell Ca_V1 channels results from both increased activity and elevated number of the channels. This offers a mechanistic interpretation of T1D serum-induced hyperactivation of β cell Ca_V1 channels.

Materials and methods

Animals

β cell-specific $Ca_V1.2$ subunit-knockout ($Ca_V1.2^{-/-}$) mice and their corresponding heterozygous ($Ca_V1.2^{+/-}$) control mice were provided by Dr. Franz Hofmann (Institut für Pharmakologie und Toxikologie, Technische Universität München, Germany) [8]. General $Ca_V1.3$ subunit-knockout ($Ca_V1.3^{-/-}$) mice and wild-type control ($Ca_V1.3^{+/+}$) mice were provided by Dr. Jörg Striessnig (Institute of Pharmacy, Pharmacology and Toxicology, University of Innsbruck, Austria) [22]. The background mouse strain is C57BL/6.

Isolation of islets of Langerhans

Islets of Langerhans were isolated from adult control and mutant mice [23]. Briefly, the mice were killed by cervical dislocation. The pancreas was quickly dissected and cut into small pieces. Subsequently, the cut pancreatic tissue was digested with collagenase A (Roche, Basel, Switzerland) under vigorous shaking for 10–12 min at 37 °C. The digested pancreatic tissue was rinsed twice with a solution containing (in mM) 125 NaCl, 5.9 KCl, 1.28 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, 0.1 % bovine albumin and 3 glucose, pH 7.4. Islets were hand picked-up under microscope.

Islet cell culture and treatments

The isolated islets of Langerhans were dispersed into single islet cells in a Ca^{2+}-free medium containing (in mM) 125 NaCl, 5.9 KCl, 2 EGTA, 25 HEPES and 1 % bovine serum albumin, pH 7.4 [24]. The cells were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum, 2 mM L-glutamine and 100 U/100 μg/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified 5 % CO_2 incubator for 2–3 days [25]. Subsequently, the cells were treated with 10 % healthy serum- or 10 % T1D serum-containing RPMI 1640 medium overnight before electrophysiological recordings.

Preparation of healthy serum or type 1 diabetic serum

Sera from healthy blood donors and T1D patients were collected and heat-inactivated at 56 °C for 30 min.

Electrophysiological recordings

Whole-cell and single Ca_V channel currents were recorded by using conventional whole-cell, perforated whole-cell and cell-attached configurations of the patch-clamp technique, respectively [4, 26–28]. Pipettes were pulled from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) on a horizontal programmable puller (DMZ Universal Puller, Zeitz-Instrumente, Augsburg, Germany) and then fire-polished and coated with Sylgard close to their tips. Typical electrode resistance was 4–6 MΩ. For conventional whole-cell recordings, pipettes were filled with a solution consisting of (in mM) 150 *N*-methyl-D-glucamine, 125 HCl, 10 EGTA, 1.2 $MgCl_2$, 3 MgATP, and 5 HEPES (pH 7.15). In perforated whole-cell patch-clamp experiments, the pipette solution contained (in mM) 76 Cs_2SO_4, 1 $MgCl_2$, 10 KCl, 10 NaCl, and 5 HEPES (pH 7.35), as well as amphotericin B (0.24 mg/ml) to permeabilize the cell membrane and allow low-resistance electrical access without breaking the plasma membrane patch. Cells used for both conventional and perforated whole-cell recordings were bathed in a solution containing (in mM) 138 NaCl, 10 tetraethylammonium chloride, 10 $CaCl_2$, 5.6 KCl, 1.2 $MgCl_2$, 5 HEPES and 3 glucose (pH 7.4). After obtaining a seal, the holding potential was set at −70 mV during the course of an experiment. Depolarizing voltage pulses (100 ms) were made from a holding potential of −70 mV to a test potential of 0 mV or several test potentials from −60 to 50 mV in 10 mV increments at 0.5 Hz. The selective Ca_V1 channel blocker nimodipine (10 μM) was used to block whole-cell $Ca_V1.3$ currents. Cell-attached single-channel recordings were made with Ba^{2+} as the charge carrier. Pipettes were filled with a solution containing (in mM): 110 $BaCl_2$, 10 TEA-Cl and 5 HEPES-$Ba(OH)_2$ (pH 7.4). A depolarizing external recording solution, containing (in mM) 125 KCl, 30 KOH, 10 EGTA, 2 $CaCl_2$, 1 $MgCl_2$, 5 HEPES–KOH (pH 7.15), was used to bring the intracellular potential to ∼0 mV. Voltage pulses (200 ms) were applied at a frequency of 0.5 Hz to depolarize cells from a holding potential of −70 mV to a membrane potential of 0 mV. Resulting currents were recorded with an Axopatch 200B amplifier (Molecular Devices, Foster City, CA), filtered at 1 kHz and digitized at 5 kHz. All recordings were made at room temperature (about 22 °C). Acquisition and analysis of data were done using the software program pCLAMP10 (Molecular Devices).

Single-cell RT-PCR

The entire islet cell was harvested with a glass pipette after a patch-clamp recording and stored in a PCR tube with 10 μl 1× Taq DNA polymerase reaction buffer containing $MgCl_2$ (Promega, Madison, WI) at −70 °C for later use. The QIAGEN OneStep RT-PCR Kit (Valencia, CA) was used to detect insulin mRNA in harvested cells according to the manufacturer's instructions. The RNasin Plus RNase Inhibitor (Promega) was added to avoid damaging mRNA. The insulin primer pair for RT-PCR analysis was synthesized by Sigma-Aldrich (St. Louis, MO) and consisted of the forward primer 5′-CAGCAAGCAGGTCATTGTTT-3′ and the reverse primer 5′-CAGTAGTTCTCCAGCTGGTAGA-3′. These primers were added at a final concentration of 0.6 μM to the reaction mix. The reaction was amplified for 35 cycles of 94 °C for 1 min, 62 °C for 1 min and 72 °C for 1 min. The amplified PCR products were detected by 2 % agarose gel electrophoresis and ethidium bromide staining.

Statistical analysis

Data are presented as mean ± SEM. The statistical significance of differences between multiple groups was assessed by one-way ANOVA, followed by least significant difference (LSD) test. The statistical difference between two groups was determined by unpaired Student's *t* test or Mann–Whitney *U* test. The significance level was set to 0.05 or 0.01.

Results

Characterization of type 1 diabetic serum

Table 1 summarizes characterization of type 1 diabetic serum. All healthy sera are negative for antibodies to glutamic acid decarboxylase (GAD), islet cells (ICA), and tyrosine phosphatase IA2 (IA-2). T1D sera were prescreened and all of them induced a higher increase in $[Ca^{2+}]_i$ upon depolarization with KCl, compared to healthy sera [20].

Table 1 Characterization of type 1 diabetic sera

Sex	Age at onset of type 1 diabetes (years)	Medication[a]	GAD	ICA	IA-2
Male	28	No	+	nd	nd
Male	34	No	+	−	−
Male	19	No	+	+	+
Female	34	No	+	+	−
Male	24	No	+	−	−
Male	27	No	+	nd	nd

+ presence, − absence, *nd* no data available, *GAD* antibodies to glutamic acid decarboxylase, *ICA* antibodies to islet cells, *IA-2* antibodies to tyrosine phosphatase IA2

[a] Insulin was the only medication administered

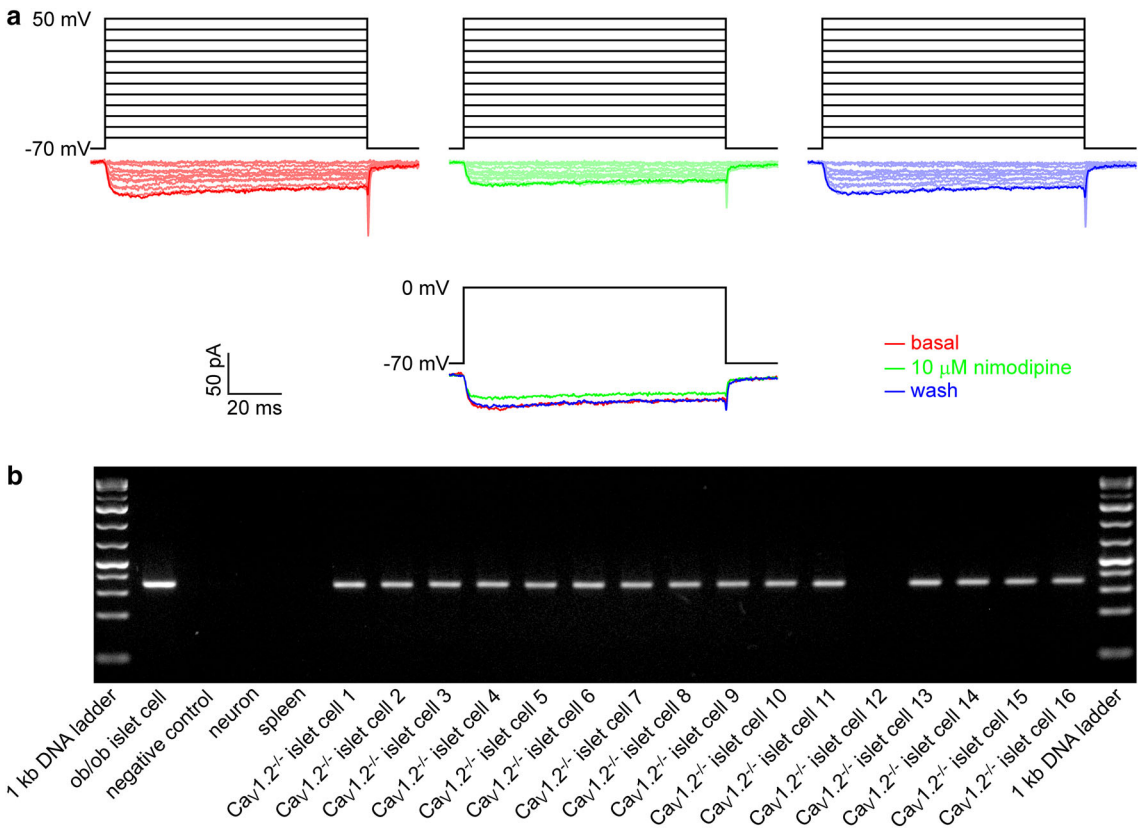

Fig. 1 $Ca_V1.3$ channels are functionally expressed in a subgroup of mouse islet $Ca_V1.2^{-/-}$ β cells. **a** Examples of whole-cell Ca_V current traces evoked by a set of depolarizing voltage pulses (*upper panel*) and those generated by single voltage pulses (*lower panel*) in an islet cell from the β cell-specific $Ca_V1.2^{-/-}$ mouse before (*red*), during exposure to 10 μM nimodipine (*green*) and after washing treatment (*blue*). **b** RT-PCR analysis of cDNA obtained from single islet cells of the β cell-specific $Ca_V1.2^{-/-}$ mouse and from the positive control ob/ob islet cell and negative controls neuron, spleen and sterile ultrapure water with specific primers for insulin (344-bp amplicon)

Functional $Ca_V1.3$ channels are present in a subgroup of mouse islet $Ca_V1.2^{-/-}$ β cells

To test for the presence of functional $Ca_V1.3$ channels in the mouse islet $Ca_V1.2^{-/-}$ β cell, we examined if Ca_V1 currents are present in the mouse islet $Ca_V1.2^{-/-}$ β cell by combining whole-cell patch-clamp analysis and single-cell RT-PCR assay. Figure 1a shows that a β cell-specific $Ca_V1.2^{-/-}$ islet cell, which is insulin mRNA-positive ($Ca_V1.2^{-/-}$ islet cell 8), displayed clear Ca_V1 currents, which were blocked by the specific Ca_V1 channel blocker nimodipine. Figure 1b shows insulin mRNA expression in single islet cells from the β cell-specific $Ca_V1.2^{-/-}$ mouse and in control samples including the positive control ob/ob islet cell and the negative controls neuron, spleen and sterile ultrapure water. Sixteen $Ca_V1.2^{-/-}$ islet cells were subjected to nimodipine-sensitive current measurements and then single-cell RT-PCR assay. These cells did not display any detectable voltage-gated Na^+ currents. Their capacitance is greater than 7 pF. Although these electrophysiological criteria strongly indicate that they are β cells, their β cell identity was still confirmed by insulin mRNA positivity. A standard RT-PCR protocol using insulin-specific primers revealed the expected 344-bp amplicon for insulin in 15 islet cells (Fig. 1b). Among them, three insulin mRNA-positive cells are sensitive to nimodipine. Hence, 20 % insulin mRNA-positive $Ca_V1.2^{-/-}$ islet cells (3 out of 15) were estimated to express functional $Ca_V1.3$ channels.

Type 1 diabetic serum increases the open probability and number of single Ca_V1 channels in mouse islet $Ca_V1.2^{-/-}$ β cells

In this set of experiments, we first validated the effectiveness of the T1D serum used on Ca_V1 channel hyperactivation in islet β cells isolated from control mice. Figure 2a shows representative Ba^{2+} currents mediated by single Ca_V1 channels in a control mouse islet β cell subjected to treatment with healthy serum and a control mouse islet β cell incubated with T1D serum. Incubation with T1D serum not only made Ca_V1 channels dwell longer in

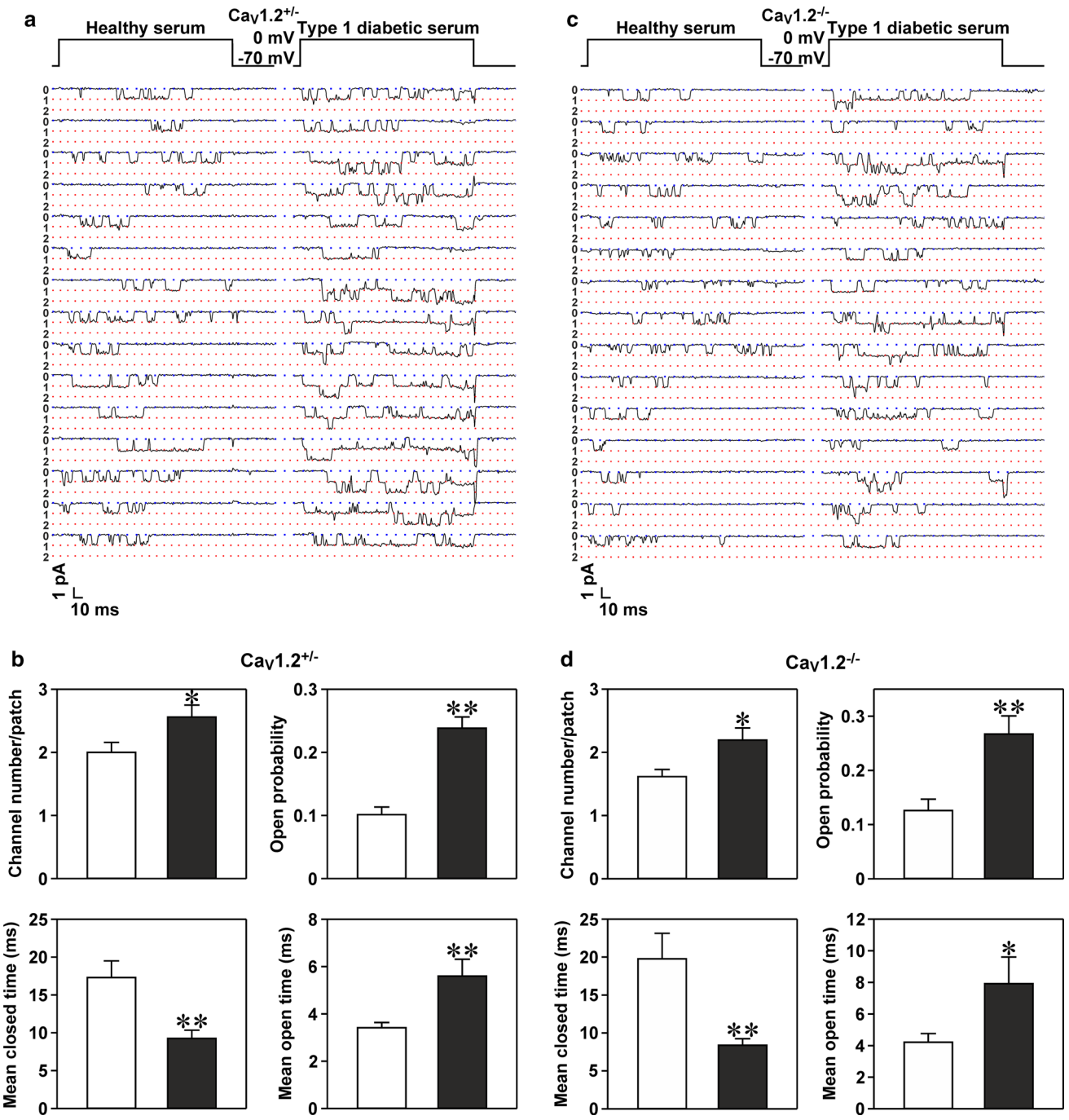

Fig. 2 Type 1 diabetic serum hyperactivates Ca_V1 channels through elevating their open probability and number in $Ca_V1.2^{+/-}$ and $Ca_V1.2^{-/-}$ β cells. **a** Sample unitary Ca_V1 channel current traces obtained from a plasma membrane patch attached to a $Ca_V1.2^{+/-}$ β cell exposed to either healthy serum or T1D serum. The plasma membrane patch was held at −70 mV and depolarized for 200 ms to a test potential of 0 mV. **b** Average number, open probability, mean closed time and mean open time of single Ca_V1 channels registered in plasma membrane patches of $Ca_V1.2^{+/-}$ β cells incubated with healthy serum (*open bars*, $n = 33$ cells) and T1D serum (*closed bars*, $n = 34$ cells), respectively. **c** Representative unitary Ca_V1 channel current traces recorded in a plasma membrane patch of a $Ca_V1.2^{-/-}$ β cell following exposure to healthy serum and T1D serum, respectively. **d** Average number, open probability, mean closed time and mean open time of single Ca_V1 channels measured in plasma membrane patches attached to $Ca_V1.2^{-/-}$ β cells exposed to either healthy serum (*open bars*, $n = 26$ cells) or T1D serum (*closed bars*, $n = 26$ cells). $*p < 0.05$ and $**p < 0.01$ vs. healthy serum

their open state, but also increased the number of functional Ca_V1 channels, reflected by more unitary conductance levels (more layers of unitary Ba^{2+} currents) (Fig. 2a). Statistical analysis revealed that treatment with T1D serum significantly altered four parameters of single Ca_V1 channels in control mouse islet β cells (Fig. 2b).

Compared to healthy serum treatment, T1D serum significantly increased the number, open probability and mean open time of Ca_V1 channels and significantly decreased the mean closed time of these channels in control mouse islet β cells (Fig. 2b).

The verification of the presence and function of $Ca_V1.3$ channels in mouse islet $Ca_V1.2^{-/-}$ β cells prompted us to clarify if T1D serum hyperactivates $Ca_V1.3$ channels in these cells. A prerequisite for tackling this issue is a simple and reliable discrimination between $Ca_V1.3$ channel-negative and $Ca_V1.3$ channel-positive β cells, since only a small proportion of mouse islet $Ca_V1.2^{-/-}$ β cells express functional $Ca_V1.3$ channels. It is difficult to identify $Ca_V1.3$ channel-positive β cells during a whole-cell patch-clamp recording. Unitary Ca_V1 channel currents, characterized by a large unitary Ba^{2+} conductance with long-lasting openings, can be convincingly recognized and discriminated from unitary Ca_V2 and Ca_V3 channel currents, exhibiting a smaller unitary Ba^{2+} conductance with shorter-lasting openings, during a cell-attached patch-clamp recording [1, 2]. The occurrence of such unitary Ca_V1 channel currents verifies the presence of functional $Ca_V1.3$ channels since the $Ca_V1.2$ channel was genetically ablated and two other members of the Ca_V1 family, i.e., $Ca_V1.1$ and $Ca_V1.4$, do not express in mouse islet β cells [1, 2]. Therefore, we performed cell-attached single-channel recording rather than whole-cell current measurements in $Ca_V1.2^{-/-}$ β cells. In the analysis, only the larger $Ca_V1.2^{-/-}$ β cells (capacitance >7 pF) displaying single Ca_V1 channel currents with a large unitary Ba^{2+} conductance and long-lasting openings were included in the analysis of $Ca_V1.3$ channel hyperactivation by T1D serum. Figure 2c represents sample unitary Ba^{2+} currents flowing through single Ca_V1 channels in a healthy serum-treated cell and in a cell exposed to T1D serum. These current traces clearly show that Ca_V1 channels stay open longer and more Ca_V1 channels appear in a plasma membrane patch attached to a cell treated with T1D serum. Summary data show that plasma membrane patches of T1D serum-treated cells display significantly more channels than those of healthy serum-treated cells (Fig. 2d). Moreover, treatment with T1D serum significantly increased the open probability, prolonged the mean open time and shortened the mean closed time of Ca_V1 channels (Fig. 2d).

Type 1 diabetic serum enhances whole-cell Ca_V currents in mouse islet $Ca_V1.3^{-/-}$ β cells

There is no doubt that every mouse islet β cell is equipped with $Ca_V1.2$ channels to mediate Ca_V1 currents. To determine if mouse β cell $Ca_V1.2$ channels undergo hyperactivation in response to T1D serum exposure, we characterized the effects of T1D serum on whole-cell Ca_V currents in mouse islet $Ca_V1.3^{+/+}$ and $Ca_V1.3^{-/-}$ β cells. As aforementioned, mouse islet $Ca_V1.3^{+/+}$ β cells were employed as positive control to assess the effectiveness of the T1D serum used in this set of experiments on Ca_V1 channel hyperactivation. Incubation with T1D serum dramatically increased whole-cell Ca_V currents in $Ca_V1.3^{+/+}$ β cells, as manifested by representative whole-cell Ca_V current traces obtained from a control $Ca_V1.3^{+/+}$ and a T1D serum-treated $Ca_V1.3^{+/+}$ β cell (Fig. 3a). Compiled data show that T1D serum significantly elevated whole-cell Ca_V current density measured at depolarizations in the range of −20 to 40 mV from a holding potential of −70 mV compared to healthy serum in $Ca_V1.3^{+/+}$ cells (Fig. 3b). Like $Ca_V1.3^{+/+}$ β cells, $Ca_V1.3^{-/-}$ β cells show that their whole-cell Ca_V1 currents are hyperactivated by T1D serum (Fig. 3). A T1D serum-treated $Ca_V1.3^{-/-}$ β cell displays larger whole-cell Ca_V currents compared to a $Ca_V1.3^{-/-}$ β cell incubated with healthy serum (Fig. 3c). Statistical analysis reveals that T1D serum-treated cells exhibit significantly higher Ca_V current density at depolarizations from −20 to 50 mV compared to cells incubated with healthy serum (Fig. 3d).

To determine if T1D serum treatment or $Ca_V1.3$ knockout alters activation of β cell Ca_V channels, the midpoint ($V_{1/2}$) of activation of whole-cell Ca_V currents was calculated by fitting whole-cell Ca_V current–voltage data with Boltzmann function. T1D serum treatment did not influence activation midpoint in either $Ca_V1.3^{+/+}$ β cells (T1D serum: -16.2 ± 1.2 mV vs. healthy serum: -16.9 ± 1.1 mV, $n = 36$ and 36, $p > 0.05$) or $Ca_V1.3^{-/-}$ β cells (T1D serum: -12.1 ± 1.3 mV vs. healthy serum: -11.8 ± 1.0 mV, $n = 35$ and 35, $p > 0.05$). However, $Ca_V1.3$ knockout slightly but significantly shifted activation midpoint to more depolarized potentials in both healthy serum-treated cells ($Ca_V1.3^{-/-}$: -11.8 ± 1.0 mV vs. $Ca_V1.3^{+/+}$: -16.9 ± 1.1 mV, $n = 35$ and 36, $p < 0.01$) and T1D serum-treated cells ($Ca_V1.3^{-/-}$:12.1 ± 1.3 mV vs. $Ca_V1.3^{+/+}$:−16.2 ± 1.2 mV, $n = 35$ and 36, $p < 0.05$).

Type 1 diabetic serum elevates the open probability and number of single Ca_V1 channels in mouse islet $Ca_V1.3^{-/-}$ β cells

The above data obtained from whole-cell patch-clamp experiments suggest that $Ca_V1.2$ channels are hyperactivated by T1D serum. To further substantiate this suggestion, we selectively characterized single Ca_V1 channels exhibiting a large unitary Ba^{2+} conductance and long-lasting openings in mouse islet $Ca_V1.3^{+/+}$ and $Ca_V1.3^{-/-}$ β cells following healthy serum and T1D serum treatments (Fig. 4a, c). We first characterized how the T1D serum used in this set of experiments affected biophysical properties of single Ca_V1 channels in mouse islet $Ca_V1.3^{+/}$

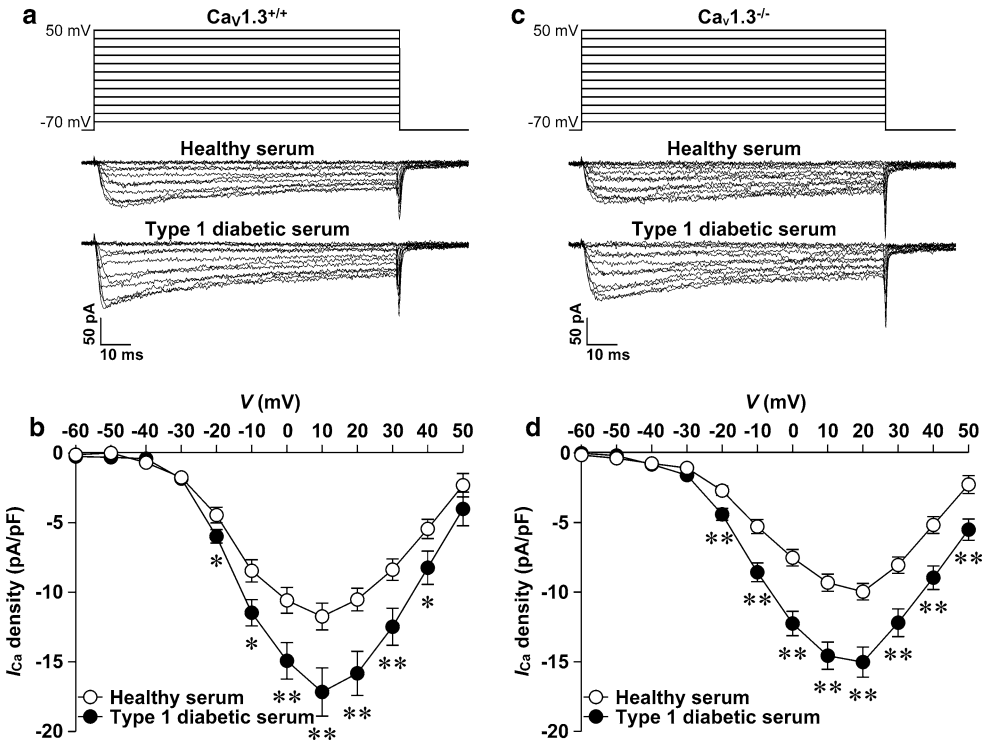

Fig. 3 Type 1 diabetic serum increases whole-cell Ca_V currents in $Ca_V1.3^{+/+}$ and $Ca_V1.3^{-/-}$ β cells. **a** Sample whole-cell Ca_V current traces from a $Ca_V1.3^{+/+}$ β cell incubated with either healthy serum (cell capacitance: 5.9 pF) or T1D serum (cell capacitance: 5.6 pF). **b** Average Ca_V current density–voltage relationships in $Ca_V1.3^{+/+}$ cells exposed to healthy serum (*open circles*, $n = 36$ cells) and T1D serum (*filled circles*, $n = 36$ cells), respectively. **c** Representative whole-cell Ca_V current traces from a $Ca_V1.3^{-/-}$ β cell following treatment with healthy serum (cell capacitance: 5.4 pF) and T1D serum (cell capacitance: 5.5 pF), respectively. **d** Average Ca_V current density–voltage relationships in $Ca_V1.3^{-/-}$ β cells subjected to exposure to either healthy serum (*open circles*, $n = 35$ cells) or T1D serum (*filled circles*, $n = 35$ cells). $^*p < 0.05$ and $^{**}p < 0.01$ vs. healthy serum

$^+$ β cells as a positive control. Figure 4a illustrates unitary Ba^{2+} currents passing through Ca_V1 channels sampled from a plasma membrane patch of a $Ca_V1.3^{+/+}$ β cell exposed to healthy serum and that incubated with T1D serum. The latter accommodated more single Ca_V1 channels, manifested by more layers of unitary Ba^{2+} currents, and longer open states (Fig. 4a). Figure 4b shows the detailed biophysical properties of the unitary Ca_V1 channels. The number, open probability and mean open time of single Ca_V1 channels following exposure to T1D serum were significantly greater than those incubated with healthy serum. The mean closed time of single Ca_V1 channels exposed to T1D serum was significantly shorter than that following healthy serum treatment (Fig. 4b).

Subsequently, we examined the effects of T1D serum on unitary Ca_V1 currents in $Ca_V1.3^{-/-}$ β cells. As above, Ca_V1 channel currents were verified with their fingerprint features, i.e., a large unitary Ba^{2+} conductance and long-lasting openings. Such features were clearly depicted in unitary Ba^{2+} current traces registered in a healthy serum-treated cell and in a cell treated with T1D serum (Fig. 4c). Figure 4c shows that unitary Ba^{2+} currents recorded in a plasma membrane patch of a T1D serum-treated cell display more layers, resulting from simultaneous opening of multiple single channels, and persist for long time periods. Detailed statistical analysis illustrated that the number, open probability and mean open time of single Ca_V1 channels significantly increased and the mean closed time of these channels significantly decreased subsequent to exposure to T1D serum (Fig. 4d).

Discussion

The presence of functional $Ca_V1.3$ channels in the mouse islet β cell remains controversial due to technical limitations [1, 2, 8, 9, 11–13]. The single-cell RT-PCR approach and immunofluorescence labeling can be used to detect $Ca_V1.3$ subunit mRNA and protein, respectively, but not functional $Ca_V1.3$ channels. In fact, we previously showed expression of $Ca_V1.3$ subunit mRNA in some mouse islet β cells, but could not clarify the presence of functional $Ca_V1.3$ channels [13]. All available anti-$Ca_V1.3$ subunit antibodies are not specific enough to solely recognize the

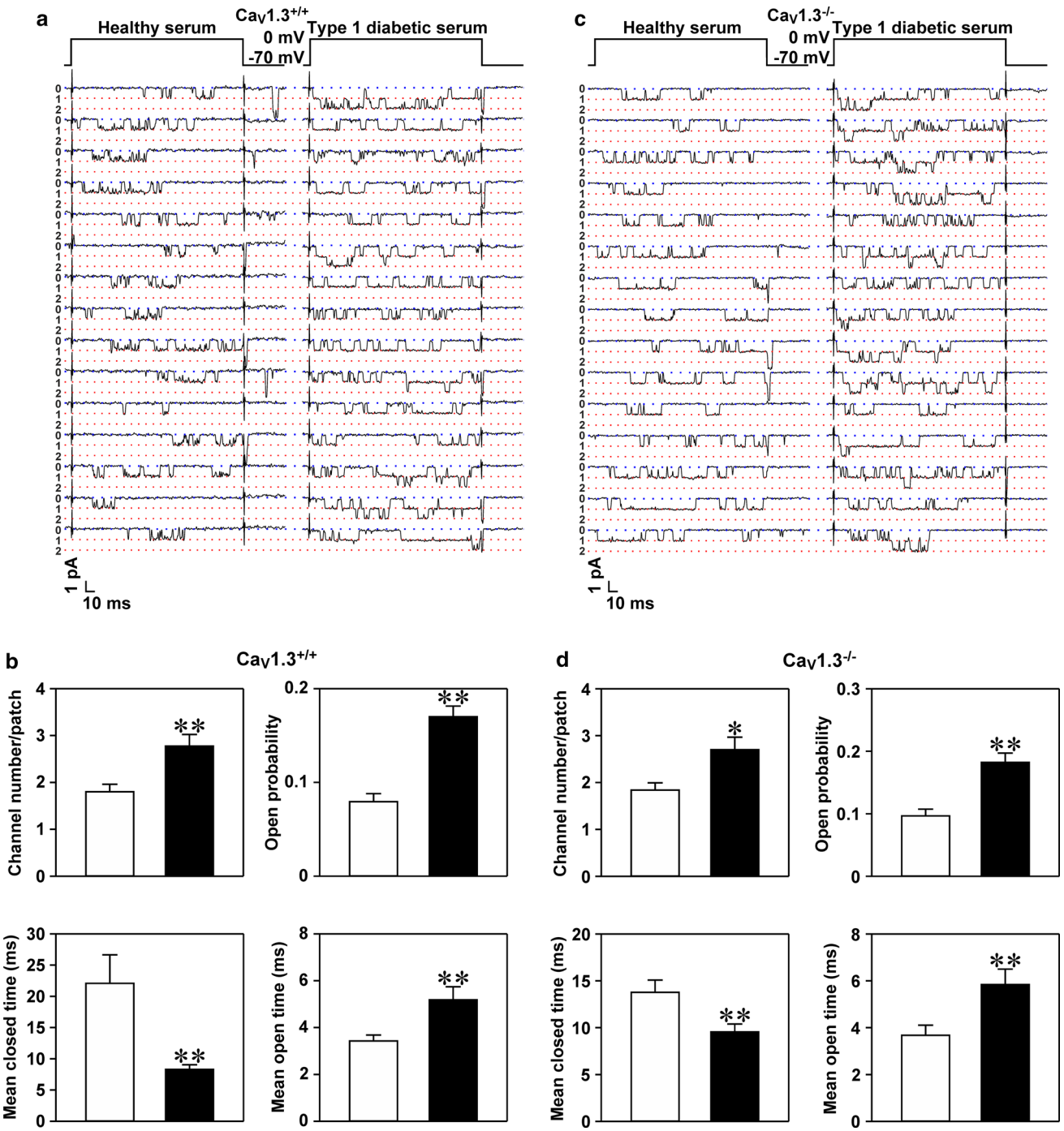

Fig. 4 Type 1 diabetic serum hyperactivates Ca_V1 channels through increasing their open probability and number in $Ca_V1.3^{+/+}$ and $Ca_V1.3^{-/-}$ β cells. **a** Examples of unitary Ca_V1 channel currents registered in a plasma membrane patch of a $Ca_V1.3^{+/+}$ β cell incubated with either healthy serum or T1D serum. The plasma membrane patch was held at −70 mV and depolarized for 200 ms to a test potential of 0 mV. **b** Average number, open probability, mean closed time and mean open time of single Ca_V1 channels acquired from plasma membrane patches attached to $Ca_V1.3^{+/+}$ β cells following incubation with healthy serum ($n = 30$ cells) and T1D serum ($n = 31$ cells), respectively. **c** Samples of unitary Ca_V1 channel currents monitored in a plasma membrane patch attached to a $Ca_V1.3^{-/-}$ β cell exposed to healthy serum and T1D serum, respectively. **d** Average number, open probability, mean closed time and mean open time of single Ca_V1 channels detected in plasma membrane patches of $Ca_V1.3^{-/-}$ β cells subjected to exposure to either healthy serum ($n = 31$ cells) or T1D serum ($n = 30$ cells). $^*p < 0.05$ and $^{**}p < 0.01$ vs. healthy serum

$Ca_V1.3$ subunit and the β cell plasma membrane only accommodates about hundred native Ca_V channels, composed only partly of $Ca_V1.3$, which are too few to be detected by confocal scanning microscopy [29]. Moreover, $Ca_V1.2$ and $Ca_V1.3$ channels differ in activation threshold and DHP sensitivity when separately expressed in heterologous cells [30, 31]. Although a combination of the patch-clamp technique with application of DHPs appears to be the best choice for identification of functional $Ca_V1.3$ channels in the mouse islet β cell, the difference in activation threshold is too subtle to be used for convincing discrimination between native $Ca_V1.2$ and $Ca_V1.3$ channels. DHPs act on both $Ca_V1.2$ and $Ca_V1.3$ channels and are not suitable to reliably differentiate $Ca_V1.3$ channels from $Ca_V1.2$ channels. The conceptual novelty of the present work is that it overcomes the aforementioned technical limitations by combining whole-cell patch-clamp analysis, application of the selective Ca_V1 channel blocker, single-cell RT-PCR detection of insulin mRNAs and the $Ca_V1.2^{-/-}$ mouse model.

The obtained data reveal that some $Ca_V1.2^{-/-}$ islet cells, which are larger in size (capacitance > 7 pF), did not display Na^+ currents, but express insulin mRNA and are equipped with the Ca_V channel having a low but true sensitivity to the selective Ca_V1 channel blocker nimodipine (10 μM). This demonstrates that a proportion of mouse islet $Ca_V1.2^{-/-}$ β cells express functional $Ca_V1.3$ channels. This makes the $Ca_V1.2^{-/-}$ mouse a convenient small animal model to clarify if T1D serum affects β cell $Ca_V1.3$ channels and to further investigate physiology and pathophysiology of these channels, which are predominantly expressed in human β cells and whose polymorphisms are closely associated with diabetes [1, 2, 16, 32].

The development of a novel treatment strategy for diabetes critically depends on understanding of the molecular pathogenesis of this disease [33–37]. We have previously shown that T1D serum and its diabetogenic factor apolipoprotein CIII selectively hyperactivate β cell Ca_V1 (L-type) channels by using the selective Ca_V1 channel blocker verapamil or nimodipine [19–21]. These studies demonstrated that T1D serum makes unphysiological amounts of Ca^{2+} enter pancreatic β cells through hyperactivation of β cell Ca_V1 channels resulting in β cell apoptosis [19]. Along the same line, we revealed that elevated apolipoprotein CIII in T1D serum serves as the diabetogenic serum factor to drive Ca^{2+}-dependent β cell destruction via selective hyperactivation of β cell Ca_V1 channels [20]. In vivo down-regulation of this apolipoprotein delays the onset of diabetes in the BioBreeding rat, a rat model for human type 1 diabetes [38]. Mechanistically, apolipoprotein CIII hyperactivates β cell Ca_V1 channels through scavenger receptor class B type I/β1 integrin-dependent coactivation of protein kinase A and Src [21]. This process aggravates the disease development on top of the T-lymphocyte-mediated autoimmune attack [2, 39, 40]. To advance our understanding of the details of hyperactivated Ca_V1 channels in diabetes development, the present study examined if either $Ca_V1.2$ or $Ca_V1.3$ channels or both are hyperactivated by T1D serum. We now demonstrate that both $Ca_V1.2$ and $Ca_V1.3$ channels in the mouse β cell are vulnerable to the attack of T1D serum and their hyperactivation underlies the molecular pathogenesis of type 1 diabetes. Importantly, this finding highlights that both Ca_V1 channel subtypes are suitable for molecular intervention in diabetes mellitus. In fact, pharmacological intervention with the Ca_V1 channel blocker verapmil has been demonstrated to ameliorate and even prevent low-dose streptozotocin-induced progressive diabetes in mice through reduction of β cell apoptosis and promotion of β cell survival and function [41].

We previously found that T1D serum significantly increases the amplitude of whole-cell and average unitary Ca_V1 currents in the β cell [19]. Such a grossly increased activity can arise from enriched density and/or increased conductivity of these channels in the β cell plasma membrane. We have now carefully analyzed how T1D serum alters Ca_V1 channel behavior at both the single-channel and whole-cell levels. Our whole-cell patch-clamp recordings show that T1D serum significantly enhances whole-cell Ca_V currents in $Ca_V1.3^{+/+}$ and $Ca_V1.3^{-/-}$ β cells. This is in line with previous findings from our group and others [19, 42–45]. More importantly, we are able to mechanistically interpret T1D serum-induced hyperactivation of β cell Ca_V1 channels by thoroughly examining the biophysical properties of single Ca_V1 channels in $Ca_V1.2^{+/-}$, $Ca_V1.2^{-/-}$, $Ca_V1.3^{+/+}$ and $Ca_V1.3^{-/-}$ β cells. The Ca_V1 channel hyperactivation results from both increased activity and elevated number of functional single Ca_V1 channels in the recorded area of the β cell plasma membrane. The former is reflected by an increased open probability attributed to the prolonged mean open time and shortened mean closed time. The latter is verified by appearance of more levels of single Ca_V1 channel conductance.

Overall, our work verifies that functional $Ca_V1.3$ channels are expressed in a proportion of mouse islet $Ca_V1.2^{-/-}$ β cells. Importantly, T1D serum hyperactivates both $Ca_V1.2$ and $Ca_V1.3$ channels by increasing their conductivity and density. Intriguingly, our findings suggest $Ca_V1.2$ and $Ca_V1.3$ channels as potential druggable targets and pave the way for a novel diabetes therapy.

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