

# Cloning of a T-Type $\text{Ca}^{2+}$ Channel Isoform in Insulin-Secreting Cells

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The T-type  $\text{Ca}^{2+}$  channel is an important determinant of electrical activity and of  $\text{Ca}^{2+}$  influx in rat and human pancreatic  $\beta$ -cells. We have identified and sequenced a cDNA encoding a T-type  $\text{Ca}^{2+}$  channel  $\alpha_1$ -subunit derived from INS-1, the rat insulin-secreting cell line. The sequence of the cDNA indicates a protein composed of 2,288 amino acids that shares 96.3% identity to  $\alpha_1\text{G}$ , the neuronal T-type  $\text{Ca}^{2+}$  channel subunit. The transmembrane domains of the protein are highly conserved, but the isoform contains three distinct regions and 10 single amino acid substitutions in other regions. Sequencing rat genomic DNA revealed that the  $\alpha_1$ -subunit we cloned is an alternative splice isoform of  $\alpha_1\text{G}$ . By using specific primers and reverse transcription-polymerase chain reaction, we demonstrated that both splice variants are expressed in rat islets. The isoform deduced from INS-1 was also expressed in brain, neonatal heart, and kidney. Functional expression of this  $\alpha_1\text{G}$  isoform in *Xenopus* oocytes generated low voltage-activated  $\text{Ba}^{2+}$  currents. These results provide the molecular biological basis for studies of function of T-type  $\text{Ca}^{2+}$  channels in  $\beta$ -cells, which is where these channels may play critical roles in diabetes. *Diabetes* 49:59–64, 2000

**G**lucose stimulation of pancreatic  $\beta$ -cells involves membrane depolarization and  $\text{Ca}^{2+}$  influx. An upsurge in cytoplasmic free  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) triggers the exocytotic machinery and subsequent insulin release.  $\text{Ca}^{2+}$  influx in  $\beta$ -cells occurs primarily through voltage-dependent  $\text{Ca}^{2+}$  channels, especially L-type  $\text{Ca}^{2+}$  channels (1). The function of T-type  $\text{Ca}^{2+}$  channels in pancreatic  $\beta$ -cells has been suggested to involve modulation of general membrane electrical activity to enhance insulin secretion (2,3). T-type  $\text{Ca}^{2+}$  channels also play an important role in regulating basal  $[\text{Ca}^{2+}]_i$  and cytokine-induced  $\beta$ -cell destruction (4).

The L-type  $\text{Ca}^{2+}$  channel, which is the most documented member of the  $\text{Ca}^{2+}$  channel family in  $\beta$ -cells, mediates stim-

ulus-secretion coupling (5). These channels are characterized by high voltage-activated persistent  $\text{Ca}^{2+}$  currents and are blocked by dihydropyridines, phenylalkylamines, and benzothiazepines. The L-type  $\text{Ca}^{2+}$  channel is a large heterooligomeric complex consisting of  $\alpha_1$ -,  $\alpha_2/\delta$ -,  $\beta$ -, and  $\gamma$ -subunits (in skeletal muscle and lung) (6).

Compared with the biology of the L-type  $\text{Ca}^{2+}$  channel, the biology of the T-type  $\text{Ca}^{2+}$  channel is not as understood. These channels are activated and inactivated at low membrane potentials and display fast inactivation and slow deactivation kinetics (7). The unique window current properties and low voltage activation of these channels also suggest that they are involved in basal  $[\text{Ca}^{2+}]_i$  regulation (8). The subunit structure of the T-type  $\text{Ca}^{2+}$  channel is unknown, but recent cloning and characterization of genes encoding neuronal and cardiac T-type  $\text{Ca}^{2+}$  channels reveal strong homology within the transmembrane regions to the  $\alpha_1$ -subunits of other members of the  $\text{Ca}^{2+}$  channel family (9,10). The regions outside the transmembrane helices vary to a great extent and determine the diversity within the family (11). Molecular identification of the T-type  $\text{Ca}^{2+}$  channel in  $\beta$ -cells will elucidate the primary structure of the channel and will allow for further pathological and genetic studies of its role in diabetes. In this article, we report the cloning and tissue distribution of an isoform of the T-type  $\text{Ca}^{2+}$  channel ( $\alpha_1\text{G-INS}$ ) derived from INS-1, the rat insulin-secreting cell line (12).

## RESEARCH DESIGN AND METHODS

**Cell culture.** INS-1 cells were cultured in RPMI-1640 medium (Gibco BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS), 25 U/ml penicillin, 25 mg/ml streptomycin, and 50  $\mu\text{mol/l}$  mercaptoethanol in an atmosphere of 5%  $\text{CO}_2$  in air at 37°C.

**Islet cell preparation.** Pancreases of Sprague-Dawley rats (Charles River Laboratory, Wilmington, MA) were removed after intrapancreatic perfusion with 2 ml of Hanks' solution (Gibco BRL) containing 4 mg/ml collagenase (Boehringer Mannheim, Indianapolis, IN), 10  $\mu\text{g/ml}$  DNase I (Sigma, St. Louis, MO), 1.28 mmol/l  $\text{CaCl}_2$ , and 1 mg/ml bovine serum albumin (Gibco BRL). The pancreatic tissue was incubated at 37°C for 20 min and then washed five times with enzyme-free Hanks' solution. Islets were picked up and treated with 0.1% pancreatin (Sigma) for 5 min at 37°C. Single cells were obtained by triturating the islets with plastic pipette tips and were then transferred into 35-mm culture dishes. Cells were cultured in RPMI-1640 medium containing 5 mmol/l glucose, 10% FBS, penicillin, and streptomycin in an atmosphere of 5%  $\text{CO}_2$  at 37°C, for 2–5 days before experiments.

**Isolation of RNA.** Total RNA was isolated from cultured INS-1 cells and from various freshly excised rat tissues by the guanidinium isothiocyanate/phenol procedure (13). Poly-A RNA was isolated from total RNA by two successive passes over an oligo (dT)-cellulose spin column (Ambion, Austin, TX).

Cloning of cDNA encoding  $\alpha_1$ -subunit of T-type  $\text{Ca}^{2+}$  channel in INS-1. First-strand cDNA was prepared using 2  $\mu\text{g}$  INS-1 cell mRNA and Moloney murine leukemia virus reverse transcriptase (Gibco BRL) with the poly-dT primers. The first 433-bp DNA fragment of the channel was deduced with polymerase chain reaction (PCR) by using the degenerate primers (forward) 5'-TNGC(A/C/T)ATGGAG

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AP, adapter primers;  $[\text{Ca}^{2+}]_i$ , cytoplasmic free  $\text{Ca}^{2+}$  concentration; FBS, fetal bovine serum; GSP, gene-specific primers; PCR, polymerase chain reaction; RT, reverse transcription; RACE, rapid amplifications of cDNA end.

(C/A)GNCC(C/T)-3' and (backward) 5'-CTT(C/G/T)CCCTTGAA(G/C)A(G/A)C(TG)-3' based on conserved voltage-dependent Ca<sup>2+</sup> channel α<sub>1</sub>-subunit sequences in domain III. By using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA), the 3'- and 5'-rapid amplifications of cDNA end-PCR (RACE-PCR) were performed to obtain the entire gene of the α<sub>1</sub>-subunit of the channel. For the 5'-RACE-PCR, the forward primer was an adaptor primer, and the backward primer was 5'-CCGCTGTCGGAGACCATGGAGACC-3'; for the 3'-RACE, the forward primer was 5'-AGCGGCCAAAATTGACCCACAG-3', and the backward primer was poly-dT. The reverse transcription-PCR (RT-PCR) products were subcloned into pT-Adv Vector (Clontech), and dideoxynucleotide sequencing assay was performed with a dsDNA Cycle Sequencing System (Gibco BRL). Tissue distribution. The gene expression of T-type Ca<sup>2+</sup> channels deduced from β-cells was examined in rat brain, heart, kidney, and liver by using an RT-PCR assay. The primers used for the RT-PCR were 5'-GAAGATGCGAGTGGACAG-3' (forward) and 5'-CTGTGGCGATGGTCACTG-3' (backward). The PCR products were detected by agarose gel electrophoresis on a 1% gel. Genome walking. The genome walker library (Clontech) was used as a template in nested PCRs with gene-specific primers (GSP) and the adapter primers (AP) that were provided with the kit. The first PCR was carried out in five tubes, each having a total volume of 50 μl: 5 μl PCR buffer (10×), 1 μl dNTP (10 mmol/l each), 2.2 μl magnesium acetate (25 mmol/l), 1 μl AP1 (10 μmol/l), 1 μl GSP1, 1 μl Advantage Genomic Polymerase Mix (50×), and 37.8 μl water. The following two-step cycle parameters were used: 1) 7 cycles of denaturing at 94°C for 25 s with annealing and extension at 72°C for 4 min and 2) 32 cycles of denaturing at 94°C for 25 s with annealing and extension at 67°C for 4 min. After the second step, the samples were held at 67°C for 4 min. The second PCR was carried out under the reaction condition similar to the first PCR, except the second reaction used AP2 and GSP2. In addition, the templates are 1 μl of 1:50 dilution for each primary PCR. The two-step cycles were similar to the first PCR, except 5 cycles were used at the first step and 22 cycles were used at the second step. Oocyte electrophysiology. cRNA transcripts were synthesized from BssH II linearized pT-Adv cDNA templates using T7 RNA polymerase (Ambion). Defolliculated *Xenopus laevis* was injected with 25 ng pT-Adv cRNA. A two-electrode volt-

age-clamp recording was performed using a Warner OC-725C amplifier (Warner Instrument, Hamden, CT) 3–5 days after injection. Data were acquired and analyzed with Pulse/PulseFit software (HEKA, Lambrecht/Pfalz, Germany). The bath solution contained the following: 40 mmol/l Ba(OH)<sub>2</sub>, 50 mmol/l NaOH, 2 mmol/l tetraethylammonium-Cl, 1 mmol/l KOH, 0.1 mmol/l EDTA, and 5 mmol/l HEPES, adjusted to pH 7.4 with methanesulphonate. Boltzmann fits were calculated using Prism (Graph Pad, San Diego, CA). Results are presented as mean ± SD unless otherwise stated.

β-Cell electrophysiological recordings. The whole-cell recordings were carried out by the standard "giga-seal" patch clamp technique. The whole-cell recording pipettes were made of hemocapillaries (Warner Instruments), pulled by a two-stage puller (PC-10, Narishige International, New York), and heat polished with a microforge (MF200-1, World Precision Instruments, Sarasota, FL) before use. Pipette resistance was in the range of 2–3 MΩ in our internal solution. The recordings were performed at room temperature (22–25°C). Currents were recorded using an EPC-9 patch-clamp amplifier (HEKA) and filtered at 2.9 kHz. Data were acquired with Pulse/PulseFit software. Voltage-dependent currents were corrected for linear leak and residual capacitance by using an online P/n subtraction paradigm.

RESULTS

Based on the conserved amino acid sequence comprising the six transmembrane segments in repeat III of the previously cloned α<sub>1</sub>-subunit (14), degenerate primers were designed to deduce the cDNA sequence of a voltage-dependent Ca<sup>2+</sup> channel from INS-1, which expresses T-type Ca<sup>2+</sup> currents (2). A 433-bp DNA fragment was obtained. We then used the RACE strategy to obtain the entire sequence of the channel. The full-length cDNA encodes a protein containing 2,288 amino acids (Fig. 1). The degenerate primers used here failed



FIG. 1. The deduced amino acid sequence of the α<sub>1</sub>-subunit of T-type Ca<sup>2+</sup> channel from INS-1 cells. Letters in parentheses below the sequence represent amino acid residues of the α<sub>1</sub>G that are different from the corresponding amino acid at the same positions. Dashed lines indicate the absence of corresponding residues. Bold regions are four transmembrane domains of the channel.

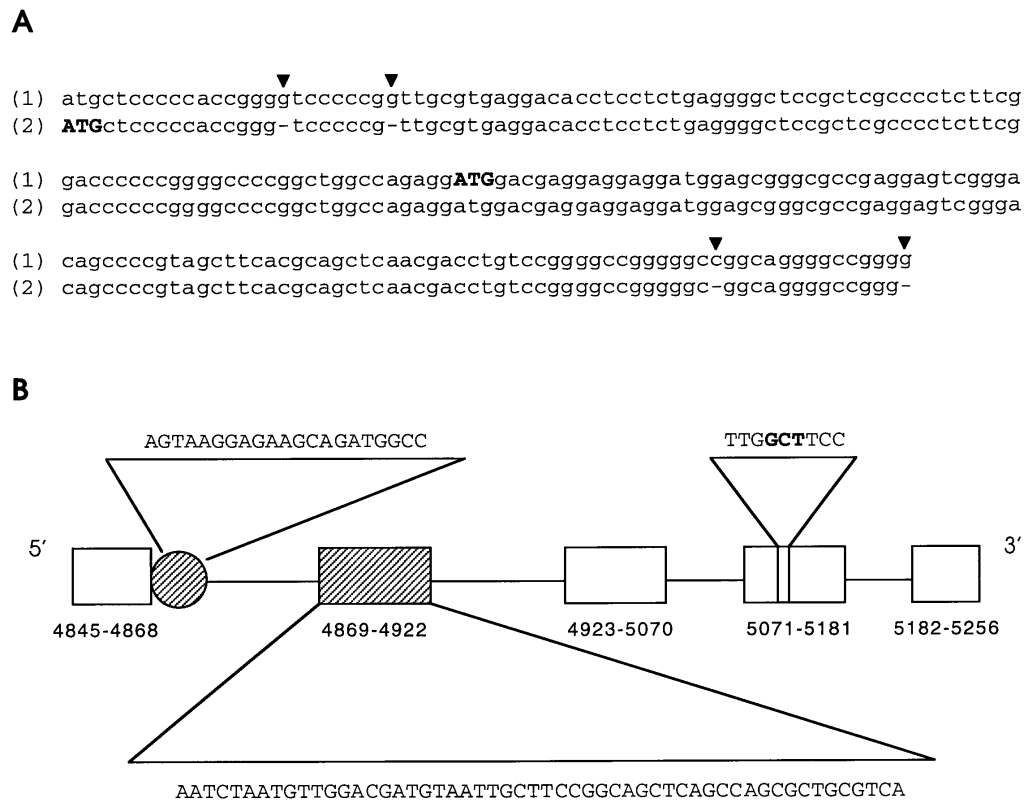


FIG. 2. A: Comparison of the nucleotide sequence of  $\alpha_1$ G-INS (1) and  $\alpha_1$ G (2) at the 5'-end regions (amino acids 1–67 of  $\alpha_1$ G). The four insertions are indicated with arrowheads. The capital ATG represents the start codon for each cDNA. B: A schematic illustration representing partial rat genomic nucleotide composition between domains III and IV. Genomic DNA contained an exon specific to  $\alpha_1$ G (shaded circle) and an exon specific to the  $\alpha_1$ -subunit of T-type  $\text{Ca}^{2+}$  deduced from INS-1 (shaded rectangle) between 4845 and 5256 of the cDNA sequence. Other exons (open rectangles) are identical between the two cDNAs. The bold letters indicate the nucleotides coding Gly-1667.

to recognize other  $\text{Ca}^{2+}$  channels, which suggests that this isoform of the T-type  $\text{Ca}^{2+}$  channel was a major one in INS-1 cells.

The T-type  $\text{Ca}^{2+}$  channel gene deduced from  $\beta$ -cells shares 96.3% amino acid identity with  $\alpha_1$ G, the neuronal isoform of the T-type  $\text{Ca}^{2+}$  channel (9). The four intramolecular homologous transmembrane domains of the  $\beta$ -cell T-type  $\text{Ca}^{2+}$  channel  $\alpha_1$ -subunit are identical (except glycine 1667) to the  $\alpha_1$ G with each repeat containing six putative membrane-spanning regions (S1–S6) and a pore-forming region (P-loop). The other highly conserved region is located at the intracellular loop between repeat I and II, where a section of histidine-rich chain is present in the  $\beta$ -cell-derived T-type  $\text{Ca}^{2+}$  channel gene and in neuronal and cardiac T-type  $\text{Ca}^{2+}$  channel genes. This structure in the loop<sub>I–II</sub> has not been observed in the protein sequences of known high voltage-activated  $\text{Ca}^{2+}$  channels.

In addition to the single amino acids that differ from  $\alpha_1$ G, the T-type  $\text{Ca}^{2+}$  channel gene derived from  $\beta$ -cells contains three unique regions that differ from the amino acid sequence of  $\alpha_1$ G. These regions are located at the  $\text{NH}_2$ -terminal amino acids (amino acids 1–34), intracellular loop L<sub>II–III</sub> (amino acids 971–994), and intracellular loop L<sub>III–IV</sub> (amino acids 1570–1588). Interestingly, all of the three regions are also observed in an isoform of  $\alpha_1$ G recently cloned from mouse brain (15).

Although the amino acid sequence of the deduced channel is entirely different from the  $\alpha_1$ G in the  $\text{NH}_2$ -terminal region (amino acids 1–34), the nucleotide sequences at this region are almost identical except for the four single nucleotide insertions that are shown in Fig. 2A. These four single

nucleotide insertions determine a different start codon and those of the amino acid sequences.

To resolve the relationship between the T-type  $\text{Ca}^{2+}$  channel isoform deduced from INS-1 and  $\alpha_1$ G, we have identified a section of Sprague-Dawley rat (*Rattus norvegicus*) genomic DNA sequence containing the introns and exons between 4845 and 5256. As shown in Fig. 2B, we found an exon that encodes the  $\alpha_1$ G fragment SKEKQMA and an exon that encodes fragment 4869–4922 of the INS-1 variant. This region also contains 8.5 kb of intron sequence. Thus, the T-type  $\text{Ca}^{2+}$  channel  $\alpha_1$ -subunit cloned from INS-1 and  $\alpha_1$ G are alternative splice isoforms of the same gene. These results suggest that transcriptional regulation may be complicated in  $\beta$ -cells.

The genomic DNA sequence was also used to examine the discrepancies of the two nucleotides between the  $\alpha_1$ G cDNA and the isoform cloned from INS-1. Our data show that the genomic nucleotide sequence encoding amino acid 1667 is GGC (glycine), which is the same as the cDNA of  $\alpha_1$ -subunit cloned from INS-1 and the corresponding residue in  $\alpha_1$ H but is different to  $\alpha_1$ G (GCG, alanine). Also of note are the nine additional single amino acid substitutions in the isoform deduced from INS-1 as compared with  $\alpha_1$ G. Of these, six correspond to the amino acids found in the analogous position of  $\alpha_1$ H: cysteine 1088, glycine 1667, alanine 1700, aspartic acid 1735, threonine 1812, and leucine 1813.

The tissue distribution of T-type  $\text{Ca}^{2+}$  channels deduced from  $\beta$ -cells and from neurones is shown in Fig. 3A. Expression of the  $\beta$ -cell T-type  $\text{Ca}^{2+}$  channel was detected in rat

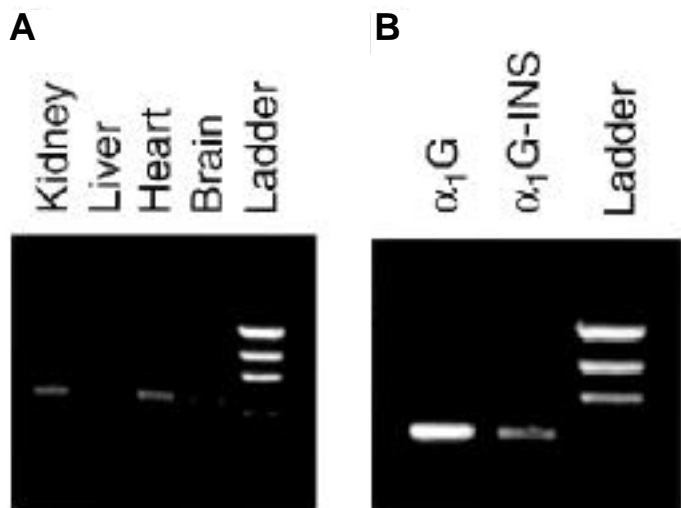


FIG. 3. A: Tissue distribution of the  $\alpha_1$ -subunit of T-type  $\text{Ca}^{2+}$  deduced from INS-1. Transcripts were found in rat kidney, liver, heart, and brain and detected by the RT-PCR method. B: Both alternative splice variants of  $\alpha_1$ G are detected in rat pancreatic islets by using the RT-PCR method.

heart and kidney, although the signal was weaker in brain. There was no expression in liver. Both  $\alpha_1$ G and the splice form were detected in rat islets and INS-1 cell preparations using RT-PCR (Fig. 3B). No  $\alpha_1$ H was detected (data not shown).

Functional expression of the T-type  $\text{Ca}^{2+}$  channels deduced from  $\beta$ -cells has been conducted in *Xenopus* oocytes by using the double-electrode voltage-clamp method. In a solution containing 40 mmol/l  $\text{Ba}^{2+}$ , a family of current traces representing T-type  $\text{Ca}^{2+}$  current characteristics was obtained (Fig. 4A). The current slowly activated at

-40 mV and peaked at -10 mV. The analysis of time constants of activation and inactivation are shown in Fig. 4B. The voltage-dependent activation (Fig. 4C) and steady-state inactivation (Fig. 4D) were fitted with the Boltzmann equation. The calculated  $V_{1/2}$  was -23.8 and -45.6 mV for activation and inactivation, respectively; and  $k$  was 5.3 and -6.0 for activation and inactivation, respectively.

#### DISCUSSION

T-type  $\text{Ca}^{2+}$  currents have been described in human pancreatic islet cells (3,16,17), rat  $\beta$ -cells (18,19), RINm5F cells (20), and dog (21) preparations. This current has not been observed in primary cultured normal (e.g., Swiss-Webster) mouse pancreatic  $\beta$ -cells (22-24). However, substantial low voltage-activated  $\text{Ca}^{2+}$  currents were observed in nonobese diabetic (NOD) mouse islet cells and in NIT-1 cells ( $\beta$ -cell line derived from NOD mouse) (8). Our results provide direct molecular biological evidence that at least two variants of  $\alpha_1$ G are present in rat pancreatic  $\beta$ -cells. It is important to know which one of these variants plays basic or special functions of T-type  $\text{Ca}^{2+}$  currents in rat islet  $\beta$ -cells. Future studies with isoform-specific antisense oligonucleotides and identification of other subunits of T-type  $\text{Ca}^{2+}$  channels should be performed to address this question.

Alternative splicing is a common mechanism in gene regulation (25,26). T-type  $\text{Ca}^{2+}$  channel splice variants of  $\alpha_1$ G have been suggested to exist by Northern blot analysis (9). Based on that data, there are at least two variants of  $\alpha_1$ G differing in their sizes of mRNA transcripts: an 8.5- and a 9.7-kb transcript, respectively. It is unclear as to the size of the INS-1-deduced T-type  $\text{Ca}^{2+}$  channel mRNA because the probe used in the Northern blot study did not discriminate between the two splice variants.

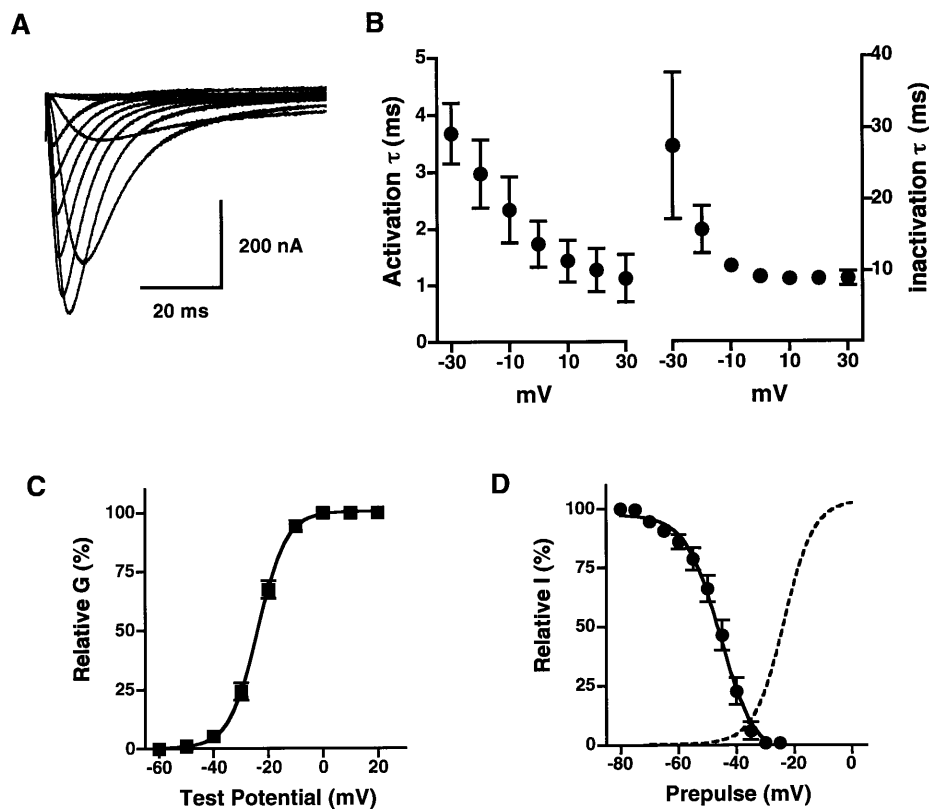


FIG. 4. Expression of  $\alpha_1$ G INS in *Xenopus* oocytes. A: 40 mmol/l  $\text{Ba}^{2+}$  inward currents elicited by depolarizing pulses from -60 to 40 mV. B: Time constants of activation and inactivation measured at test potentials between -30 and 30 mV. The time constants of activation were obtained by fitting the increasing portion (activation) of currents with the Hodgkin-Huxley equation where the  $m$  value was designated as four ( $n = 6$ ). The time constants of inactivation were obtained by single exponential fitting ( $n = 6$ ). Voltage-dependent conductance (C) ( $n = 7$ ) and steady-state inactivation (D) ( $n = 3$ ) of expressed currents in oocytes are shown. Conductances were calculated by using the equation  $G_T = I_T / (V - V_{rev})$ . A value of +70 mV was used as the reversal potential ( $V_{rev}$ ) in our calculations. The holding potential for C and D was -80 mV. The currents in D were measured at -10 mV after varying 1,000-ms prepulse potentials. Peak currents were normalized to the maximum current and then averaged (error bars represent SE). The dashed line in D represents the activation curve from C.

At the moment, we do not know why it is necessary for pancreatic  $\beta$ -cells to have multiple splice forms of T-type  $\text{Ca}^{2+}$  channels. A dysregulation of  $\beta$ -cell T-type  $\text{Ca}^{2+}$  channels occurs in disease models of diabetes. It has been documented that  $\beta$ -cells from GK rats and from neonatally streptozotocin-induced diabetic rats have an increased density of T-type  $\text{Ca}^{2+}$  currents (27,28) and that high glucose promotes proliferation of rat islet cells (29–31) and INS-1 cells (32–34). The intracellular signaling pathways responsible for glucose-mediated  $\beta$ -cell proliferation are not well understood (30). However, it has been shown that in other cell systems, the expression of T-type  $\text{Ca}^{2+}$  currents are associated with cell cycle changes (35–37). In  $\beta$ -cells, therefore, the increased expression of T-type  $\text{Ca}^{2+}$  currents may be related to high glucose-induced  $\beta$ -cell proliferation. Alternative splicing may thus provide an economical way for the  $\beta$ -cell to regulate the levels of different isoforms. It is worth noting that the  $\alpha_1\text{G}$  isoform from INS-1 had similar kinetics to  $\alpha_1\text{G}$  isolated from brain (9). The insertions in the INS-1 isoform may thus be sites for interaction with other  $\beta$ -cell-specific proteins.

The biophysical kinetics of T-type  $\text{Ca}^{2+}$  channels recorded from endocrine cells are different from those of neurones. The  $\alpha_1\text{G}$  isoform deduced from INS-1 shows a less negative and steeper steady-state inactivation that is similar to native  $\beta$ -cells (16) and other endocrine T-type  $\text{Ca}^{2+}$  currents (38,39). These may favor the "window current" properties of T-type  $\text{Ca}^{2+}$  channels and may thereby allow these channels to effectively regulate basal  $[\text{Ca}^{2+}]_i$  in these cells because the resting membrane potential of endocrine cells is generally more positive than that of neurones. Nonetheless, cloning of the T-type  $\text{Ca}^{2+}$  channel from  $\beta$ -cells will help elucidate the channel's pathophysiological role in the process of diabetes.

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The nucleotide sequence reported in this article has been submitted to the GenBank Database (accession number AF125161).

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