Identification and Electrophysiological Characteristics of Isoforms of T-type Calcium Channel $\text{Ca}_v3.2$ Expressed in Pregnant Human Uterus

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Key Words

Human T-type Ca^{2+} channels • $\alpha 1H$ spliced isoforms • $\text{Ca}_v3.2$ • III-IV linker • Uterus

Abstract

Electrophysiological characteristics were compared among four cloned human $\alpha 1H$ isoforms transcribed by alternative splicings of exons 25B and 26 [$\Delta 25B/+26$ (native form; $\alpha 1H-a$), $\Delta 25B/\Delta 26$ ($\alpha 1H-b$), $+25B/\Delta 26$, and $+25B/+26$] in the intracellular loop between domains III and IV (III-IV linker) of a human T-type calcium channel ($\text{Ca}_v3.2$). The native isoform $\Delta 25B/+26$ predominated in ovary and non-pregnant uterus, while isoform $\Delta 25B/\Delta 26$ ($\alpha 1H-b$) predominated in pregnant uterus and testis. Expressions of the newly identified $+25B/\Delta 26$ and $+25B/+26$ isoforms were greater in the uterus at gestation than in the non-pregnant uterus. When expressed in *Xenopus laevis* oocytes, all isoforms produced transient inward currents with low voltage-dependent activation and inactivation characterized in typical T-type Ca^{2+} currents. Each isoform possessing exon 25B ($+25B/\Delta 26$ or $+25B/+26$) showed current activation and inactivation at a more negative membrane potential than the respective isoform ($\Delta 25B/\Delta 26$ or $\Delta 25B/+26$)

lacking it. Moreover, the current activation and inactivation rates were faster for the two isoforms possessing exon 25B than for the respective isoforms lacking it. By itself, exon 26 seemed not to affect any electrophysiological characteristics. Increasing the net positive charge (relative to the native form), as occurred in isoforms $\Delta 25B/\Delta 26$, $+25B/\Delta 26$, and $+25B/+26$, caused recovery from short-term inactivation to become faster. Our results show that molecular-structure variations within the III-IV linker influence the voltage-dependence and kinetics of both activation and inactivation. Although the role of T-type Ca^{2+} channels in uterine tissue remains unknown, changes in the uterine expression of these $\alpha 1H$ isoforms may influence physiological functions during pregnancy.

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Introduction

T-type Ca^{2+} channels are activated by small depolarizations near the resting membrane potential, and this allows them to generate specific cell events, such as low-threshold Ca^{2+} spikes. Although the roles performed by these channels in smooth muscle cells remain

unknown, their existence in mammalian tissues has been confirmed by electrophysiological and molecular biological experiments [1-3]. Three types of T-type channel $\alpha 1$ subunits [namely, $\alpha 1G$ ($Ca_v3.1$), $\alpha 1H$ ($Ca_v3.2$), and $\alpha 1I$ ($Ca_v3.3$)] have been cloned [4-7]. While sharing the common characteristics typical of T-type channels, such as low-voltage activation and inactivation, they exhibit divergency in, for example, their activation and inactivation kinetics and permeability to divalent cations. This divergency might conceivably explain the significant functional differences among the T-type currents recorded from native tissues, particularly in their inactivation kinetics and the voltage-dependence of their steady-state inactivation [8], although there is also evidence of differences in tissue expression among these three T-type channels [9, 10].

Alternative splicing is an important mechanism for the production of physiological diversity. Indeed, alternative splicings of the $\alpha 1C$ gene determine the tissue-specific dihydropyridine sensitivity of cardiac and vascular smooth muscle L-type Ca^{2+} channels [11, 12]. Many splice variants of the high voltage-activated (HVA) Ca^{2+} channels, L-, N-, and P/Q-types, have been found in various tissues, and cloning has made clear both their functional significance and the physiological diversity that results from the existence of multiform channels following alternative splicings [13-15]. On the other hand, research into alternative splicings of LVA Ca^{2+} channels has only recently begun [16, 17]. In particular, for the splice isoform $Ca_v3.2$ ($\alpha 1H$) there is no report of molecular cloning, although PCR has identified a splice variation designated $\alpha 1H-b$ in humans [18]. In the present study, we found two more $\alpha 1H$ isoforms in the human uterus. These were generated by alternative splicings of exons 25B and 26 (namely, +25B/ Δ 26 and +25B/+26) situated in the intracellular loop between domains III and IV (III-IV linker) of $Ca_v3.2$, as are the native form $\alpha 1H-a$ (Δ 25B/+26) and $\alpha 1H-b$ (Δ 25B/ Δ 26) isoforms. In the experiments reported here, we cloned these isoforms, and compared their electrophysiological characteristics with those of the native isoform ($\alpha 1H-a$; Δ 25B/+26), for which molecular cloning of the corresponding cDNA was described a few years ago [19]. We also investigated the expressions of these isoforms among non-neuronal human tissues.

Materials and Methods

Cloning of human $Ca_v3.2$ $\alpha 1$ subunit

For the human $Ca_v3.2$ $\alpha 1$ subunit, $\alpha 1H$ isoforms were cloned by the PCR cloning method (dividing the full length into four parts with overlapping sequences). For this, we used the DNA polymerase (KOD-Plus-; TOYOBO, Tokyo, Japan) showing the highest fidelity, and primer sequences derived from the known $\alpha 1H$ subunit $Ca_v3.2$ sequence (Genbank accession number: AF073931). Template cDNA was synthesized [using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA)] from human uterus total RNA derived from normal tissue (BD Biosciences Clontech, Palo Alto, CA, USA). These genes were subcloned into the TOPO[®] TA cloning vector (pcDNA3.1/V5/His-TOPO; Invitrogen). As a result of the sequencing of a number of clones by dideoxy chain-termination and cycle-sequencing methods, four alternatively spliced isoforms were discovered. Each gene excised by appropriate restriction enzymes was sequentially ligated to generate a full-length clone.

Oocyte expression

Capped cRNA was synthesized from a plasmid linearized with XhoI using T7 RNA polymerase, then a 50-100 poly(A) tail was added (mMESSAGEMACHINE T7 Ultra; Ambion, Austin, TX). The synthesized cRNA was purified using a MEGAclear Purification Kit (Ambion). Oocytes were prepared from *Xenopus laevis* (Seac Yoshitomi, Fukuoka, Japan) using standard technique. Each oocyte was injected with 15-25 ng of cRNA in a volume of 25 nl. The results shown were obtained from seven batches of oocytes derived from four individual animals. For control experiments, oocytes were injected only with the elution solution used for cRNA purification. These control oocytes did not show any Ba^{2+} currents.

Electrophysiological analysis

Oocytes were voltage-clamped using a two-microelectrode voltage-clamp amplifier (GeneClamp 500B; Axon Instruments, Foster City, CA). The standard bath solution contained the following (in mM): 10 $Ba(OH)_2$, 90 NaOH, 1 KOH, 5 HEPES and 0.1 EDTA, adjusted to 7.4 with methansulfonic acid (195 mosmol kg^{-1} H_2O). Voltage and current electrodes (1.5-1.8 M Ω tip resistance) were filled with 3M KCl. Data were filtered at 2 kHz using the pCLAMP system (Digidata 1322A and pCLAMP 8.0; Axon Instruments).

Tissue distribution analysis of $\alpha 1H$ isoforms by RT-PCR

A set of first-strand cDNAs generated from human tissues (Human MTC Panel II; Clontech, Palo Alto, CA) was screened using specific primers designed to amplify all four isoforms of the $\alpha 1H$ subunit. The cDNA Panels were normalized to allow more accurate assessment of tissue specificity and of the relative abundance of target mRNAs. In addition to these, cDNA samples were prepared both from human uterus total RNA derived from normal tissue (BD Biosciences Clontech) and from human uterus total RNA obtained at gestation. The uterus

Fig. 1. Diagrammatic representation and amino acid sequences of human $\text{Ca}_v3.2$ isoforms. The marks under the sequences represent: positively charged amino acids (○), negatively charged amino acids (●), and hydrophobic amino acids (*).

tissue was obtained from three patients who were undergoing cesarean operations at the Fukuoka University Hospital. After gross removal of connective tissue, bundles of smooth muscle cells were isolated. All specimens were blended (so that individuals could not be identified), and then total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Approval was obtained from the local ethics committees (#222, Fukuoka University; and #48, Fukuoka Dental College). To obtain semi-quantitative data, the PCR was performed under non-saturating conditions. The PCR was performed as recommended in the manufacturer's protocol for the cDNA Panels. As advised in the protocol, the PCR was terminated at 22 cycles for the housekeeping gene G3PDH. The PCR cycles for the $\alpha 1\text{H}$ subunit were determined as follows: the PCR was stopped every two cycles at 22 - 38 cycles, and 5- μl samples were removed to run on a gel. The PCR products were electrophoresed on 3 % agarose gel, and stained with SYBR[®] Gold. The fluorescence intensity of each band was determined by means of a scanning densitometer (Image Reader and Image Gauge V3.1; Fuji Photo Film, Tokyo, Japan), and the reaction cycle-intensity curves were plotted. Thus, 32 cycles within the exponential phase were selected for the gene amplification of the $\alpha 1\text{H}$ isoforms. For uterus (which was not included in Human MTC Panel II), the amount of cDNA to be used for the PCR was determined in advance on the basis of the expression level of G3PDH obtained by measuring the fluorescence intensity. The primers were as follows: forward, 5'-TGTCGACCAGCAGCCTGTGCAGAA-3'; reverse, 5'-TGGCTGGTGCACAGCGAGTGAATG-3'. These primers are

located at the 5' site and 3' site of the sequences of exon 25B and exon 26, and are located at the regions of least conservation among the three T-type channels. The fragments were sequenced for identification of each isoform.

Data analysis

Electrophysiological data were analyzed using Clampfit software (Axon Instruments) and Excel (Microsoft). Fitting and graphing of the data were achieved using KaleidaGraph (Abelbeck Software). Current-voltage curves (I - V curves) were fitted using a combined Boltzmann and linear ohmic relationship, in which $I = G_{\text{max}} \times (V_m - V_{\text{rev}}) / (1 + \exp^{(V_m - V_{0.5})/k})$, where I is the amplitude of the inward current recorded at the membrane potential V_m , G_{max} is the maximum value of the chord conductance, V_m is the membrane potential, V_{rev} is the reversal potential, $V_{0.5}$ is the membrane potential at which the half value of the maximum current amplitude is elicited, and k is the slope factor. To minimize the consequences of current rectification near the reversal potential for the determination of conductance, current values greater than +20 mV were not considered for the fit. Voltage-dependent inactivation curves were fitted using $I/I_{\text{max}} = 1 - 1 / (1 + \exp^{(V_{0.5} - V_m)/k})$, where I_{max} is the maximum amplitude of the inward current, and the other terms are as noted above. The kinetics of the recovery from inactivation were examined using a double-exponential expression: $\Delta I = A_1 (1 - \exp^{-V_m/\tau_1}) + A_2 (1 - \exp^{-V_m/\tau_2})$, where ΔI is the relative amplitude of the current, A_1 and A_2 are the relative amplitudes of each exponential, and τ_1 and τ_2 are their respective time constants. The rate of activation was taken as the time required for the currents to

rise from 10 % to 90 % of the peak amplitude (termed Rise 10-90), to minimize the measurement error caused by the inactivation process. The rate of inactivation was estimated from the half time ($t_{1/2}$) of the decay of the peak current.

A one-way ANOVA was combined with Sheffé's *t*-test to compare the different values among isoforms, with significance being accepted at $p < 0.05$. Results are presented as the mean $\pm$ SEM, with *n* indicating the number of oocytes used.

Results

We cloned the first full-length DNA fragments of alternatively spliced isoforms of human $\text{Ca}_v3.2$ $\alpha 1\text{H}$. These had sequence variations within the intracellular loop between domains III and IV by virtue of various combinations of exon 25B and exon 26 (Fig. 1). Here, we call these splicing variants the $\Delta 25\text{B}/\Delta 26$ ($\alpha 1\text{H-b}$), +25B/ $\Delta 26$, and +25B/+26 isoforms. We also studied the native $\Delta 25\text{B}/+26$ isoform ($\alpha 1\text{H-a}$). Fig. 2A shows, for the native $\Delta 25\text{B}/+26$ ($\alpha 1\text{H-a}$) isoform, typical recordings of currents elicited by depolarizing pulses (from -90 to $+40$ mV) of 100 ms duration from a holding potential of -110 mV in 10 mM Ba^{2+} . When we tested for functional expressions of these newly cloned- $\alpha 1\text{H}$ isoforms in oocytes, all the above isoforms produced typical T-type currents. The current-voltage relationships were then compared among the various isoforms. This investigation indicated that the current was activated at around -65 mV for the +25B/ $\Delta 26$ (peaked at -25.6 ± 0.6 mV) and +25B/+26 (peaked at -26.3 ± 0.9 mV) isoforms, whereas activated at around -60 mV for the $\Delta 25\text{B}/\Delta 26$ ($\alpha 1\text{H-b}$) (peaked at -19.6 ± 1.0 mV; $p < 0.05$ vs. +25B/ $\Delta 26$) and native $\Delta 25\text{B}/+26$ (peaked at -20.8 ± 0.6 mV; $p < 0.01$ vs. +25B/+26) isoforms (Fig. 2B). Examination of the steady-state voltage-dependent activation of the currents (using the chord conductance) showed that the $V_{0.5}$ value (membrane potential needed to elicit the half-value of the maximum current amplitude) for the +25B/ $\Delta 26$ current or +25B/+26 current was statistically different from that for the $\Delta 25\text{B}/\Delta 26$ current ($p < 0.05$) or native $\Delta 25\text{B}/+26$ current ($p < 0.001$), respectively (Fig. 3A and Table 1). Indeed, the currents for those isoforms possessing exon 25B (+25B/ $\Delta 26$ and +25B/+26) were activated at more negative membrane potentials than the currents for the isoforms lacking it [$\Delta 25\text{B}/\Delta 26$ (by 3.3 mV) and $\Delta 25\text{B}/+26$ (by 5.5 mV)]. The values obtained for the slope factor (*k*) for the voltage-dependent activation curve did not differ among the four isoforms.

Steady-state voltage-dependent inactivation curves were obtained by application of a 5 s conditioning pulse of various amplitudes followed by a 100 ms test pulse to -30 mV (Fig. 3B). Comparison of the half-maximal values ($V_{0.5}$) revealed that for the isoforms possessing exon 25B (i.e., +25B/ $\Delta 26$ and +25B/+26), the current was inactivated at a significantly more negative membrane potential than those obtained for the respective isoforms lacking it [i.e., $\Delta 25\text{B}/\Delta 26$ (by 3 mV, $p < 0.01$, vs. +25B/ $\Delta 26$) and $\Delta 25\text{B}/+26$ (by 3.9 mV, $p < 0.001$, vs. +25B/+26)]. By contrast, the $V_{0.5}$ values were similar between the $\Delta 25\text{B}/\Delta 26$ and native $\Delta 25\text{B}/+26$ isoforms, and also between the +25B/ $\Delta 26$ and +25B/+26 isoforms, indicating that the insertion encoded by exon 26 may not affect the inactivation process of the $\alpha 1\text{H}$ currents. No significant difference among the isoforms was observed in the values obtained for the slope factor for the voltage-dependent inactivation curve (Table 1).

Fig. 4A shows, for the four isoforms, the inward currents elicited by depolarizing pulses to either -35 mV or -15 mV. Both the activation kinetics (Rise 10-90 values) and inactivation kinetics ($t_{1/2}$ of the decay) were voltage-dependent for all isoform channels, within the negative range of membrane potentials (Fig. 4B). The activation rates of the inward currents elicited by a depolarizing pulse to -35 mV or -15 mV were significantly different between the $\Delta 25\text{B}/\Delta 26$ and +25B/ $\Delta 26$ isoforms (for -35 mV, $p < 0.05$; for -15 mV, $p < 0.01$), and also between the native $\Delta 25\text{B}/+26$ and +25B/+26 isoforms (for -35 mV, $p < 0.01$; for -15 mV, $p < 0.01$). However, there was no such difference between the $\Delta 25\text{B}/\Delta 26$ and native $\Delta 25\text{B}/+26$ isoforms, or between the +25B/ $\Delta 26$ and +25B/+26 isoforms (Table 1). Thus, the presence of exon 25B in the +25B/ $\Delta 26$ and +25B/+26 isoforms accelerated the activation rate, while the presence of exon 26 had no influence over it.

Similarly, the currents obtained for the +25B/ $\Delta 26$ and +25B/+26 isoforms (having exon 25B) exhibited significantly faster inactivation rates than those obtained for the $\Delta 25\text{B}/\Delta 26$ ($p < 0.01$, vs. +25B/ $\Delta 26$) and native $\Delta 25\text{B}/+26$ ($p < 0.01$, vs. +25B/+26) isoforms at a more negative membrane potential (i.e., at -35 mV) (Table 1). By contrast, no such difference was observed between those isoforms possessing exon 26 and those isoforms lacking it ($\Delta 25\text{B}/+26$ vs. $\Delta 25\text{B}/\Delta 26$; +25B/+26 vs. +25B/ $\Delta 26$). At a more positive membrane potentials (i.e., at -15 mV), inactivation kinetics tended to normalize (except for the $\Delta 25\text{B}/\Delta 26$ isoform, which displayed a slower inactivation rate). Collectively, these data indicate that the sequence encoded by exon 25B located in the III-IV

Fig. 2. Native $\alpha 1H$ T-type inward current ($\Delta 25B/+26$; $\alpha 1H-a$) expressed in an oocyte (A) and the current-voltage relationships obtained for four types of $\alpha 1H$ isoforms (B). Depolarizing pulses (100 ms) with amplitudes varied in 5 mV steps were applied at 15 s intervals from a holding potential of -110 mV. Data, which are normalized with respect to the peak current observed in each oocyte, represent the mean of 9-13 observations with SEM.

linker region is involved in the activation and inactivation kinetics of the $\alpha 1H$ currents.

To assess influences on the time course of recovery, paired pulses were applied with various intervals. As shown in Fig. 5, the time course of recovery from short-term inactivation was well fitted with a double exponential function. The time constant of the first component was significantly faster for the $\Delta 25B/\Delta 26$ isoform than for the other isoforms, that for the native $\Delta 25B/+26$ isoform being slower than all the others. Although no significant difference was observed in the values obtained for the time constant of the second component among the $\Delta 25B/\Delta 26$, $+25B/\Delta 26$, and $+25B/+26$ isoforms, that for the native

Fig. 3. Voltage-dependent activation (A) and inactivation (B) of $\alpha 1H$ isoforms. (A) Reversal potential was estimated by drawing the regression line for the values obtained for the inward current between -10 and $+15$ mV by the least squares method, and chord conductance was calculated from the I-V relationship (Fig. 2B). The maximum conductance value obtained for each isoform was normalized as 1.0, and the other values were then expressed in a relative manner. (B) Amplitude of the inward current evoked by a 100 ms test pulse (to -10 mV from a holding potential of -110 mV) was plotted against the stable membrane potential achieved during the conditioning pulse (5 s). The inward current evoked by a test pulse with no conditioning pulse was normalized as 1.0, and all other values were expressed in a relative manner. Typical traces of native isoform ($\Delta 25B/+26$) currents are presented in the inset.

isoform was significantly slower than the others (Fig. 5A, B).

Fig. 4. Inward currents evoked in four isoforms by depolarizing pulses to -35 or -15 mV from a holding potential of -110 mV (A), together with relationships between activation rates (B) or half-decay time (C) and amplitude of depolarizing pulse. (A) Traces were superimposed after matching their peak heights.

(B) The rate of activation (Rise 10-90) was taken as the time required for the currents to rise from 10 % to 90 % of the peak amplitude. (C) Rate of current inactivation was estimated from half-time of current decay. In B and C, each symbol represents mean of 7-13 observations with SEM.

Using an $\alpha 1H$ -specific primer pair flanking the alternatively spliced region in the III-IV linker, we examined the expressions of all four mRNAs by means of semi-quantitative PCR in several human tissues (Fig. 6). PCR products for $\Delta 25B/+26$ ($\alpha 1H$ -a) (297 bp) and $\Delta 25B/\Delta 26$ (279 bp) were observed in colon, ovary, testis, and prostate, as well as in the uterus, but very few were noted in spleen, thymus, or leukocytes (when the number of PCR cycles was increased, more were observed). When compared the fluorescence intensity of each band between these two isoforms, the native type $\Delta 25B/+26$ appeared to predominate in ovary and non-pregnant

uterus, but the $\Delta 25B/\Delta 26$ type predominated in testis and pregnant uterus. Only in pregnant uterus were +25B/ $\Delta 26$ (312 bp) and +25B/+26 (330 bp) additionally expressed to any great extent.

Discussion

Two isoforms, $\Delta 25B/+26$ ($\alpha 1H$ -a) and $\Delta 25B/\Delta 26$ ($\alpha 1H$ -b), of the $\alpha 1H$ Ca^{2+} channel have already been reported, although no functional characteristics have previously been identified for the $\Delta 25B/\Delta 26$ isoform [18].

In the present study, we newly identified, through cDNA cloning experiments, two additional isoforms (+25B/ Δ 26 and +25B/+26) in the pregnant human uterus that had sequence variations in the III-IV linker region.

The presence of eleven amino acids derived from exon 25B in α 1H channels seems to have significant effects on the steady-state voltage-dependency of the current. The presence of this amino acid chain in the +25B/ Δ 26 and +25B/+26 isoforms (Fig. 1) shifted the voltage-dependent activation and inactivation curves toward negative membrane potentials as compared with those for the respective isoform lacking exon 25B (namely, Δ 25B/ Δ 26 and Δ 25B/+26, respectively) (Table 1). Thus, an exon 25B-encoded insertion makes the currents activate and inactivate at more negative potentials. In addition, the α 1H +25B/ Δ 26 and +25B/+26 channels showed significantly faster rates of activation and inactivation than those observed following deletion of these eleven amino acids (i.e., in the Δ 25B/ Δ 26 and Δ 25B/+26 channels). Exon 25B expresses one positively charged and nine hydrophobic amino acids. If it is the presence of an extra positively charged residue that influences these channel properties, it is not easy to understand why the presence of an additional positive charge in the double-deletion (Δ 25B/ Δ 26) isoform at the beginning of exon 27 (Fig. 1) did not alter its voltage-dependency (as compared with that of the native Δ 25B/+26 isoform). At present, we suspect that the hydrophobic region following the positively charged residue may have a more important influence over the kinetics and voltage-dependence of activation and inactivation.

Exon 26 expresses six amino acids (STFPSP), three of which are hydrophobic. In the native isoform (Δ 25B/+26), the first S is directly bound to an RRRR sequence of exon 25A. By alternative splicing of exon 26, the first amino acid on exon 27 is changed to a positively charged lysine residue in the Δ 25B/ Δ 26 isoform (from a negatively charged glutamate residue). When we compared the exon 25B-deletion isoforms, we found that the voltage-dependent activation and inactivation obtained for the Δ 25B/ Δ 26 (α 1H-b) channel were very similar to those obtained for the native Δ 25B/+26 (α 1H-a) channel. Activation and inactivation kinetics were also similar between these two channels, a significant difference being observed only in the decay rate at -15 mV (Table 1). Correspondingly, the inward currents obtained for the exon 25B-insertion isoforms (+25B/ Δ 26 and +25B/+26) were almost identical to each other (except in their voltage-dependent activation). Thus, there was no consistent statistical difference between those isoforms

Fig. 5. Time course of recovery of inward current from short-term inactivation. (A) A two-pulse protocol (to -30 mV from a holding potential of -110 mV) separated with an interpulse interval of variable duration was used to measure the fast recovery rates (as shown in the inset). Data were normalized with respect to the peak current amplitude of the first pulse. These recovery plots were fitted with a double exponential function. Each symbol represents mean of 7-10 observations with SEM. (B) Values obtained for the time constants of the first and second components. Cross-hatched columns: first component; open columns: second component. Dotted lines: comparison of the first components; solid lines: comparison of the second components. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

possessing exon 26 and those lacking it, indicating that exon 26 has less influence than exon 25B over the kinetics and voltage-dependence of activation and inactivation.

The time course of recovery from short-term inactivation was significantly faster for the Δ 25B/ Δ 26 isoform than for any of the other isoforms, and it was

					† exon25B Δ or +		‡ exon26 Δ or +			
	Δ25B/Δ26	Δ25B/+26	+25B/Δ26	+25B/+26	Δ/Δ : +/Δ	Δ/+ : +/+	Δ/Δ : Δ/+	Δ/+ : +/+		
Voltage-dependent activation	V0.5 (mV)	-33.04 ± 0.516 (11)	-34.41 ± 0.454 (11)	-36.36 ± 0.746 (11)	-39.94 ± 0.582 (12)	*	***	-	*	-
	slope (mV)	5.31 ± 0.145 (9)	5.23 ± 0.100 (11)	4.93 ± 0.159 (11)	4.90 ± 0.096 (12)	-	-	-	-	-
Steady-state inactivation	V0.5 (mV)	-53.73 ± 0.599 (9)	-54.91 ± 0.535 (11)	-56.75 ± 0.660 (11)	-58.77 ± 0.359 (12)	**	***	-	-	***
	slope (mV)	-3.72 ± 0.055 (9)	-3.85 ± 0.086 (11)	-3.91 ± 0.078 (11)	-3.69 ± 0.031 (12)	-	-	-	-	-
Rise 10-90 value for activation rate	-35 mV (ms)	7.21 ± 0.269 (7)	7.60 ± 0.244 (9)	5.91 ± 0.242 (10)	5.40 ± 0.204 (13)	*	**	-	-	**
	-15 mV (ms)	2.85 ± 0.046 (7)	2.71 ± 0.069 (9)	2.23 ± 0.053 (10)	2.17 ± 0.047 (13)	**	**	-	-	**
Half-time of decay from peak current	-35 mV (ms)	33.85 ± 1.495 (7)	33.83 ± 1.424 (9)	27.34 ± 0.793 (10)	23.75 ± 0.381 (13)	**	**	-	-	**
	-15 mV (ms)	16.60 ± 0.236 (7)	14.51 ± 0.221 (9)	14.07 ± 0.177 (10)	13.96 ± 0.107 (13)	**	-	**	-	**

Table 1. Electrophysiological characteristics of the various $\alpha 1H$ isoforms. Each value is expressed as mean $\pm$ s.e. and (n) is the number of oocytes used. †comparison between isoforms having or not having exon 25B, ‡comparison between isoforms

having or not having exon 26. Statistical analysis among isoforms was performed using a one-way ANOVA followed by Scheffé's test (— :no significance, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

Fig. 6. Expressions of $\alpha 1H$ isoforms in human tissues. Arrows A, B, C, and D indicate, respectively, the positions of the $\Delta 25B/\Delta 26$ (279 bp), native $\Delta 25B/+26$ (297 bp), +25B/ $\Delta 26$ (312 bp), and +25B/+26 (330 bp) isoforms of the $\alpha 1H$ channel. The fluorescence intensity of each band (A, open columns: $\Delta 25B/\Delta 26$; B, shaded columns: native $\Delta 25B/+26$) measured by means of a scanning densitometer was plotted in the middle panel. The expression level of the housekeeping gene G3PDH in each tissue is shown in the lower panel. The PCR products were electrophoresed on 10 % polyacrylamide gel (for $\alpha 1H$ channels) or on 3.5 % gel (for G3PDH), and stained with ethidium bromide.

significantly slower for the native form $\Delta 25B/+26$ than for any of the other isoforms. In contrast to the situation existing for the other channel properties mentioned above, neither exon 25B nor exon 26 alone seems to contribute to this phenomenon. However, as can be seen in Figure 1, the $\Delta 25B/\Delta 26$ isoform (which acquired a net positive charge by the replacement of E by K) showed the fastest recovery rate, while the native form $\Delta 25B/+26$ (which kept the negatively charged E) had the slowest rate, and the +25B/ $\Delta 26$ and +25B/+26 isoforms (each of which have net charge 0 due to the presence of both K and E)

displayed intermediate recovery rates. In addition to the presence of consecutive plural positively charged amino acids in neighboring regions in the $\alpha 1H$ isoform, an increase in the net positive charge resulting from a combination of these two exons may contribute to the observed alteration in the process of recovery from short-term inactivation.

Although the least conserved regions among the T-type $\alpha 1$ subunits are their intracellular loops, the III-IV linker is an exception, with 75 % identity among them [5, 17], indicating that this region may have a common

physiological function among all T-type channels. In the $\alpha 1G$ channel, the presence of similar splicing in the same region has been reported [16]. Unfortunately, the current associated with the $\alpha 1G +25B/+26$ isoform has not been reported, so any discussion about the involvement of exon 25B must be limited. Although the $\alpha 1G \Delta 25B/+26$ ($\alpha 1G$ -bc) isoform has a longer amino acid sequence in exon 26 (expressing 17 amino acids) than that in $\alpha 1H \Delta 25B/+26$ ($\alpha 1H$ -a), these two appear to be suitable counterparts for a comparison of electrophysiological characteristics. In the study by Chemin et al. [16], the $\alpha 1G \Delta 25B/\Delta 26$ ($\alpha 1G$ -b) isoform was found to acquire a net positive charge, like the $\alpha 1H \Delta 25B/\Delta 26$ ($\alpha 1H$ -b) isoform, by losing exon 26 (containing two negatively charged aspartates) while keeping the first lysine residue in exon 27. Those authors found that this $\alpha 1G$ isoform showed a faster time course of recovery than the $\alpha 1G \Delta 25B/+26$ and $\alpha 1G +25B/\Delta 26$ isoforms, a similar result to that observed here for the $\alpha 1H$ isoforms. Further, they noted no change due to exon 26 in either the inactivation rate or voltage-dependent activation between the $\alpha 1G \Delta 25B/\Delta 26$ and $\Delta 25B/+26$ isoforms (again, much as we observed for the $\alpha 1H$ isoforms). However, whereas $\alpha 1G \Delta 25B/\Delta 26$ showed both a shift in the voltage-dependent inactivation curve in the positive direction and a slower activation rate (as compared with the $\alpha 1G \Delta 25B/+26$ isoform), we did not observe such phenomena in the case of the corresponding $\alpha 1H$ isoforms. Possibly, this difference may reflect the presence of some other factor in the $\alpha 1G$ channel. In any event, our results suggest that the molecular structure of the III-IV-linker region is important for the gating properties of the T-type $\alpha 1H$ channels, as previously indicated for the $\alpha 1G$ channels.

The results of our RT-PCR experiments on several human tissues indicated that the $\alpha 1H$ channel is distributed both in muscle and in non-muscle tissues. Although the dominant isoform expressed differs among these tissues, either the $\Delta 25B/+26$ ($\alpha 1H$ -a) or $\Delta 25B/\Delta 26$ ($\alpha 1H$ -b) isoform predominated in all the tissues examined. In the present experiments, no additional isoform was clearly identified in any of the tissues examined (except that in the pregnant human uterus (i) the two other isoforms examined here were also detected, and (ii) the double-deletion clone $\Delta 25B/\Delta 26$ became predominant in late pregnancy). LVA currents have been described in smooth muscle cells from bronchi, ileum, colon, bladder, and uterus [10]. The roles played by T-type channels in smooth muscle function are still obscure, although these channels are believed to contribute to the spontaneous electrical activities involved in the generation of the pace-maker

potential in cardiac cells. Using $\alpha 1H$ -deficient mice, Chen et al. [1] found that a lack of $\alpha 1H$ channels increased coronary artery tone and prevented relaxation being induced by several agents in coronary arterioles. These findings may indicate that T-type Ca^{2+} channels play an important role in the regulation of muscle excitability, rather than in the direct regulation of muscle contraction. The presence of T-type Ca^{2+} channels in the uterine smooth muscle cells of pregnant women has already been reported [2]. Moreover, coinciding with the commencement of spontaneous uterine activities during pregnancy an elevation of the expression levels of $\alpha 1H$ and $\alpha 1G$ mRNAs has been observed in the pregnant rat in addition to an elevation of L-type $\alpha 1C$ mRNA [3]. The results obtained for pregnant human uterus are particularly interesting in that the T-type currents were larger than the L-type currents [20]. In that previous study, the authors also suggested that the uterine myocyte T-type current might be primarily involved with action potential transmission and the L-type currents primarily with increasing intracellular free calcium by bulk calcium transport [20].

It would appear that $\alpha 1H$ channels and $\alpha 1G$ channels exist to a similar extent in human uterine smooth muscle (confirmed by PCR, data not shown). This suggests that the T-type currents recorded in uterine myocytes result from the presence of a mixture of these two subtypes (and of their many spliced isoforms). Hence, it is difficult to obtain direct evidence, first that the four $\alpha 1H$ isoforms alter uterine physiological function, and second exactly how these isoforms might contribute to the electrophysiological functions of the uterus. Be that as it may, the various changes we observed in the uterine expression of these $\alpha 1H$ isoforms may have some physiological meaning for the function of the uterus, perhaps for the excitation-contraction mechanism of smooth muscle.

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