a1D (Cav1.3) Subunits Can Form L-type Ca\(^{2+}\) Channels Activating at Negative Voltages*

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**In cochlea inner hair cells (IHCs), L-type Ca\(^{2+}\) channels (LTCCs) formed by a1D subunits (D-LTCCs) possess biophysical and pharmacological properties distinct from those of a1C containing C-LTCCs.** We investigated to which extent these differences are determined by a1D itself by analyzing the biophysical and pharmacological properties of cloned human a1D splice variants in tsA-201 cells. Variant a1D\(_{8A}\) containing exon 8A sequence in repeat I, yielded a1D protein and L-type currents, whereas no intact protein and currents were observed after expression with exon 8B. In whole cell patch-clamp recordings (charge carrier 15–20 mm Ba\(^{2+}\)), a1D\(_{8A}\) - mediated currents activated at more negative voltages (activation threshold, -45.7 versus -31.5 mV, p < 0.05) and more rapidly (\(\tau_{act}\) for maximal inward currents 0.8 versus 2.3 ms; \(p < 0.05\)) than currents mediated by rabbit a1C. Inactivation during depolarizing pulses was slower than for a1C (current inactivation after 5-s depolarizations by 90 vs 99%, \(p < 0.05\)) but faster than for LTCCs in IHCs. The sensitivity for the dihydropyridine (DHP) L-type channel blocker isradipine was 8.5-fold lower than for a1C. Radioligand binding experiments revealed that this was not due to a lower affinity for the DHP binding pocket, suggesting that differences in the voltage-dependence of DHP block account for decreased sensitivity of D-LTCCs. Our experiments show that a1D\(_{8A}\) subunits can form slowly inactivating LTCCs activating at more negative voltages than a1C. These properties should allow D-LTCCs to control physiological processes, such as diastolic depolarization in sinoatrial node cells, neurotransmitter release in IHCs and neuronal excitability.

L-type Ca\(^{2+}\) channels (LTCCs)\(^{1}\) form a family of voltage-gated Ca\(^{2+}\) channels with high sensitivity to dihydropyridine (DHP) Ca\(^{2+}\) channel modulators. Their Ca\(^{2+}\)-selective pore is formed by different DHP-sensitive a1 subunit isoforms (a1S, a1C, a1D, a1F) together with auxiliary subunits, including a2- and b subunits (1–3). Whereas a1S and a1F expression is restricted to skeletal muscle and the retina, respectively, LTCCs formed by a1C (C-LTCCs) and a1D (D-LTCCs) subunits are widely expressed in neuronal and (neuro)endocrine cells as well as in electrically excitable cells in the cardiovascular system (4–9). In most cases, both channel types are even found in the same cells, with D-LTCCs usually being the much less abundant isoform (7).

Using D-LTCC-deficient mice, we have previously demonstrated that inward currents through a1D form LTCCs with biophysical and pharmacological properties distinct from C-LTCCs (4, 6). These include a more negative range of current activation and slower current inactivation during depolarizations, allowing these channels to mediate long lasting Ca\(^{2+}\) influx during weak depolarizations. Such properties allow LTCCs to control tonic neurotransmitter release in hair cells (5, 10), diastolic depolarization in the sinoatrial node (11), and electrical excitability of neurons (12–15).

In addition to these biophysical properties, it has also been postulated that n-LTCCs may exhibit a lower sensitivity to DHP channel blockers (4, 16). However, this has never been proven or quantified in a comparative study of the DHP sensitivity of D- and C-LTCCs. At present it is also unclear to which extent these biophysical and pharmacological differences are determined by the a1D subunit itself, by known accessory Ca\(^{2+}\) channel subunits, or by other Ca\(^{2+}\)-channel-associated proteins.

By analyzing the biophysical and pharmacological properties of cloned human a1D splice variants in tsA-201 cells, we provide evidence that most of the biophysical differences described above are determined by a1D. We also demonstrate that alternative splicing of exon 8 is critically affecting the expression of functional a1D protein in tsA-201 cells. Our analysis also revealed that, in functional experiments, D-LTCCs display lower sensitivity for DHPs than a1C, despite similar affinity for the DHP binding pocket.

**EXPERIMENTAL PROCEDURES**

Cloning of Human a1D (Cav1.3) Subunits—a1D cDNAs were obtained by reverse transcription-polymerase chain reaction from human pancreas poly(A\(^{+}\)) RNA (CLONTECH) using proofreading Pfu Turbo\(^{\text{TM}}\) DNA polymerase (Stratagene). First strand cDNA was synthesized using 1 \(\mu\)g of poly(A\(^{+}\)) RNA employing Ready-To-Go\(^{\text{TM}}\) T-promoted first-strand reaction kit (Amersham Pharmacia Biotech). Five a1D fragments with different native or artificial restriction sites were amplified from the first strand cDNA using suitable primer pairs. Fragments were as follows (nucleotide numbering according to Ref. 9; asterisks indicate artificial restriction sites introduced by polymerase chain reaction): F1, SalI*-SpeI (nucleotides 1–758), F2, SpeI-SphI (nucleotides 758–1405), F3/4 ClaI-HindIII (nucleotides 1353–3993), F5 HindIII-XhoI* (nucleotides 3993–5340), and F6 XhoI*-BamHI* (nucleotides 1–1405).
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5340–6498). Fragments were subcloned into the expression vectors pBluescript SK + (Stratagene) or pSoup1-2 (Life Technologies, Inc.). Two F2 fragments containing different splice variants (8A and 8B; Ref. 9) were obtained and used to construct a1D8A and a1D8B. The sequence integrity of all fragments was determined by DNA sequencing. The complete a1D cDNA was constructed by generating subclone F5/6 by coligating the HindIII-XhoI fragment (F5) and the XhoI-BamHI fragment (F6) into pBluescript SK+ . F1 and F2 fragments were coligated into the corresponding SalI/SphI RE sites of the plasmid containing fragment F3/4. The resulting SalI-HindIII fragment (F1–4) was ligated into the SalI/HindIII restriction sites of subclone F5/6. For heterologous expression studies, the a1D cDNA was cloned into the mammalian expression plasmids pGFP– and pGFP + (17). Cloning into pGFP + generates a fusion protein of a1D with N-terminally located green fluorescent protein (GFP).

Transient Expression of a1D Splice Variants in tsA-201 Cells—tsA-201 cells were maintained in Dulbecco’s modified Eagle’s medium/Coon’s F-12 medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Sebaby), 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin at 37 °C and 7% CO2. For transient Ca2+ channel expression, cells were grown to 80% confluence and plated at dilutions of 1:10 on glass coverslips 12 h before transfection by lipofection with (−) [3H]isradipine were performed in a final assay volumes of 0.5 ml. Binding experiments with (−) [3H]isradipine and BayK8644 were employed as their racemic mixtures.

Transfected cells were visualized, and post-inactivated GFP fluorescence Ca2+ currents in IHCs were measured as previously described in cochlea isolated from 2–4-day-old animals (4). Immuno blotting and preparation of affinity-purified sequence-directed antibodies was carried out as described (4, 26) using antibodies described in the legend to Fig. 1.

**RESULTS**

We cloned two different cDNAs encoding full-length a1D subunits (Fig. 1A) from human pancreatic tissue, containing alternatively spliced exons 8A or 8B (a1D8A, a1D8B), which results in a six-amino acid difference in the pore region of repeat I. Their sequence is identical to a previously cloned cDNA (9) but both splice variants lack exons 32 and 44. Heterologous expression in tsA-201 cells together with auxiliary subunits yielded DHP-sensitive L-type currents for a1D8A (100 of 154 patched GFP-expressing cells gave measurable I(f, b) but not for a1D8B. Similarly, no current was measured under our experimental conditions after transfection with rat a1D cDNA (18), which also contains exon 8B. To determine if the absence of Ca2+ current resulted in the expression of a non-functional subunit or was due to the absence of a1D protein, a1D subunit expression was quantified by immunoblot analysis of transfected tsA-201 cell membranes (Fig. 1B). A full-length form of a1D protein was only detected for a1D8A but not for rat and human a1D8B. After transfection of cells with a1D8B, a1D immunoreactivity was only associated with polypeptides smaller than the expected full-length form (Fig. 1), suggesting that a1D8B protein underlies proteolytic degradation in tsA-201 cells. As a cloning artifact was ruled out by DNA sequencing (see “Experimental Procedures”), our data suggest that the six-amino acid residue difference prevents effective a1D Ca2+ subunit expression in tsA-201 cells.

Next we tested if a1D8A could give rise to L-type currents with the characteristics described recently in chick and mouse cochlea (4, 5, 16). These include low activation threshold, fast activation kinetics, slow inactivation, and lower apparent DHP antagonist sensitivity. After transfection using Ca2+ phosphate precipitation, the a1D current density was similar to a1C-a mediated currents (Table 1). With 15 mM Ba2+ as the charge carrier, the threshold of activation for a1D8A was found to be at over 15 mA more hyperpolarized potentials (−45.7 ± 0.5 mV; \( n = 38 \)) as compared with a1C-a (−31.5 ± 0.5 mV; \( n = 16 \)) (Table I, Fig. 2A). The maximum of the current-voltage rela-
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Fig. 1. A, position of alternatively spliced exons 8, 32, 44 in a1D subunits. The expressed a1D cDNAs (a1D\(_{63}\) and a1D\(_{50}\)) are identical to the cDNA reported by Williams et al. (9) but lack exons 32 and 44 (position indicated by arrows). They contain exon 8A or 8B in repeat I (bold line and black box). A sequence alignment of the predicted amino acid sequences encoded by exon 8A and exon 8B is also shown. B, expression of a1D subunits in tsA-201 cells. Heterologous expression of human a1D\(_{63}\), a1D\(_{50}\), and rat a1D\(_{50}\) in tsA-201 cells is shown. Cells were transfected with a1 together with \(\alpha_6\) and \(\beta_3\) subunit DNA as described under “Experimental Procedures.” a1D subunit expression was analyzed in immunoblots of membranes prepared from lysed cells after separation on 8% SDS-polyacrylamide gels using sequence-directed antibodies raised against a1 subunits. A1D\(_{30–48}\) (anti-\(\alpha_1\)D) and 2121–2137 (anti-\(\alpha_1\)D\(_{2111–2137}\)) (9) or an antibody recognizing all a1 subunits (\(\alpha_1\)max) raised against residues 1352–1400 of a1B. Arrows indicate specific a1D immunoreactivity absent in mock transfected cells. One representative experiment (of \(n > 2\)) is shown.

TABLE I

Biophysical properties of D- and C-LTCCs expressed in tsA-201 cells

The half-maximal voltage for activation (\(V_{0.5,\text{act}}\)), the slope for activation (\(k_{\text{act}}\)), the maximum of the current-voltage relationship (\(V_{\text{max}}\)), and the slope for inactivation (\(k_{\text{inact}}\)) were obtained by fitting the data as described under “Experimental Procedures.” The activation threshold is defined in the legend to Fig. 2. The time course of current activation was fitted using a single exponential function. \(\tau_{\text{act}}\) is given for \(I_{\text{Ba}}\) elicited by test pulses to \(V_{\text{max}}\). Data are given as mean ± S.E., * statistically significant difference (\(p < 0.05\)).

|          | \(V_{0.5,\text{act}}\) | \(k_{\text{act}}\) | \(V_{\text{max}}\) | Current density | Activation threshold | \(\tau_{\text{act}}\) | \(V_{0.5,\text{inact}}\) | \(k_{\text{inact}}\) |
|----------|-----------------------|--------------------|-------------------|-----------------|---------------------|----------------|------------------------|-----------------|
| a1D      | -17.5 ± 0.9*          | -8.9 ± 0.6         | -5.5 ± 0.9*       | 24.3 ± 5.7      | -45.7 ± 0.5*        | 0.8 ± 0.06* | -42.7 ± 1.6*          | 6.6 ± 0.8*      |
| \(n = 38\) | n = 38               | n = 38             | n = 38            | n = 38          | n = 38              | n = 24       | n = 6                  | n = 6           |
| \(n = 16\) | n = 16               | n = 16             | n = 16            | n = 16          | n = 16              | n = 4        | n = 4                  | n = 4           |
| a1C      | -3.9 ± 1.3            | -7.8 ± 0.2         | 8.8 ± 0.9         | 24.5 ± 6.6      | -31.5 ± 0.5         | 2.3 ± 0.39  | -27.6 ± 2.7           | 13.8 ± 1.7      |
| \(n = 16\) | n = 16               | n = 16             | n = 16            | n = 16          | n = 16              | n = 4        | n = 4                  | n = 4           |

The midpoint voltage for activation of DHP\(_{15}\) was not complete during the 5-s conditioning pulses. Steady-state inactivation of \(I_{\text{Ba}}\) was compared after coexpression of \(\beta_2\) subunits with 15 mM Ca\(^{2+}\) as the charge carrier. The midpoint voltage of the steady-state inactivation curve for a1D\(_{63}\) was about 10 mV more negative (−42.7 ± 1.6 mV, \(n = 6\)) compared with a1C\(_{63}\) (−27.6 ± 2.7 mV, \(n = 4\)) (Fig. 3D). Note that inactivation of a1D was not complete during the 5-s conditioning pulses.

The more negative activation threshold (a1D\(_{63}\): −37.2 ± 0.98, \(n = 13\); a1C\(_{63}\): −27.6 ± 1.3, \(n = 16\)) was also observed when 15 mM Ca\(^{2+}\) were used as the charge carrier. a1D\(_{63}\) showed pronounced Ca\(^{2+}\)-induced inactivation kinetics. During test pulses to \(V_{\text{max}}\) about 70% of \(I_{\text{Ba}}\) inactivated within less than 400 ms (\(n = 6\)). Inactivation of a1D\(_{63}\) current was slower and less complete than a1C\(_{63}\) (data not shown).

Previous experiments suggested that the DHP antagonist sensitivity may be lower for D- than for C-LTCCs. However, this has never been proven by a systematic comparison of DHP...
antagonist effects on α1D and α1C-a currents under identical experimental conditions. We therefore measured the inhibition of both channel types by the DHP antagonist isradipine at various concentrations and at different holding potentials. At −90 mV holding potential, 300 nM isradipine completely inhibited α1C-a current (98.3 ± 1.7%, n = 3) but only 30–40% of α1D8A (Fig. 4). The concentration dependence of block revealed that isradipine sensitivity for α1D8A was 8.5-fold lower (Fig. 4B) at this holding potential. Isradipine sensitivity increased by an order of magnitude at a more positive holding potential (−50 mV; Fig. 4B), demonstrating a voltage-dependent mechanism of DHP block as described previously for α1C-a currents (28, 29).

To determine if this sensitivity difference was due to a lower affinity of isradipine for α1D8A, its affinity for the DHP binding pocket was determined in membranes prepared from transfected cells in radioligand binding experiments with (±)-[3H]isradipine (Fig. 5). Under identical experimental conditions, similar affinities were measured for α1D8A (K_D = 0.42 ± 0.06 nM, n = 10) and α1C-a (K_D = 0.68 ± 0.12 nM, n = 8) (Fig. 5A). Therefore, different affinities for the DHP drug binding pocket cannot account for the lower sensitivity observed in our functional studies. Dissociation of bound (±)-[3H]isradipine occurred about 8-fold faster from α1D8A (k_−1 = 32.6 ± 4.8 × 10^{-3} min^{-1}, n = 3) than from α1C-a (k_−1 = 4.2 ± 0.1 × 10^{-3} min^{-1}, n = 2) (Fig. 5B). This suggests that, despite similar binding affinity, binding kinetics differ between the two α1 subunit isoforms.

\( \alpha1D8A \) Ca\(^{2+} \) channels coexpressed with β3 and α2δ in tsA-201 cells also exhibited the typical current modulation by the Ca\(^{2+} \) channel activator BayK8644 (Fig. 4, C and D) similar to its actions in IHCs (4). BayK8644 increased the maximal I_Ba, produced a slight hyperpolarizing shift in the IV relationship and slowed current deactivation of the tail current induced by repolarization of the cell to the holding potential (Fig. 4D). The extent of maximal I_Ba stimulation was more pronounced in tsA-201 cells as compared with IHCs (7.5 ± 1.8 -fold, n = 6), versus 2.9-fold; Ref. 4).

**DISCUSSION**

Here we present the first detailed patch clamp analysis of the biophysical properties of whole cell currents through Ca\(^{2+} \) channels formed by α1D subunits after heterologous expression in mammalian cells. Our studies revealed that these subunits can form L-type Ca\(^{2+} \) channels with properties similar to D-LTCCs previously described in IHCs (4). We clearly demonstrate lower DHP antagonist sensitivity, more rapid activation kinetics, and slower inactivation for the α1D8A splice variant as compared with α1C-a currents. Another novel finding was that the lower DHP antagonist sensitivity is not due to lower binding affinity for the DHP binding pocket. Instead, it is only
current traces elicited by 30-ms depolarization for a holding potential of −90 mV (filled circles) or −50 mV (open circles) before and during superfusion of the cell with isradipine-containing solution. Maximal $I_{Na}$ is plotted against time. The inset shows representative current traces (holding potential −50 mV) in the absence (control, at 40 s) and presence of 300 nM isradipine (at 120 s). B, the concentration-response relationship of peak $I_{Na}$ inhibition of α1D$_{8A}$ (circles) and α1C-a (triangles) by isradipine was determined at holding potentials of −90 mV (filled symbols) and −50 mV (open symbols; α1D$_{8A}$ only). C, normalized IV curves for α1D$_{8A}$ in the absence (control, open squares) and presence of the Ca$^{2+}$ channel activator BayK8644 (5 μM, filled squares). D, corresponding peak current traces elicited by 30-ms depolarizations to $V_{max}$ in the absence and presence of BayK8644.

**Fig. 5.** Reversible binding of (+)-[3H]isradipine to α1D and α1C-a expressed in tsA-201 cells. A, equilibrium saturation studies. Increasing concentrations of (+)-[3H]isradipine were incubated (37 °C, 120 min) with membrane protein (α1D, 33 μg/ml; α1C, 23 μg/ml) prepared from tsA-201 cells transfected with α1D or α1C-a together with α2-δ + β DNAs. Nonspecific binding was determined in the presence of 1 μM isradipine. Specific binding data were fitted to a monophasic saturation isotherm yielding the following binding parameters: α1D$_{8A}$, $K_D = 334$ pm and $B_{max} = 1.72$ pmol/mg; α1C, $K_D = 503$ pm and $B_{max} = 1.11$ pmol/mg. B, dissociation experiments. Membrane protein from cells expressing α1D (25 μg/ml) or α1C-a (25 μg/ml) were incubated with 0.19 nM (+)-[3H]isradipine until equilibrium ($B_e$) was reached. Dissociation was induced by rapid addition of a final concentration of 1 μM unlabeled isradipine. A semilogarithmic representation of the time-dependent decay of specific (+)-[3H]isradipine binding (B) is illustrated. Linear regression analysis ($r > 0.98$) yielded the following $k_d$ values: for α1D$_{8A}$, 27.8 × 10$^{-3}$ × min$^{-1}$; for α1C, 4.3 × 10$^{-3}$ × min$^{-1}$.

![Diagram](http://www.jbc.org)
possible that alternative splicing in this region also affects the activation threshold of a1D. In contrast to our constructs, a previously cloned human a1D (9) contains exon 32 (and exon 44). Unfortunately, its biophysical and pharmacological properties have not been directly compared with a1C under identical experimental conditions. Stable expression of this cDNA in HEK-293 cells (together with \( \beta_3 \) and \( \alpha_{28} \), 20 mM \( \text{Ba}^{2+} \) as charge carrier) yielded channel currents with \( V_{\text{m}} \), of +1.8 mV and \( V_{\text{m}} \), of +20 mV (33). However, correction for junction potential would shift these values to more negative potentials by about 23 mV, resulting in activation parameters nearly identical to our a1D8A subunit. Minor effects of exon 32 on channel gating cannot be ruled out and will require a more detailed analysis.

The only property not mimicked by our splice variant was the very slow inactivation of IHC currents during a depolarizing pulse. a1D8A currents inactivate slower than a1C-a but faster than \( I_{\beta_3} \) in IHCs. As compared with a1C-a, the slower inactivation of a1D8A can be explained by an increase of the contribution and the time constant of a slow component of \( I_{\beta_3} \) inactivation. It remains to be determined if alternative splicing or the presence of another auxiliary subunit is responsible for the slow inactivation of class D currents in IHCs.

The relatively weak channel block of \( I_{\beta_3} \) through class D LTCCs by DHP antagonists was now directly confirmed in our experiments. We describe for the first time that DHP inhibition of a1D is voltage-dependent and favored at more positive voltages. As for a1C-a, this indicates higher affinity for inactivated channels (28, 29) or induction of inactivated channel states (25). a1D8A showed an approximately 10-fold lower sensitivity for block by the DHP antagonist isradipine than a1C-a. This finding nicely explains the absence of major side effects expected from block of class D channels in humans at therapeutic plasma concentrations. This includes the lack of bradycardic actions expected from the block of class D channels in sinoatrial node cells as well as the absence of hearing disturbances resulting from the block of this channel type in cochlear IHCs (4). Instead, DHPs preferentially block class C LTCCs in the cardiovascular system (34). The higher DHP sensitivity of the smooth muscle a1C splice variant (a1C-b) as compared to the cardiac splice variant (a1C-a; Ref. 28) in part explains the selectivity of DHPs for arterial smooth muscle, resulting in therapeutic antihypertensive effects in the absence of cardiodepression (34). The higher sensitivity of a1C-b is also not due to a higher affinity of DHP antagonists for its DHP binding pocket (28). Instead, it could be explained by differences in the voltage dependence of a1C block and is due to amino acid divergence between the two splice variants in transmembrane segment IS6. We propose that a similar mechanism must explain the lower DHP antagonist sensitivity of a1D8A as compared with a1C-a because the affinity for the DHP binding pocket is the same in these subunits.

Although we and others (9) found evidence for alternative splicing in segment IS6 (exons 8A and 8B, respectively), the role of exon 8B for a1D function and DHP modulation could not be assessed. This was due to the absence of intact a1D protein and currents after expression of our human a1D construct in which exon 8A sequence was exchanged for exon 8B (a1D8B). The inhibitory role of exon 8B on a1D expression is further supported by our inability to transiently express full-length rat a1D, which also contains exon 8B, despite the abundant presence of mRNA in the transfected cells. In accordance with our interpretation, the functional expression of exon 8B containing a1D cDNAs isolated from rat brain (35) and chicken cochlea (36) has not yet been reported. Further studies must determine which of the 6 amino acids differing between these alternative exons account for this effect. It is possible that alternative splicing in IS6 serves as a molecular switch to modulate a1D subunit expression on the post-transcriptional level.

Our experiments provide convincing evidence that a1D8A-mediated I-type currents activate at slightly more positive voltages than recombinant T-type channels (37) but at more negative voltages than C-LTCCs as reported here. In this respect they resemble a1E-mediated currents (37), which were originally classified as low voltage-activated (38). Although we cannot exclude that alternative splicing or biochemical modulation (e.g. phosphorylation) also allows other I-type a1 subunit isoforms (including a1C) to activate at such “intermediate” voltages, our data provide direct evidence that D-LTCCs can account for the "low voltage-activated" I-type currents described previously in neurons (15). We have developed mutant mice in which the DHP sensitivity of a1C-subunits is dramatically reduced (39). Such animal models will allow direct determination of the role of D-LTCCs for I-type current components in various tissues.

Taken together, the evidence demonstrates that expression of a1D subunits should enable cells to slowly inactivating voltage-gated Ca\(^{2+}\) influx in response to rather weak depolarizations. This property allows them to participate in important physiological functions such as tonic neurotransmitter release in IHCs (10) and control of diastolic depolarization in sinoatrial node (4). These properties also make them ideally suited to contribute to subthreshold Ca\(^{2+}\) signaling (e.g. in hippocampal pyramidal cells; Ref. 14) and to the plateau potential underlying bistable membrane behavior (e.g. in motoneurons; Refs. 12 and 13).

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Note Added in Proof—In a report, which will be published later this year (Scholze et al., Mol. Endocrinol., in press), similar biophysical properties are reported for a Ca, I.3 splice variant cloned from HIT-T15 cells after expression in Xenopus laevis oocytes.

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