Journal of Bioenergetics and Biomembranes, Vol. 30, No. 4, 1998

Molecular Characterization of a Novel Family of Low Voltage-Activated, T-Type, Calcium Channels

Edward Perez-Reyes[1]

Received April 30, 1998

Low voltage-activated, T-type, calcium channels are thought to be involved in pacemaker activity, low threshold Ca^{2+} spikes, neuronal oscillations and resonance, and rebound burst firing. Mutations in T-type channel genes may be a contributing factor to neurological and cardiovascular disorders, such as epilepsy, arrhythmia, and hypertension. Due to the lack of selective blockers, little is known about their structure or molecular biology. This review discusses our recent findings on the cloning, chromosomal localization, and functional expression, of two novel channels, α1G and α1H. The biophysical properties of these cloned channels (distinctive voltage dependence, kinetics, and single channel conductance) demonstrates that these channels are members of the T-type Ca^{2+} channel family.

KEY WORDS: Molecular cloning; calcium channel; low voltage-activated; T-type; brain; heart; heterologous expression.

The activity of T-type Ca^{2+} channels was first described as a paradox (Andersen *et al.*, 1964; Huguenard, 1996; Llinas and Jahnsen, 1982). Why did neurons fire action potentials after hyperpolarizing pulses designed to mimic inhibitory post-synaptic potentials (IPSP)? This behavior was called post-anodal excitation, or rebound burst firing. The paradox was resolved by studying the gating behavior of T-type Ca^{2+} channels. In particular, T-type channels have three unique properties: one, they inactivate near the resting membrane potential, two, they recover quickly from inactivation, and three, they open near the resting membrane potential. Therefore, T-type channels can open after an IPSP, causing further depolarization of the membrane potential to where either Na^+ or high voltage-activated (HVA) Ca^{2+} channels can open.

The existence of low voltage-activated (LVA) Ca^{2+} channels was established in the mid-80's in studies on dorsal root ganglion neurons (Carbone and Lux, 1984; Bossu *et al.*, 1985; Fedulova *et al.*, 1985; Nowycky *et al.*, 1985), cardiac myocytes (Nilius *et al.*, 1985; Bean, 1985), and GH3 pituitary cells (Matteson and Armstrong, 1986). These studies established the following properties to distinguish LVA from HVA Ca^{2+} channels: (1) they opened at lower voltages, (2) they inactivated more rapidly (FI), (3) they inactivated at more negative potentials, (4) they deactivated more slowly (SD), (5) they had a smaller single-channel conductance in Ba^{2+}, (6) they were more resistant to rundown, and (7) they were less sensitive to classical Ca^{2+} channel blockers, such as the dihydropyridines. These distinctive gating and permeability properties define T-type channels as a subset of low voltage-activated Ca^{2+} channels. Subsequent studies demonstrated that T-type Ca^{2+} channels were responsible for generating low threshold spikes (LTS) spikes (Coulter *et al.*, 1989; Crunelli *et al.*, 1989; Hernandez-Cruz and Pape, 1989; Suzuki and Rogawski, 1989). Physiological roles for T-type channels have been proposed in both neural and peripheral tissues. In neurons they appear to control pacemaker activity, rebound burst firing, low threshold spikes, and neuronal oscillations. In peripheral tissues T-type channels may be involved in pacing of the heart (Hagiwara *et al.*, 1988), smooth muscle contraction (Akaike *et al.*, 1989), adrenal hor-

[1] Department of Physiology, and Cardiovascular Institute, Loyola University Medical Center, Maywood, Illinois 60153.

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mone secretion (Cohen *et al.*, 1988; Enyeart *et al.*, 1993), and fertilization (Arnoult *et al.*, 1996). Genetic abnormalities in T-type channel genes have been proposed in absence epilepsy (Tsakiridou *et al.*, 1995) and cardiomyopathy (Sen and Smith, 1994).

The molecular biology of voltage-activated Ca^{2+} channels began with the cloning of the skeletal muscle dihydropyridine receptor, α1S (Tanabe *et al.*, 1987). Dihydropyridines, which are useful in the treatment of hypertension, bind to high voltage-activated, L-type channels with such high affinity to allow their purification. In contrast, there are no high-affinity ligands for T-type channels. Using a combination of low-stringency hybridization and polymerase chain reaction (PCR) techniques, five other α1 subunits of Ca^{2+} channels were cloned (Perez-Reyes and Schneider, 1995). All six of these α1 subunits form high voltage-activated Ca^{2+} channels. Original studies on a rat α1E clone (Soong *et al.*, 1993) suggested that it belonged to the low voltage-activated family. However, mouse (Williams *et al.*, 1994), rabbit (Wakamori *et al.*, 1994), and human (Bezprozvanny and Tsien, 1995; Schneider *et al.*, 1995; Williams *et al.*, 1994) α1E clones form high voltage-activated channels. Strong evidence that α1E is not part of T-type channels was provided by studies showing it had a 2-fold higher single-channel conductance (12–14 pS) (Bourinet *et al.*, 1996; Schneider *et al.*, 1995; Wakamori *et al.*, 1994; Williams *et al.*, 1994). However, α1E does share two properties with T-type channels: one, they both inactivate near the resting membrane potential, and two, they are equally permeable to Ca^{2+}, Ba^{2+}, and Sr^{2+} (Bourinet *et al.*, 1996; Shuba *et al.*, 1991).

After years of trying to clone T-type channels using PCR-based strategies, I decided to try an *in silico* strategy, and clone using a computer chip to surf the Genbank DNA database. This strategy had been successfully used to identify a novel K^+ channel (Ketchum *et al.*, 1996). In this study they used the program BLAST, or the *b*asic *l*ocal *a*lignment *s*earch *t*ool (Altschul *et al.*, 1990), which can be accessed through the internet (http://www.ncbi.nlm.nih.gov). This progam allows one to probe the database with either a nucleotide or protein sequence (tblastn). The limitation is that one must predict which region might be conserved in the novel gene. My alternative cloning strategy was to use a text-based search (http://www2.ncbi.nlm.nih.gov/genbank/query__form.html) of the Genbank to find novel sequences that had homology to any region of cloned Ca^{2+} channels. In particular, I focused on the *e*xpressed *s*equence *t*agged (EST) division, which contains partial cDNA fragments cloned from normalized cDNA libraries (Soares *et al.*, 1994), and partially sequenced by the IMAGE Consortium (Lennon *et al.*, 1996). One clone, H06096, was chosen based on its 30% identity to the carp α1S (Genbank #P22316), and sequenced in its entirety (#AF029228). The first 957 nucleotides of this clone appear to encode a voltage-gated Ca^{2+} channel, as the deduced amino acid sequence contains readily identifiable motifs including an S4 region and a pore loop clone. After residue 957, the open reading frame is lost, suggesting this clone contains an intron.

Using the deduced amino acid sequence of AF029228 I searched the Genbank and found #U37548, which contains a putative protein found in the genomic DNA (cosmid C54D2.5) of *Caenorhabditis elegans* (Wilson *et al.*, 1994). Hydropathy analysis of the *C. elegans* sequence suggested it had the four-domain structure typical of voltage-gated Na^+ and Ca^{2+} channels. AF029228 aligned best with the third domain (58% sequence identity). These results suggested the existence of a novel four-domain Ca^{2+} channel that was common in both nematodes and man.

Using the *Bam*H1 fragment of H06096 as probe (#162-977), we screened both rat brain and human heart λgt10 cDNA libraries. A full-length rat brain cDNA, referred to as α1G (Genbank #AF027984), was assembled from five overlapping clones (Perez-Reyes *et al.*, 1998). Screening of the human heart library was performed at moderate stringency, allowing Dr. Cribbs to isolate a related sequence, α1H (Genbank #AF051947), derived from a distinct gene (Cribbs *et al.*, 1998). Sequence identity among the Ca^{2+} channel α1 subunits is highest in the putative membrane spanning regions, with most changes being conservative with respect to structure (Fig. 1). Charged residues are particularly conserved, with many charges being conserved across all domains and in voltage-gated Na^+ and K^+ channels (Jan and Jan, 1990). The charged residues of the S4 regions are also conserved, consistent with its role as a voltage sensor (Stuhmer *et al.*, 1989). The cation selectivity of Ca^{2+} channels requires a ring of negative charge provided by glutamate residues found at similar locations in each domain (Yang *et al.*, 1993; Tang *et al.*, 1993). In both α1G and α1H, two of these glutamates are replaced by aspartate, suggesting an altered selectivity. In contrast, there is little conservation of the sequences that link these regions within a domain, and even less between the intracellular loops that connect the domains. Notably missing are the motifs involved in binding the β sub-

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                S1                       S2                       S3                       S4
Unit I
   α1G      PWFERVSMLVILLNCVTLGMFRP FDDFIFAFFAVEMVVKMVALGI TWNRLDFFIVIAGMLEYSLD FSAVRTVRVLRPLRAINRVPSMRI
   α1H      PWFEHVSMLVIMLNCVTLGMFRP FDAFIFAFFAVEMVIKMVALGL TWNRLDFFIVVAGMMEYSLD LSAIRTVRVLRPLRAINRVPSMRI
   SCDcon   KPFeiiILLtIFANCVALAiYIP vEYlFLiiFtvEaflKvIAYGl GWNlLDFiIVvvGlFsaILE VKALRAFRVLRPLRLVSGVPSLQV
   ABEcon   PPFEYMILATIIANCIVLALEQH TEPYFIGIFCFEAGIKIvALGF GWNVMDFiVVLsGILATaGT LRTLRAVRVLRPLKLVSGIPSLQi
Unit II
   α1G      KYFGRGIMIAILVNTLSMGIEYH SNIVFTSLFALEMLLKLLVIGP PYNIFDGVIVVISVWEIVGQ LRTFRLMRVLKLVRFLPALQRQLV
   α1H      KYFSRGIMMAILVNTLSMGVEYH SNIVFTSMFALEMLLKLLVYGP PYNIFDGIIVVISVWEIVGQ LRTFRLLRVLKLVRFLPALRRQLV
   SCDcon   nvFYWLVIflVfLNTLtIaSEHy ANkaLLaLFTaEMLlKMYSLGL lFNRFDCFiVCgGIlEtiLV lRCvRLLRIFKiTryWnSLsNLVA
   ABEcon   QvFYWiVLslVALNTaCVAiVHh AEFlFLGLFllEMslKMYGmGp SFNCFDfGVtvGSiFEVvWA LRALRLLRIFKiTKYWaSLRNLVV
Unit II
   α1G      KMFDHVVLVIIFLNCITIAMERP SNYIFTAVFLAEMTVKVVALGW SWNVLDGLLVLISVIDILVS LRVLRLLRTLRPLRVISRARGLKL
   α1H      KMFDHVVLVFIFLNCVTIALERP SNYIFTAIFVAEMMVKVVALGL SWNLLDGLLVMVSLVDIVVA LRVLRLLRTLRPLRVISRAQGLKL
   SCDcon   tiFTNlILfFIlLSSisLAAEDP FDivFTtiFTiEIaLKMTayGA YFNiLDlLVVsVSLiSfGiq VKILRVLRVLRPLRAINRAKGLKH
   ABEcon   RYFEmcILlVIAaSSIALAAEDP fDYvFTGVFTFEMVIKMIDqGL LWNILDFvVVvGALVAFAla IKSLRVLRVLRPLKTIKRLPKLKA
Unit IV
   α1G      HYLDLFITGVIGLNVVTMAMEHY CNYIFTVIFVFESVFKLVAFAF RWNQLDLAIVLLSIMGITLE IRIMRVLRIARVLKLLKMAVGMRA
   α1H      HYLDLFITFIICVNVITMSMEHY CNYVFTIVFVFEAALKLVAFGF RWNQLDLAIVLLSLMGITLE IRIMRVLRIARVLKLLKMATGMRA
   SCDcon   tyFEYlMFvLIlLNTiCLaMQHY LNmlFTglFTvEMiLKlIAFKp pWNvFDfLIVIGSIIDViLS itFFRLFRVMRLvKLLSRgEGiRT
   ABEcon   PsFEYtIMAMIALNTvVLMMKyY lNIaFTmvFSlECvLKviAFGf tWNiFDFiTViGSITkIvlT mSFLkLFRAARLIKLLRQGYTIRI

                S5                      P Loop                   S6
Unit I                                               #
   α1G      MLGNVLLLCPFVFFIFGIVGVQLWAGL FDNIGYAWIAIFQVITLEGWVDIMY FYNFIYFILLIIVGSFFMINLCLVVIATQF
   α1H      MLGNVLLLCPFVFFIFGIVGVQLWAGL FDNTCYAWIAIFQVITLEGWVDIMY FYNFIYFILLIIVGSFFMINLCLVVIATQF
   SCDcon   PLlHIALLVLFviIIYAIIGLELFmGK FDNFaFaMLTVfQCITMEGWTDVLY ElPWvYFVsLvIfGSFFvLNLVLGVLSGEF
   ABEcon   PLLQIGLLLFFAILmFAIIGLEFYsGK FDNILFAvLTVFQCITMEGWTtvLY TWNWLYFIPLIIIGSFFvLNLVLGVLSGEF
Unit II                                              #
   α1G      NVATFCMLLMLFIFIFSILGMHLFGCK FDSLLWAIVTVFQILTQEDWNKVLY SWAALYFIALMTFGNYVLFNLLVAILVEGF
   α1H      NVATFCTLLMLFIFIFSILGMHLFGCK FDSLLWAIVTVFQILTQEDWNVVLY SWAALYFVALMTFGNYVLFNLLVAILVEGF
   SCDcon   SiASLLLLLFLFIIIFsLLGMQLFGGk FDNFPQaLltVFQiLTGEDWNsVMY mlVCIYFIILFiCGNYILLNVFLAIAVDNL
   ABEcon   SIISLLFLLFLFIVVFALLGMQLFGGr FDTFPAAImTVFQILTGEDWNeVMY MwsavYFIVLTLFGNYTLLNVFLAIAVDNL
Unit III                                             #
   α1G      PIGNIVVICCAFFIIFGILGVQLFKGK FDNLGQALMSLFVLASKDGWVDIMY PWMLLYFISFLLIVAFFVLNMFVGVVVENF
   α1H      PIGNIVLICCAFFIIFGILGVQLFKGK FDNLGQALMSLFVLSSKDGWVNIMY PWMLLYFISFLLIVSFFVLNMFVGVVVENF
   SCDcon   TIGNIviVTTLLQFMFACIGVQLFKGK FDNVLaAMMaLFTVSTFEGWPeLLY VEisIFFIIYIIIiAFFMMNIFVGFVIVTF
   ABEcon   NVFNILIVYkLFMFIFAVIAVQLFKGK YDNiiWALLTLFTVSTGEGWPqVLq MEmSIFYVVYFVVFPFFFVNIFVALIIITF
Unit IV                                              #
   α1G      QVGNLGLLFMLLFFIFAALGVELFGDL FRNFGMAFLTLFRVSTGDNWNGIMK VISPIYFVSFVLTAQFVLVNVVIAVLMKHL
   α1H      QVGNLGLLFMLLFFIYAALGVELFGRL FSNFGMAFLTLFRVSTGDNWNGIMK ALSPVYFVTFVLVAQFVLVNVVVAVLMKHL
   SCDcon   ALPYVALLIvMLFFIYAVIGMQvFGKi FQTFPQAVLLLFRCATGEAWQdImL sFAvfYFISFYMLCAFLIINLFVAVIMDNF
   ABEcon   ALPYVCLLIAMLFFIYAIIGMQVFGNI FRsFfgaLMLLFRSATGEAWqkIML dlAYvYFVSFIFfCSFLMLNLFVAVIMDNF
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Fig. 1. Alignment of the putative membrane spanning regions of α1G, α1H, and the consensus sequences of HVA channels. A consensus sequence of the L-type family (SCDcon) was made from the sequences of α1S (L33798), α1C (L04569), and α1D (M76558), while the non-L-type HVA (ABEcon) consensus was made from the sequences of α1A (X99897), α1B (M94172), and α1E (L27745). Amino acids that are conserved in all eight mammalian α1 subunits within a domain are boldfaced. The highly conserved amino acids in the pore loop that determine Ca^{2+} selectivity in high voltage-activated Ca^{2+} channels (Yang *et al.* 1993; Tang *et al.* 1993) are marked with a hash.

unit (Pragnell *et al.*, 1994) and Ca^{2+} (de Leon *et al.*, 1995).

Using this alignment, an evolutionary tree was constructed (Fig. 2). This analysis shows that T-type channels form a distinct branch of the Ca^{2+} channel superfamily. Also shown is the partial sequence of α1F deduced from genomic DNA derived from the X-chromosome (Fisher *et al.*, 1997). Although α1F is highly related to L-type channels, proof that it is an L-type channel will require cloning and expression of its full-length cDNA.

Northern analysis of α1G mRNA distribution indicated that it was expressed at high levels in the brain, in particular the thalamus, cerebellum, and amygdala, and it was expressed in the heart (Perez-Reyes *et al.*, 1998). In contrast, α1H mRNA showed a broader distribution in peripheral tissues such as kidney and liver (Cribbs *et al.*, 1998).

Considerable evidence suggests a link between T-type Ca^{2+} channels and epilepsy (Tsakiridou *et al.*, 1995; Coulter *et al.*, 1990; Kelly *et al.*, 1990; Todorovic and Lingle 1998). Mutations in the α1A (P/Q-type)

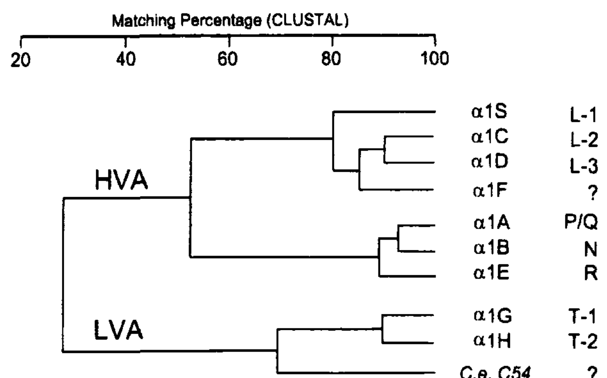

Fig. 2. Evolutionary tree analysis based on alignment of the membrane spanning regions as shown in Fig. 1. Matching percentage was calculated using CLUSTAL (Higgins and Sharp, 1988). The *C. elegans* sequence (*C.e.* C54) was modified from the cosmid sequence (U37548) to include an exon encoding Domain III S2–S4. The Genbank accession numbers of the sequences are given in the legend to Fig. 1, except for α1F, which is from U93305.

gene have been linked to ataxic and epileptic phenotypes in both mice (Fletcher *et al.*, 1996; Doyle *et al.*, 1997) and men (Ophoff *et al.*, 1996). Similarly, the genetic defect in the ataxic and seizure-prone mouse, lethargic (*lh*), has been linked with the HVA auxiliary subunit, β_4 (Burgess *et al.*, 1997; Castellano *et al.*, 1993). To explore such a link our collaborators at the Rayne Institute mapped the genes encoding T-type channels. The α1G gene, *CACNA1G*, was mapped to the human chromosome 17q22 and the mouse chromosome 11 (Perez-Reyes *et al.*, 1998). The α1H gene, *CACNA1H*, was mapped to human chromosome 16p13.3 and mouse chromosome 17 (Cribbs *et al.*, 1998). A mouse neurological mutant with an ataxic phenotype, teetering (*tn*), maps close to the *Cacna1g* locus (Montgomery *et al.*, 1997; Meier, 1967).

Functional expression of α1G was measured in *Xenopus* oocytes; however, no current could be measured from oocytes injected with α1H. In contrast, HEK-293 cells transfected with α1H displayed robust currents. Figure 3a shows a representative family of current traces elicited by depolarizing pulses from an α1G-injected oocyte. Figure 3b shows current traces from an HEK-293 cell transfected with α1H. Inward Ba^{2+} currents activated slowly near threshold potentials (−55 mV in 10 mM Ba^{2+}), while stronger depolarizations produced a current that activated and inactivated quickly. Randall and Tsien have called such crossing of successive currents a signature pattern of classical T-type channels (Randall and Tsien, 1997). To illustrate this pattern (Fig. 3c) we recorded *bona fide* T-type currents from undifferentiated NIE-115 mouse neuroblastoma cells (Shuba *et al.*, 1991). In contrast, currents from oocytes injected with α1E have a distinct pattern, since activation is uniformly fast (Fig. 3d). Uninjected oocytes had no detectable (> 10 nA) inward Ba^{2+} currents. Figure 3e shows the normalized peak current–voltage curves for α1G, α1H, and α1E (10 mM Ba^{2+}). These results demonstrate that α1G and α1H are low voltage-activated channels that gate 30 mV more negative than α1E channels. In addition, α1G and α1H currents activate and inactivate with similar kinetics as observed for T-type channels (reviewed in Huguenard, 1996; and Chen and Hess, 1990).

One of the defining features of low voltage-activated Ca^{2+} channels is their slow deactivation (tail current) after a test pulse (Matteson and Armstrong, 1986). Figure 3f illustrates the time and voltage dependence of deactivation. Deactivation kinetics were voltage dependent (Fig. 3g) as demonstrated for native T-type tail currents (Chen and Hess, 1990; Randall and Tsien, 1997; Santi *et al.*, 1996).

A final defining characteristic of T-type Ca^{2+} channels is that their unitary conductance is tiny (Carbone and Lux, 1984; Nilius *et al.*, 1985). Measurement of this conductance is complicated by the low probability of channel opening at negative potentials where the driving force is stronger. Therefore, we used tail current protocols as shown in Fig. 3f to enhance channel opening at negative potentials. Figure 3h shows representative channel openings from a single patch. The average single channel conductance was 7.5 pS (Perez-Reyes *et al.*, 1998). An average of the values reported in the literature for neuronal T-type channels was 7.7 pS (reviewed in Huguenard, 1996). A slightly smaller conductance (5.3 pS) was measured for α1H in HEK-293 cells (Cribbs *et al.*, 1998). We are currently investigating if the small differences in the observed biophysical properties between α1G and α1H can be ascribed to the different expression systems used (oocyte vs. mammalian cells).

These results demonstrate that we have identified two members of the low voltage-activated T-type Ca^{2+} channel family. Functional expression of α1G in oocytes and α1H in HEK-293 cells allowed us to measure their biophysical properties. Based on the following criteria we unambiguously demonstrate that they encode T-type Ca^{2+} channels: they open near the resting membrane potential of most cells (LVA), they open slowly near threshold, they inactivate relatively quickly giving rise to transient currents, they close

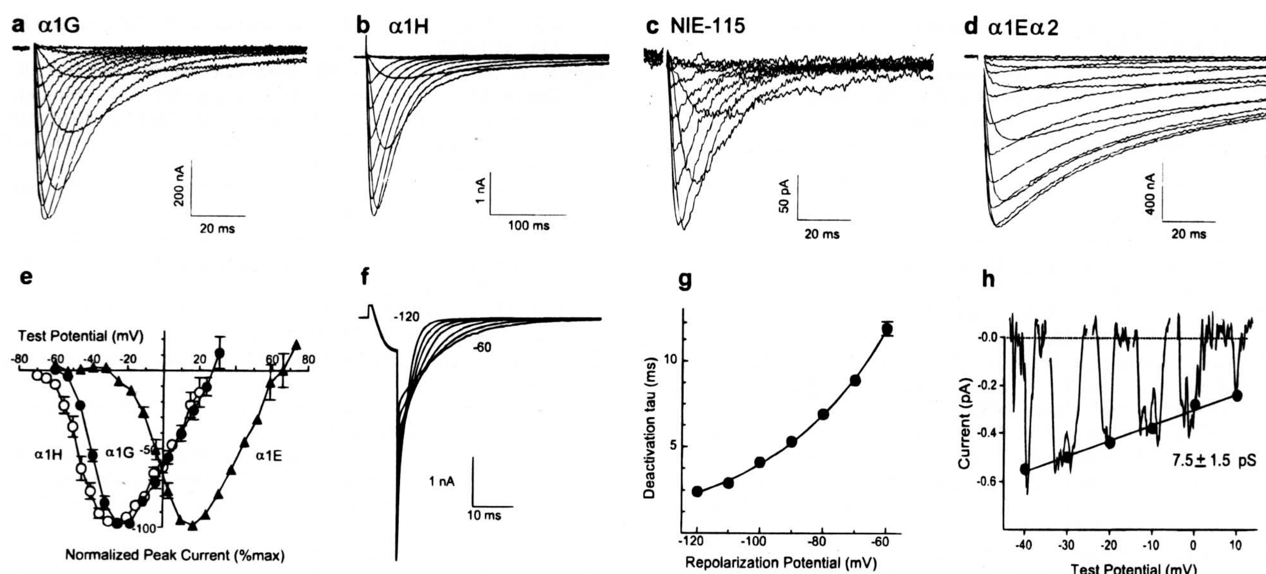

Fig. 3. Biophysical properties of cloned α1G and α1H. (a) Cloned α1G currents were recorded in oocytes using 40 mM Ba^{2+} as charge carrier. (b) Cloned α1H currents were recorded in HEK-293 cells using 10 mM Ba^{2+} as charge carrier. (c) Currents from an undifferentiated mouse neuroblastoma cell were recorded using the nystatin-perforated patch method (10 mM Ba^{2+}). (d) Cloned α1E plus α2 currents were recorded from an oocyte injected with 50 nl of 25 mM BAPTA (2 mM Ba^{2+}). (e) Average *IV* curves recorded in 10 mM Ba^{2+}. Peak currents for each cell were normalized to the maximum current observed, then averaged. Error bars represent the s.e.m. from α1G (filled circles), α1H (open circles), or α1E (filled squares). (f) Representative tail currents from α1H at varying repolarization potentials. (g) Voltage-dependence of deactivation; the data were fit with a single exponential, then plotted as a function of the repolarization potential. (h) Representative single-channel openings are superimposed on the slope conductance plot. Single channels were measured in oocytes expressing large α1G currents (> 500 nA). The voltage protocol included a 5 ms step to +20 mV followed by repolarization to the indicated potentials. The current through the main open state of the channel was measured at each potential, then plotted versus the test potential (mean ± s.d.). The slope of the line was calculated by linear regression.

slowly giving rise to slow tail currents (SD), and they have a small unitary conductance in Ba^{2+}.

Since functional expression was obtained either after injection of α1G alone in oocytes or transfection of α1H alone in HEK-293 cells, this suggests that other subunits are not required. In our study of the effects of β subunits on endogenous oocyte Ca^{2+} channels, we reported single-channel evidence suggesting that HVA β subunits could interact with endogenous T-like channels (Lacerda *et al.*, 1994). However, there was no evidence for LVA channels at the whole cell level. Using antisense oligonucleotides to knock down the expression of HVA β subunits in nodose neurons, Lambert *et al.*, showed that only HVA currents were affected (Lambert *et al.*, 1997). Preliminary experiments in oocytes failed to show any effect of HVA β subunits on the activity of α1G (Lee and Perez-Reyes, unpublished observations). My tentative conclusion is that T-type channels may be formed by a single subunit; however, it is highly likely that both oocyte and HEK-293 cells may be supplying additional subunits, as in the case of HVA channels (Tareilus *et al.*, 1997; Singer-Lahat *et al.*, 1992).

ACKNOWLEDGMENTS

I would like to thank the members of my lab who made this work possible: Leanne Cribbs, Jung-Ha Lee, Asif Daud, and Jie Wang. I would also like to acknowledge the valuable contributions of our collaborators, in particular Antonio E. Lacerda, Jonathin Satin, and Jane Barclay. This work was supported by grants from the National Heart, Lung, and Blood Institute, the Potts Foundation, and the American Heart Association.

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