T-Type Calcium Channel α1G and α1H Subunits in Human Retinoblastoma Cells and Their Loss After Differentiation

KAZUYUKI HIROOKA,1,2 GABRIEL E. BERTOLESI,1,3 MELANIE E. M. KELLY,2,3 EILEEN M. DENOVAN-WRIGHT,3 XIAOLU SUN,1,2 JAWED HAMID,4 GERALD W. ZAMPONI,4 ALEXANDER E. JUHASZ,4 LAWRENCE W. HAYNES, AND STEVEN BARNES1,2

1Department of Physiology and Biophysics, 2Department of Ophthalmology, and 3Department of Pharmacology, Dalhousie University, Halifax, Nova Scotia B3H 4H7; and 4Neuroscience Research Group, University of Calgary, Calgary, Alberta T2N 4N1, Canada

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INTRODUCTION

Human retinoblastoma cells have been studied extensively at the cellular and molecular levels and provide a valuable means for investigating growth and differentiation of retinal precursor cells in culture (Gallie et al. 1999; Siegel 1999). Retinoblastoma cell lines are derived from a heritable retinoblastoma that lacks an active RB1 tumor suppressor gene (Kyritsis et al. 1993a; Kelly et al. 2000). Alterations in the expression of transient inward current have been documented in Y-79 cells undergoing differentiation (Gomez et al. 1993a). Molecules have identified voltage-gated calcium channel (Ca channel) α1 subunits as the products of at least 10 different genes that correspond to 6 different Ca channel types. These have been grouped according to biophysical and pharmacological properties into low-voltage-activated (LVA), T-type Ca channels (α1G, α1H, α1I), and high-voltage-activated (HVA) L-type (α1C, α1D, α1F, α1S), N-type (α1B), and P/Q-type (α1A) Ca channels. R-type Ca channels (probably α1E) show properties of LVA and HVA channels (Davia 1999).

Functionally unique Ca channels allow for temporal and spatial control of intracellular calcium ([Ca2+]i) and support regulation of cellular activity. T-type Ca channels have more negative activation ranges and inactivate more rapidly than other Ca channels. When the range of membrane potentials for activation and inactivation overlap, these channels can undergo rapid cycling between open, inactivated, and closed states, giving rise to continuous calcium influx in a range of negative membrane potentials where HVA channels are not normally activated. The membrane depolarizing influence of T-type Ca channel activation can become regenerative and produce calcium action potentials and oscillations.

Increases in [Ca2+]i, occurring in part via activation of voltage-dependent T-type Ca channels, are important for the orderly progression of the cell cycle and may contribute to the regulation of cell proliferation and growth (Berridge et al. 1998; Ciapa et al. 1994; Guo et al. 1998). Alterations in the density of T-type Ca channel currents and oscillations in [Ca2+]i have been described in a variety of organisms (Day et al. 1998; Kono et al. 1996; Kuga et al. 1996; Mitani 1985). In the retina, T-type Ca channels have been described in terminally differentiated retinal cell types. The functional activity of T-type Ca channels appears to decrease during development (Brinngam et al. 2000; Rothe et al. 1999), consistent with a role for the T-type Ca channels in embryonic tissue.

In the present work, we demonstrate that undifferentiated...
retinoblastoma cells proliferating in cell suspension express two distinct T-type Ca channel subtypes, α1G and α1H, and we assess the biophysical and pharmacological properties of the resultant transient inward current. Differentiation of retinoblastoma cells results in a decrease in the mRNA levels of α1G and α1H subunits and a reduction of T-type Ca channel current. These results suggest that T-type Ca channels have roles in proliferative retinoblastoma cells but are no longer essential in cells induced to differentiate.

**METHODS**

Y-79 and WERI human retinoblastoma cell lines were obtained from the American Type Culture Collection (Rockville, MD) and were maintained per the distributor’s instructions as previously described (Barnes and Haynes 1992). Briefly, cells were grown in 75-cm\(^2\) culture flasks containing RPMI Medium (GIBCO, Toronto) with 2 mM glutamine, 1.5 g/l NaHCO\(_3\), 4.5 g/l glucose, 10 mM HEPES, 1 mM sodium pyruvate, and 10% fetal bovine serum. Antibiotics were not added to the culture medium. Cultures were grown in a humidified atmosphere of 5% CO\(_2\) at 37°C and were split approximately twice per week when the culture medium became slightly acidic and/or when aggregates of cells were 1–2 mm diam. In experiments where cells were induced to undergo differentiation, approximately 10\(^5\) cells/ml were plated on polylysine (100 μg/ml) glass coverslips and maintained in RPMI with N2 neuronal supplement (Sigma Chemical, St. Louis, MO) for 7–10 days. The compositions of the extracellular and internal solutions used in most electrophysiological measurements were (in mM) 150 NaCl, 5 KCl, 20 BaCl\(_2\), 1 MgCl\(_2\), and 5 HEPES, pH 7.4; and 155 CsCl, 5 HEPES, and 5 EGTA, pH 7.2, respectively. The recordings in Fig. 1 were performed with an external solution containing 2 mM CaCl\(_2\) and no BaCl\(_2\), while the pipette contained 155 KCl instead of CsCl. All solutions were prepared with distilled-deionized water. Chemicals were purchased from Fisher Scientific (Toronto, Ontario), Sigma Chemical Company (Missauga, Ontario) and from BDH (Toronto, Ontario) except in cases noted otherwise. Pipette solutions were filtered through a 0.22-μm membrane. Tetrodotoxin (TTX), NiCl\(_2\), and CdCl\(_2\) were dissolved in water and added to external solutions to achieve the appropriate concentrations. Amiloride (ICN Biochemicals, Cleveland, OH), nifedipine (ICN Biochemicals), α-conotoxin GVIA, α-conotoxin MVIIIC, mibefradil and pimozide were diluted in dimethyl sulfoxide (DMSO) and dispersed in external solutions so that the final ratio of DMSO never exceeded 1:1,000. This concentration of DMSO was tested and found not to influence cell membrane conductances. Mibefradil was provided kindly by Hoffman-La Roche (Basel, Switzerland).

Culture medium (~0.5 ml) containing Y-79 retinoblastoma cells was transferred from a culture dish or flask to a glass-bottomed Lucite recording chamber mounted on the stage of an inverted microscope equipped with Hoffman Modulation optics (Nikon Diaphot). The cell suspension was gently triturated with a Pasteur pipette to disperse cell aggregates into single cells. The cells were then allowed to attach to the glass bottom of the recording chamber before a flow of solution was started. Solutions were superfused by gravity at a rate of 0.5–2 ml/min. Cells were superfused for 10–15 min to remove the culture medium. The whole cell configuration of the patch-clamp technique was used to record membrane potentials and currents. Pipettes were drawn from thin wall borosilicate capillary tubes (ID, 1.1–1.2 mm: wall, 0.2 mm, Micro-Hematocrit, Drummond). Silicone elastomer (Sylgard)—coated pipettes were heat polished and, when filled with internal solution, had resistances ranging from 5 to 15 MΩ. The apparent seal resistances obtained on cells ranged from 500 MΩ to 20 GΩ. The series resistance was typically in the range of 10–20 MΩ after rupturing the membrane patch at the pipette tip. Since the maximum voltage error resulting from the voltage drop across the series resistance is 4 mV for transient inward currents with a magnitude of approximately 200 pA, series resistance compensation was not routinely used. An Axopatch-1D patch-clamp amplifier was used for both current-clamp and voltage-clamp recordings. The cell-membrane capacitance and series resistance were estimated from the transient compensation dial settings on the amplifier after minimizing the capacitative transient in response to a 10-mV voltage step. Current-clamp and voltage-clamp protocol generation, data acquisition, and plotting were controlled by BASIC-Fastlab programs (Indec Systems, Sunnyvale, CA). Analysis in some experiments was done with Sigmaplot (Jandel Scientific, Corte Madera, CA) or Origin (Microcal Software, Northampton, MA). Signals were filtered at 2 kHz and digitized at 1 kHz. Membrane potentials and currents were displayed on an analog oscilloscope (Tektronix 5251, Beaverton, OR) and digitized for storage (Indec Systems, Sunnyvale, CA).

Total cellular RNA was isolated from cultured undifferentiated (UD) or differentiated (D) retinoblastoma cells using Trizol reagent (GIBCO BRL). RNA samples were treated with Dnase (Promega) to remove trace genomic DNA and then converted to single-stranded cDNA as previously described (Denovan-Wright et al. 1999). Single-stranded cDNA from the retinoblastoma samples was used as a template for PCR reactions with primers complementary to bases 1,336 of the human α1G cDNA (Genbank accession number AF129133), bases 5,552–5,968 of human cyclophilin template for PCR reactions with primers complementary to bases 5,573 and 5,987 of the human α1H cDNA (Genbank accession number AF051946), bases 742–761 and 949–968 of human α1I (Genbank accession number AF129133), and bases 46–67 and 395–416 of human cyclophilin (Genbank accession number BC005520). The PCR conditions were as follows: 1) 1 min at 94°C, 2) 30 s at 94°C, 3) 30 s at 63°C, 4) 1 min at 72°C, and 5) 10 min at 72°C; repeating step 2 to step 4 35 times. For α1I, step 3 was 30 s at 55°C (35 repetitions) and for cyclophilin, step 3 was 30 s at 50°C (28 repetitions). PCR products of 395, 436, and 227 bp were obtained in the reactions using α1G, α1H, and α1I Ca channel primers, respectively, and a 371-bp product was obtained using cyclophilin-specific primers. The 395- and 436-bp PCR product was designated to represent the α1I cDNA.
for α1G and α1H obtained using cDNA from UD retinoblastoma cells and a 227-bp product obtained using cDNA from D retinoblastoma cells were cloned into pGEM-T vector. Plasmid DNA was isolated from selected transformants using spin columns (Qia-gen), and the sequence of the cloned inserts was determined using M13 universal forward and reverse primers and the T7 sequencing kit (Pharmacia). Sequence identity was confirmed using the National Institutes of Health Blast program (Altschul et al. 1997). The ethidium bromide–stained PCR products were fractionated by agarose gel electrophoresis and visualized using a Gel doc (BioRad) apparatus. The optical density of the α1G-, α1H-, α1L-, and cyclophilin-specific products was determined using Scion Image (Scion Corporation).

Total cellular RNA was isolated from both UD and D retinoblastoma cells using Trizol reagent (GIBCO BRL) and the manufacturer’s protocol. A northern blot was then prepared by fractionating 10 μg of total RNA. The cloned α1G and cyclophilin cDNA inserts were isolated following digest and gel electrophoresis. Twenty-five nanograms of insert was radio-labeled using α-32PdATP (3000C/mm mol; Amersham) and used in northern hybridization analysis as previously described (Denovan-Wright et al. 1999). To demonstrate that equivalent amounts of RNA were loaded, blots were stripped and reprobed following hybridization of α1G and rehybridized with a cyclophilin-specific probe.

RESULTS

Active membrane properties in human retinoblastoma cells

Retinoblastoma cells are electrically excitable and can produce membrane potential oscillations and regenerative activity. Figure 1A shows a cell recorded under current clamp in which a 50-pA injection of depolarizing current exceeded threshold to initiate a broad action potential of about 20 ms duration at half-width. Figure 1B shows the voltage-clamped current-voltage (I-V) relation for the cell, and the inward current presumably responsible for the spike can be seen. Positive to −40 mV, a transient inward current could be detected near the onset of the depolarizing voltage steps. This inward current is plotted against command potential in solid circles. Positive to −20 mV, the same depolarizing voltage steps elicited more slowly developing, sustained outward currents. These are plotted in the I-V relation with solid diamonds. In the experiments to follow, we blocked the outward currents with internal Cs+ and enhanced the transient inward current with 20 mM Ba2+. We show that the transient inward current is carried in T-type Ca channels in undifferentiated retinoblastoma cells.

Activation and inactivation of transient inward current in retinoblastoma cells

T-type Ca channel activity in a human Y-79 retinoblastoma cell is shown in Fig. 2. The cell was voltage clamped in 20 mM Ba2+-containing bath solution with microelectrodes containing the CsCl intracellular solution to minimize outward currents. When the cell was depolarized positive to −40 mV from a holding potential of −80 mV, a transient inward current appeared (Fig. 2A). Currents decayed during voltage steps with time constants close to 25 ms. The I-V relation made from the peak inward currents during each step from the experiment is shown in Fig. 2B. Current begins to activate at −40 mV and reaches a peak at −10 mV. Figure 2C shows currents recorded in response to a voltage protocol designed to investigate inactivation properties of the transient current. Conditioning steps to voltages of −70 up to −20 mV were applied for 200 ms, and then the cell was stepped to a voltage of −20 mV and the current recorded. Current was largest following the step to −70 mV and completely inactivated following the step to −20 mV. D: activation curve derived from data in A and B and inactivation curve derived from data in C. Boltzmann functions gave an activation midpoint of −24.2 mV (with slope factor of 6.1 mV), and an inactivation midpoint of −38.1 mV (with slope factor −4.7 mV) for this cell.

\[ I = \frac{g_{Na} E_{Na}}{1 + \exp\left(\frac{V - V_0}{k}\right)} \]
Divalent cation permeation of the T-type Ca channel in retinoblastoma cells

T-type Ca channels have been characterized by the properties of their permeation and block by divalent cations. We first compared the permeation of Ca\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\) in Y-79 retinoblastoma T-type Ca channels. In most neurons, Ca\(^{2+}\) and Sr\(^{2+}\) carry current better than Ba\(^{2+}\) in T-type Ca channels, and this is the case for expressed α1G and α1H subtypes, although it has been reported that the α1H subtype does not exhibit preference for Ca\(^{2+}\) over Ba\(^{2+}\) (McRory et al. 2001). The records in Fig. 3A show peak whole cell currents obtained in experiments where equimolar replacement Ba\(^{2+}\) was made with Ca\(^{2+}\) (left panel) or Sr\(^{2+}\) (right panel). The I-V relations for current recorded in 20 mM Ba\(^{2+}\), 20 mM Ca\(^{2+}\), and 20 mM Sr\(^{2+}\) are shown in Fig. 3B and indicate that the peak current was greater in Ca\(^{2+}\) than in Ba\(^{2+}\) (39.4 ± 7.1%, n = 4) and when Sr\(^{2+}\) replaced Ba\(^{2+}\) (44.4 ± 1.7%, n = 4).

We confirmed that removal of Ca\(^{2+}\) from the external Ringer abolished the T-type Ca channel current in retinoblastoma cells (Gomez et al. 1993b). Figure 4A shows whole cell currents activated by stepping the membrane potential from −60 to −10 mV in the presence of 2 mM Ca\(^{2+}\) and in the absence of external Ca\(^{2+}\). The I-V relations derived under these conditions are shown in Fig. 4B. Inward current was completely eliminated in the Ca\(^{2+}\)-free solution but was restored when Ca was included in the external perfusate. These results demonstrate that in the absence of Ca\(^{2+}\) no current flows in the T-type Ca channels. A report describing sodium permeation in T-type Ca channels in divalent-free conditions (Bringmann et al. 2000) does not contradict our result, since in our test 1 mM Mg\(^{2+}\) was present that, in the absence of Ca\(^{2+}\), blocks the channels.

In some types of Ca channels, permeant divalent cations compete for binding sites within the channel and anomalous mole-fraction behavior results (e.g., current is larger in pure divalent baths and is reduced in mixtures) (Hess and Tsien 1984). Using Ba\(^{2+}\) as charge carrier, we investigated the effects of altering the external Ca\(^{2+}\) concentration on T-type Ca channel current magnitude and gating. Figure 4C shows the results of changing Ca\(^{2+}\) concentration between 0, 0.1, 1, and 5 mM when T-type Ca channel current is supported with 20 mM Ba\(^{2+}\) externally. Anomalous mole fraction behavior resulted as Ca\(^{2+}\) replaced the 20 mM Ba\(^{2+}\) bathing solution (n = 4). At low concentrations of Ca\(^{2+}\), maximum peak current was reduced while for complete replacement of Ba\(^{2+}\) by Ca\(^{2+}\), current increased (see also Fig. 3A). As the Ca\(^{2+}\) was increased, current magnitudes were reduced mildly. This result is consistent with a competition between Ca\(^{2+}\) and Ba\(^{2+}\) as charge carrier of T-type Ca channel current.

Inorganic cations such as Ni\(^{2+}\) and Cd\(^{2+}\) have been widely reported to block T-type Ca channels. IC\(_{50}\)'s for these cations vary considerably for channels in native tissues and for recombinant channels in various mammalian expression systems, partly as a function of the permeating divalent present (reviewed in Hofmann et al. 1999; Lacinová et al. 2000a). Ni\(^{2+}\) and Cd\(^{2+}\) each blocked the T-type Ca channel current in Y-79 retinoblastoma cells in a dose-dependent manner, typical of the actions of these divalent currents carried in T-type Ca channels (Kostyuk 1999; Lacinová et al. 2000b; Lee et al. 1999b; Williams et al. 1999). Figure 5A shows representative current traces recorded in a Y-79 cell at −10 mV from a holding potential of −60 mV with 20 mM Ba\(^{2+}\) as the charge carrier and in the presence of increasing concentrations of Ni\(^{2+}\). The dose-response curves for Ni\(^{2+}\) and Cd\(^{2+}\) shown in Fig. 5B revealed that in the cells tested, the half-maximal block for Ni\(^{2+}\) occurred at 160 μM (n = 6), while this value for Cd\(^{2+}\) was 167 μM (n = 6). The dose-response relationship for Ni\(^{2+}\) was broader than that for Cd\(^{2+}\), particularly in the lower concentration range, and a multiple partial F-test showed that a weighted, biphasic dose-response relation having IC\(_{50}\)'s of 22 μM (30%) and 352 μM (70%) was a significant improvement.
over the fit obtained using a single IC$_{50}$ of 160 μM (0.001 < P < 0.005).

On the basis of their observation that replacement of Na$^+$ reduced the transient current, Gomez et al. (1993a) concluded that a unique class of channel, permeable to both Na$^+$ and Ca$^{2+}$ and capable of being gated only if Ca$^{2+}$, or some other divalents, were present in Y-79 retinoblastoma cells. We further tested whether extracellular Na$^+$ and voltage-dependent Na channels make a contribution to the transient inward current. The currents shown in Fig. 6A were generated in response to a voltage step to −10 mV and reveal an identical transient inward current whether Na$^+$ was present or whether it was replaced with equimolar N-methyl-$\delta$-glucamine (NMDG). The I-V relation shown in Fig. 6B showed that the T-type Ca channel current recorded in the presence of 20 mM Ba$^{2+}$ was nearly identical when 155 mM NMDG or 155 mM Na$^+$ was in the bathing solution. In a total of four cells tested in this manner, the peak current in NMDG was larger by 0.2 ± 1.8%, an insignificant effect. Figure 6C shows that when 150 mM NaCl was replaced with 150 mM TMACl, peak currents recorded at −10 mV were also unaffected (0.9 ± 0.5%, n = 4). To rule out the presence of voltage-gated Na channels in this experimental paradigm, Fig. 6D shows that application of 100 mM TTX had no effect on peak current amplitude with the average change in current being −1.3 ± 0.8% (n = 4). Taken together these results rule out Na$^+$ as a charge carrier in the transient inward current in retinoblastoma cells.

Pharmacological properties of T-type Ca channels in retinoblastoma cells

T-type Ca channel currents in native tissues and expressed α1G, α1H, and α1I channels are sensitive to inhibition by micromolar and nanomolar concentrations, respectively, of the organic blocker mibebradil (Clozel et al. 1997; Lacinová et al. 2000b). We examined the effect of mibebradil as well as the neuroleptic drug, pimozide, which has also been shown to block T-type Ca channels (Arnoult et al. 1998), on the T-type Ca channel current in retinoblastoma cells. Figure 7A shows currents recorded during voltage-clamp steps to −10 mV from a holding potential of −60 mV before and during application of 1 μM mibebradil. At this potential, mibebradil blocked about two-thirds of the T-type Ca channel current. In Fig. 7B, the I-V relation shows that mibebradil reduced the T-type Ca channel current at all potentials, with some reduction in block at more depolarized potentials. Figure 7C summarizes the block of T-type Ca channel currents by mibebradil (1 μM) and pimozide (1 μM). Overall, mibebradil blocked 53.0 ± 6.7% (n = 5) of the T-type Ca channel current at −10 mV, whereas pimozide blocked 43.2 ± 8.9% (n = 4) of the current at this potential.

Blockers of L-, N-, and P/Q-type Ca channels had no effect on the peak T-type Ca channel currents in retinoblastoma cells. Figure 8 shows T-type Ca channel currents elicited at −10 mV in the absence and presence of various organic Ca channel blockers. Neither nifedipine (L-type channel blocker: 10 μM; −0.3 ± 1.8%, n = 4), ω-conotoxin GVIA (N-type channel blocker: 0.3 μM; 0.4 ± 1.8%, n = 4), ω-agatoxin IVA (P-type channel blocker: 200 nM; −1.7 ± 2.7%, n = 4), or ω-conotoxin MVIIC (P-type channel blocker: 250 nM; −1.3 ± 4.6%, n = 3) reduced current carried by 20 mM Ba$^{2+}$ in the channels.
Furthermore, ethosuximide (5 mM; 0.7 ± 1.1%, n = 4) and amiloride (100 μM; −5.8 ± 1.4%, n = 4), agents reported to reduce some neuronal T-type Ca channel currents (Williams et al. 1999), were mostly ineffective on the retinoblastoma T-type Ca channel current.

**Differentiation of retinoblastoma cells reduces T-type Ca channel currents**

Transient Ca channel current disappeared following differentiation of retinoblastoma cells. Figure 9A shows phase-contrast photomicrographs of undifferentiated Y-79 retinoblastoma cells. Undifferentiated Y-79 cells proliferate as a suspension culture, usually in clusters. Figure 9B shows cells after 8 days of growth on a poly-δ-lysine/laminin–coated substrate in defined medium. Once attached and growing on the poly-δ-lysine substrate, these differentiated cells either form glia or express neuronal phenotypes and extend short neuritic processes. Similar morphology for differentiated retinoblastoma cells has been previously described (Gomez et al. 1993b). Figure 9C shows whole cell current traces recorded from a representative cell shown 11 days after differentiation was induced. Using 20 mM Ba^2+ as the charge carrier with CsCl in the pipette, voltage-clamp steps from a prepulse potential of −80 mV to potentials of −40 through 0 mV elicited only small inward currents in differentiated Y-79 cells. Similarly, in cells tested at 5–11 days cultured under differentiating conditions, only a small amount of a transient inward current could be detected. Inward current measured at −10 mV was 0.56 ± 0.15 pA/pF in differentiated cells (n = 6), which had on average the same capacitance as undifferentiated cells. In contrast, at the same potential, the inward current was 5.9 ± 1.1 pA/pF in 14 undifferentiated cells (P = 0.0031).

**FIG. 7.** Mibefradil and pimozide reduce transient current. A: currents recorded during voltage-clamp steps to −10 mV from a prepulse potential of −80 mV before and during application of 1 μM mibefradil in 20 mM Ba^2+ solution. The drug blocked about 63% of the inward current at this potential. B: current-voltage relation for the cell shown in A. C: mibefradil (1 μM) blocked 53.0 ± 6.7% (n = 5) of the transient inward current, while pimozide (1 μM) blocked 43.2 ± 8.9% (n = 4).

**FIG. 8.** Blockers of L-, N-, and P/Q-type Ca channels have no effect on the peak transient Ca channel currents recorded in 20 mM Ba^2+ solution. Neither nifedipine (L-type channel blocker: 10 μM; −0.3 ± 1.8%, n = 4), ω-conotoxin GVIA (N-type channel blocker: 0.3 μM; 0.4 ± 1.8%, n = 4), or ω-agatoxin IVA (P-type channel blocker: 200 nM; −1.7 ± 2.7%, n = 4), or ω-conotoxin MVIIIC (P-type channel blocker: 250 nM; −1.3 ± 4.6%, n = 3) reduced current carried by 20 mM Ba^2+ in the channels. Ethosuximide (5 mM; 0.7 ± 1.1%, n = 4) and amiloride (100 μM; −5.8 ± 1.4%, n = 4), agents reported to reduce some neuronal T-type Ca channel currents, were ineffective.

**FIG. 9.** Photomicrographs of undifferentiated and differentiated human Y-79 retinoblastoma cells. A: undifferentiated cells in suspension culture. B: neuron-like differentiated cells. Scale bar for A and B is 40 μm. C: T-type Ca channel current disappears following differentiation of retinoblastoma cells. The cell shown was recorded in 20 mM Ba^2+ solution, but 11 days after differentiation was induced by growth on a laminin-coated coverslip in serum free medium. In this cell, approximately 20 pA of peak transient current was observed.
Endogenous $\alpha$1G and $\alpha$1H Ca channel subunits decrease following differentiation

Three T-type Ca channel subunits, $\alpha$1G, $\alpha$1H, and $\alpha$1I, have been identified (Klöckner et al. 1999). Of these, the $\alpha$1G and $\alpha$1I subtype appear to be the predominant types distributed in the CNS with lower expression of $\alpha$1G in some peripheral tissues (Perez-Reyes et al. 1998; Talley et al. 1999). We examined the expression of T-type Ca channel subunits in undifferentiated and differentiated Y-79 cells to determine which subunits were present, and whether the differences observed in the T-type Ca channel current were due to alterations in Ca channel subunit expression following differentiation. We used RT-PCR with primers specific for $\alpha$1G, $\alpha$1H, $\alpha$1I Ca channel subunit mRNA to identify T-type Ca channel subtypes in undifferentiated Y-79 cells and cells that had been growing on poly-d-lysine/laminin substrate for 6–12 days in defined medium.

We obtained PCR products for $\alpha$1G and $\alpha$1H T-type Ca channels using cDNA made from the mRNAs of undifferentiated Y-79 cells as the template (Fig. 10A). We amplified very little product for $\alpha$1I (227 bp) from undifferentiated retinoblastoma cells, but the product increased in differentiated Y-79 cells (Fig. 10B). A constitutive housekeeping gene, cyclophilin, was used as an internal control for PCR reactions and produced a 371-bp PCR product (Denovan-Wright et al. 1999). The PCR products obtained from the undifferentiated retinoblastoma cells using $\alpha$1G and $\alpha$1H primers were cloned and sequenced. The cloned insert for the 395-bp product corresponded to the nucleotide sequence for the human $\alpha$1G (Perez-Reyes et al. 1998). The cloned insert for the 436-bp product was identical to the nucleotide sequence for the human $\alpha$1H Ca channel subunit (Cribbs et al. 1998). Thus our molecular findings are consistent with our electrophysiological data and confirm that $\alpha$1G and $\alpha$1H subunits likely underlie T-type Ca channel current in undifferentiated Y-79 cells. The lack of PCR product for $\alpha$1I in undifferentiated Y-79 cells and the slower activation and inactivation kinetics reported for cloned $\alpha$1I Ca channels (Lee et al. 1999a; Monteil et al. 2000b) suggest that this subunit does not contribute to the transient current in undifferentiated cells. The decrease in T-type Ca channel current in differentiated Y-79 cells is also supported by loss of mRNA expression for $\alpha$1G and/or $\alpha$1H Ca channel subunit.

We used northern blot analysis to demonstrate that the differences in the relative amounts of Ca channel subunit mRNA determined by nonquantitative RT-PCR reflected actual quantitative changes in $\alpha$1G mRNA levels. For this analysis, the 395-bp $\alpha$1G cloned cDNA insert was radio-labeled and used as a hybridization probe in northern blot analysis of total RNA isolated from undifferentiated and differentiated Y-79 retinoblastoma cells. Figure 11A demonstrates that the probe annealed with an mRNA of approximately 9.5 kb in the undifferentiated cells (lane U). In the differentiated cells, we did not observe any hybridization with the $\alpha$1G-subunit specific Ca channel probe (Fig. 11A, lane D). The northern blot was stripped and rehybridized with a 371-bp probe for human
cyclophillin, demonstrating that equivalent amounts of RNA were loaded for both samples (Fig. 11B). This result demonstrated that the levels of α1G Ca channel subunit mRNA were decreased in differentiated versus undifferentiated retinoblastoma cells and confirmed that the RT-PCR analysis of α1G, and by extrapolation α1H, reflect a measurable decrease in these Ca channel mRNA subunits. These findings are consistent with the functional electrophysiological studies showing a loss of the T-type Ca channel current in differentiated retinoblastoma cells.

**DISCUSSION**

This report examines biophysical, pharmacological, and molecular properties of the ion channels responsible for the transient inward calcium current in human retinoblastoma cells and begins to probe the regulation of the T-type Ca channels responsible for this current. We showed that the current has properties common to other T-type Ca channel currents and that these channels are predominant in undifferentiated, mitogenic cells and disappear in cells chemically induced to exit the cell cycle and differentiate. Our molecular studies identified the T-type Ca channels in retinoblastoma cells to be the α1G and α1H subtype, and we found that, consistent with reduction of channel current, levels of mRNA for these subunits decrease in differentiated, noncycling retinoblastoma cells.

**Properties of T-type Ca channels in retinoblastoma cells are similar to those of expressed T-type Ca channels**

The transient current in retinoblastoma cells resembles T-type Ca channel currents in other cells in terms of channel gating, permeation, and pharmacology (Kostyuk 1999). The most striking difference is that the current we recorded in retinoblastoma cells gates at relatively positive potentials compared with endogenous and expressed T-type Ca channels. In retinoblastoma cells recorded in 20 mM Ba\(^{2+}\), activation was half complete at −25 mV, and inactivation was half complete at −40 mV. Native T-type Ca channel currents have been reported to activate with midpoints near −60 mV, while inactivation midpoints span the range from −92 to −83 mV (Kostyuk 1999). The activation midpoints of expressed α1G and α1H lie in the range of −51 to −28 mV, while inactivation is half complete in the range of −75 to −68 mV. Consideration must of course be made for divalent concentration. Our activation and inactivation curves were derived in 20 mM Ba\(^{2+}\), and surface charge effects could be expected to shift the curves up to 15–20 mV in the positive direction (Hille 2001).

Inactivation of T-type Ca channel current in retinoblastoma cells occurs with a time constant of about 24 ms at −20 mV, similar to reports for α1G and α1H channels. This time constant is much faster than time constants reported for expressed α1I T-type Ca channels (Lee et al. 1999a).

T-type Ca channel current in retinoblastoma cells is carried by Ca\(^{2+}\) and Sr\(^{2+}\) preferably over Ba\(^{2+}\), it is not carried by Na\(^{+}\), and there is no current in the absence of Ca\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\). We showed that, as in other reports on Ca channels, current is carried well in the presence of Ba\(^{2+}\) or Ca\(^{2+}\) alone, but that low concentrations of Ca\(^{2+}\) block the conduction of Ba\(^{2+}\), consistent with an anomalous mole fraction effect. Ni\(^{2+}\) and Cd\(^{2+}\) both blocked the channels with an IC\(_{50}\) of about 160 μM, close to the value reported for other T-type Ca channels (Lacinová et al. 2000b; Williams et al. 1999). The dose-response relation for Ni\(^{2+}\) was broader than that of Cd\(^{2+}\) and was fit best with the sum of two Michaelis-Menton relations, one having an IC\(_{50}\) at 22 μM and another at 352 μM. These values are particularly interesting considering reports that Ni\(^{2+}\) blocks Ba\(^{2+}\) current flow in α1G and α1H channels with IC\(_{50}\) of 470 μM (Lacinová et al. 2000b) and 6.6 μM (Williams et al. 1999), respectively. Although our data may not offer adequate resolution to resolve the issue, the double fit could be indicative of two populations of channels being blocked at different concentrations of Ni\(^{2+}\). Were they to be interpreted in this manner, our results might suggest that about 30% of the channels could be α1H while the remaining 70% could be α1G.

The similarities between the T-type Ca channels in retinoblastoma and other cell types are strong pharmacologically. Mibefradil and pimozide blocked the transient current with IC\(_{50}\) in the range of 1 μM each, values in general agreement with other reports investigating expressed α1G and α1H channels (Martin et al. 2000; Monteil et al. 2000a), although it must be noted that these agents are not particularly selective for T-type Ca channel currents (Bezprozvanny and Tsien 1995; Enyeart et al. 1990).

**Comparison with previous assessments of transient current in retinoblastoma cells**

Previous work by Gomez et al. (1993a) concluded that a single and unique type of sodium and calcium conducting channel exists in undifferentiated retinoblastoma cells. While their work showed that the expression of channels carrying transient inward current changes dramatically with differentiation, a different conclusion was reached with regard to the nature of the transient inward current in the undifferentiated cells. Gomez et al. found that Na\(^{+}\) replacement reduced transient current magnitude. On this basis, these authors concluded that retinoblastoma cells express a unique channel that was permeable to both Na\(^{+}\) and Ca\(^{2+}\), but that its gating was permitted only if Ca\(^{2+}\), or some other divalent, were present. Since we invariably obtained contrasting results in an uncomplicated manipulation of the ionic conditions (e.g., Na\(^{+}\) replacement had no effect on current magnitude), we conclude nothing more extravagant than that the transient inward current is carried in Ca channels.

A second current component frequently became evident at +10 mV following extended periods of dialysis with intracellular solution. This component, which could only be detected when Ba\(^{2+}\) was used in the bath, was sustained, and since its activation range began at least 40 mV more positive than that of the T-type Ca channel current, it was considered likely to arise from the activity of HVA Ca channels. Owing to the positive activation range of this current component, our recordings were never contaminated with sustained Ca channel activity.

**Undifferentiated retinoblastoma cells express α1G and α1H T-type Ca channels**

Here we show that the predominant T-type Ca channel subunit present in undifferentiated retinoblastoma cells are α1G and α1H, and that α1I has very little presence, consistent
with the biophysical characterizations. Our data suggest that transient inward current flows in a combination of \( \alpha \text{G} \) and \( \alpha \text{H} \) channels, which, when expressed in HEK-293 cells, appear to have much the same biophysical phenotype (Criibs et al. 1998; Lee et al. 1999a). As discussed in the section above, a distinguishing feature is that, under conditions where \( \text{Ba}^{2+} \) is the charge carrier, \( \text{Ni}^{2+} \) blocks \( \alpha \text{G} \) and \( \alpha \text{H} \) channels with broadly different IC\(_{50}\). Ca channels in retinoblastoma cells follow this scheme, since \( \text{Ni}^{2+} \) exhibited a biphasic inhibition curve. We did not find one population of cells with the IC\(_{50}\) for \( \text{Ni}^{2+} \) block in a low range and a second population of cells with an IC\(_{50}\) in a much higher range, suggesting that all cells in our culture expressed both \( \alpha \text{G} \) and \( \alpha \text{H} \) channels.

**Loss of T-type Ca channels with differentiation**

Following differentiation of Y-79 retinoblastoma cells, T-type Ca channel activity was reduced to very low levels. In agreement with this functional loss, there was a marked decrease of both \( \alpha \text{G} \) and \( \alpha \text{H} \) mRNA levels. Although T-type Ca channel current was reduced in some cases to undetectable levels, products corresponding to \( \alpha \text{G} \) and \( \alpha \text{H} \) were detected, but were much less abundant, in the cDNA samples prepared from differentiated versus undifferentiated cell cultures. Based on the molecular analysis of Ca channel mRNA and the biophysical analysis of the current, it does not appear that \( \alpha \text{H} \) subunits contribute to the transient current in differentiated or undifferentiated cells, although the small increase of \( \alpha \text{H} \) mRNA following differentiation may suggest that a different mechanism regulates expression of these subunits. It is important to note that the cells in which T-type Ca channels were recorded with patch-clamp electrodes were selected for their signs of differentiation and may have represented a pure population of differentiated cells, whereas the RT-PCR reactions used cDNA derived from total RNA of a large number of cells cultured under the same differentiating conditions. It is possible that not all of the cells in the culture had undergone full differentiation, and therefore mRNA from undifferentiated cells may have contaminated our result.

**T-type Ca channels in the cell cycle**

Transient increases in \([\text{Ca}]_i\) have been reported to be essential for progress through specific stages of the cell cycle in different mammalian cells (Ciapa et al. 1994), and inhibition of calcium influx using mibefradil has been shown to prevent normal cell cycle progression in endothelial cells (Nilius et al. 1997). However, a recent study found that overexpression of human \( \alpha \text{G} \) and \( \alpha \text{H} \) subunits in HEK-293 cells did not affect DNA synthesis during the cell cycle (Chemin et al. 2000). Differences in signaling pathways or calcium compartmentalization in native cells, rather than frank expression, could limit comparison of native cell physiology with expression systems such as HEK cells.

Ca channels have also been implicated in cancer development, where alterations of calcium signaling potentially affect cell proliferation and apoptosis (Yao and Kwan 1999). Pimozide, a neuroleptic drug that binds sigma receptor sites in the nervous system and also inhibits T channels, has been demonstrated to be effective in decreasing the growth of breast cancer cells (Strobl et al. 1998). With the decrease in \( \alpha \text{G} \) and \( \alpha \text{H} \) T-type Ca channel expression and T-type Ca channel current now documented in differentiated cells, further studies with the retinoblastoma cell line may help define the role of T channels in the cell cycle and in cell proliferation and may aid in the identification of mechanisms by which T-type Ca channels are regulated during development.

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T-TYPE Ca CHANNELS IN RETINOBLASTOMA CELLS


