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Inhibition of T-type calcium channels disrupts Akt signaling and promotes apoptosis in glioblastoma cells

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ABSTRACT

Glioblastoma multiforme (GBM) are brain tumors that are exceptionally resistant to both radio- and chemotherapy regimens and novel approaches to treatment are needed. T-type calcium channels are one type of low voltage-gated channel (LVCC) involved in embryonic cell proliferation and differentiation; however they are often over-expressed in tumors, including GBM. In this study, we found that inhibition of T-type Ca^{2+} channels in GBM cells significantly reduced their survival and resistance to therapy. Moreover, either T-type selective antagonists, such as mibefradil, or siRNA-mediated knockdown of the T-type channel alpha subunits not only reduced cell viability and clonogenic potential, but also induced apoptosis. In response to channel blockade or ablation, we observed reduced phosphorylation of Akt and Rictor, suggesting inhibition of the mTORC2/Akt pathway. This was followed by reduction in phosphorylation of anti-apoptotic Bad and caspases activation. The apoptotic response was specific for T-type Ca^{2+} channels, as inhibition of L-type Ca^{2+} channels did not induce similar effects. Our results implicate T-type Ca^{2+} channels as distinct entities for survival signaling in GBM cells and suggest that they are a novel molecular target for tumor therapy.

1. Introduction

Glioblastoma multiforme (GBM) are malignant tumors of the brain with an exceedingly poor prognosis (median of 10–12 months with radiation and chemotherapy, 14.6 months overall [1]). Current treatment consists of surgical excision followed by chemo- and radiotherapy. However, a majority of GBM are phosphatase and tensin homolog (PTEN)-deficient, many harbor mutations in p53, epidermal growth factor receptor (EGFR) and other genetic alterations, which confer resistance to apoptosis and therapy [2]. Consequently, there is a need for new approaches to therapy.

Calcium (Ca^{2+}) is a crucial secondary messenger that regulates many cellular processes, among which are cell proliferation and survival [3,4]. Voltage-dependent Ca^{2+} channels provide one of the pathways for influx of calcium into cells, and channel activation leads to transient elevation in the concentration of Ca^{2+} in the cytosol. Among these channels, the low voltage-gated Ca^{2+} channel family (LVCC or Cav3, commonly called T-type Ca^{2+} channels) are functionally linked to many physiological processes [5]. For example, T-type channels are normally expressed in the brain and regulate neuronal excitablility and sensory transmission [6]. On the other hand, T-type Ca^{2+} channels are often over-expressed in GBM and other cancer cells [7,8], and are thought to support tumor proliferation and progression [9,10]. According to Human Protein Atlas (http://www.proteinatlas.org), majority of GBM tumor samples obtained from patients expressed LVCC Cav3.1 (IHC staining: strong 9%, moderate 18%, weak 55%, negative 18%), while 27% expressed Cav3.2 (IHC staining: weak 27%, negative 73%); Cav3.3 expression was not determined. Therefore, LVCC pose an attractive potential target for GBM tumor therapy. Therefore, T-type channels pose an attractive potential target for cancer therapy, either using specific antagonists as a single agent, or in combination with standard chemo- or radiotherapy [8,11].

Among the several known calcium channel antagonists available, the majority are either those with broad specificity or

Abbreviations: CACNA1C, L-type channel subunit Cav1.2; CACNA1G, T-type channel subunit Cav3.1; CACNA1H, T-type channel subunit Cav3.2; GBM, glioblastoma multiforme; LVCC, low voltage gated calcium channel.

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with specificity for L-type channels [12]. Only a few specific T-type channel antagonists exist. Mibefradil (Posicor/Ro 40-5967, Hoffmann-La Roche) was originally developed for treating hypertension and chronic angina pectoris [13,14] and is a potent inhibitor of T-type channel currents with 10 to 20 times higher selectivity for T-type over L-type Ca^{2+} channels [15]. Mibefradil was FDA-approved and extensively used but later recalled from the market because of concerns over potential toxicity due to drug-drug interactions [16]. Nonetheless, it is well-tolerated and has high efficacy as a T-type channel antagonist. Thus, there has been recent interest in re-purposing mibefradil as an anti-cancer compound [11].

Here we examine the function of T-type Ca^{2+} channels in glioblastoma cells. We show that inhibition of T-type channels with mibefradil, or down-regulation of T-type channel subunits expression with siRNA, leads to an increase in apoptosis and sensitizes glioblastoma cells to ionizing radiation *in vitro*. We also demonstrate reduced mTORC2/Akt signaling in the cells following inhibition of T-type Ca^{2+} channels. Our results raise the possibility that T-type channel inhibitors may be useful as novel and effective therapeutics for glioblastomas.

2. Materials and methods

2.1. Cell lines and reagents

Human GBM cell lines, U251, U87 and T98G, were purchased from the American Type Culture Collection (ATCC; Rockville, MD) and maintained in a 37 °C/5% CO_2 humidified chamber in RPMI-1640 medium supplemented with 5% FBS (U251), or in MEM medium with 10% FBS (U87 and T98G). All cell culture materials and supplies were purchased from Life Technologies GIBCO (Grand Island, NY). No authentication of cell lines was done by the authors. Mibefradil and TTL1177 (formerly TH1177, [17]) were generous gifts from TAU Therapeutics LLC, (Charlottesville, VA); verapamil, nifedipine and z-VAD-FMK (z-VAD) were purchased from Sigma (Sigma–Aldrich, St. Louis, MO). The following antibodies were used: Akt, phospho-Akt (Ser473, Thr308, Thr450), Bad, phospho-Bad (Ser136), caspase-3, caspase-8, caspase-9, PARP, cleaved PARP, survivin, Bcl-2, Mcl-1, mTOR, phospho-mTOR (Ser2481), phospho-PDK1 (Ser241), Rictor, phospho-Rictor (Thr1135) from Cell Signaling Technology (Beverly, MA); Cav3.1, phospho-DNA-PK (Ser2056) from Abcam (Cambridge, MA); Cav 3.1, Cav3.2 and Cav1.2 were from UC Davis/NIH NeuroMab Facility (Davis, CA); PDK1 from Thermo Scientific (Waltham, MA); and actin from Sigma (St. Louis, MO). Secondary antibodies conjugated to IR dyes were purchased from Li-COR (Lincoln, NE). Electrophoresis supplies and PCR reagents were obtained from Bio-Rad (Hercules, CA).

2.2. Drug treatment and irradiation

Incubations with antagonists and other agents were conducted at 37 °C for times indicated in Section 3 (usually 0–24 h). An equivalent volume of drug vehicle (DMSO or PBS) was added to the control dishes (final concentration $\leq$ 0.01%). Standard settings consisted of 1×10^6 cells in 100 mm dish/10 mL of medium, treated with 1–10 μmol/L drug, and were appropriately re-scaled to other culture dishes. In the irradiation experiments, cells were irradiated using a 220 keV X-ray irradiator (3.3 Gy/min) at room temperature.

2.3. Cell viability and clonogenic survival assays

To assess proliferation and metabolic viability, cells were plated in a 96-well plate, allowed to attach, treated with drugs as described in the Results section and proliferation/viability was determined by AlamarBlue (Life Technologies, Grand Island, NY). To assess the fraction of live cells with intact membranes the cells were grown in 6-well plates, treated as described, and harvested by trypsinization. Following a 5 min incubation with 0.4% trypan blue, the total cell number and the fraction of trypan blue-excluding cells were assessed simultaneously using a TC10 cell counter (Bio-Rad). Clonogenic survival assays were conducted as described previously [18]. In brief, exponentially growing cells were treated with drugs, or sham-treated with vehicle, for 1 to 24 h. Cells were trypsinized, counted, and appropriate numbers were plated in fresh, drug-free medium. Colonies consisting of >50 cells were counted after 10–14 days. All data points were determined in triplicate and experiments were conducted at least three times.

2.4. RNA interference (RNAi)

SMARTpool siRNA targeting CACNA1G, CACNA1H, and CACNA1C, as well as control, non-targeting siRNA (SMARTpool #2) were purchased from Dharmacon (Lafayette, CO). Additionally, two individual siRNAs targeting CACNA1C and CACNA1G were designed and obtained from Invitrogen (Carlsbad, CA) (CACNA1C, accession no. NM_000719.2, 5′-GACAGAAAUUUAAGGGAAA-3′ and CACNA1G, accession no. NM_018896, 5′-GGAACAAAGUCCUCUACAA-3′). Transfections were performed with RNAiMAX (Invitrogen) (Carlsbad, CA) according to the manufacturer's instructions, and the cells were assayed for mRNA and protein expression after 72–96 h.

2.5. RNA isolation and reverse transcription PCR (RT-PCR)

Total cellular RNA was isolated from exponentially growing cells using a RNAeasy kit (Qiagen, Valencia, CA). Reverse transcription was performed with 2 μg of total RNA using iScript reagent (Bio-Rad). The primers were based on previously published work [19] (CACNA1G-F, 5′-AAAGAGGCTGGTGAAGACGA-3′; CACNA1G-R, 5′-TCCTGGTCAACACACTCAGC-3′, CACNA1H-F, 5′-TCGAGGAGGACTTCCACAAG-3′; CACNA1H-R, 5′-TGCATCCAGGAATGGTGAG-3′; CACNA1C-F, 5′-CAAGAGTTGGTGGAGAAGCC-3′; CACNA1C-R, 5′-TGAAGCTCAGAGAGTGGTCG-3′). The PCR amplification was carried out using 25 pmol of specific primers and JumpStart REDTaq polymerase mix (Sigma). The thermal profile consisted of initial denaturation at 95 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 60 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 7 min. PCR products were resolved on a 1% agarose gel, stained with SYBR Safe and quantified with ImageJ. The GAPDH-specific product was used to normalize the signals.

2.6. Caspase-3/7 activation assay

Cells were plated in 96-well plate, allowed to attach, treated with drugs for 0–24 h, or mock-treated with equal volume of DMSO, and in some experiments irradiated (X-rays, 6 Gy). Following 24 h incubation, the activation of cellular caspases-3/7 was measured by the fluorescence-based Apo-ONE Homogeneous Caspase-3/7 Assay (Promega, Madison, WI). Multiple (4–12) wells per treatment condition were used to obtain average fluorescence signals and normalized to sham-treated controls.

2.7. Annexin V/PI staining

The AnnexinV/PI apoptosis staining kit was purchased from Becton-Dickinson (San Jose, CA). Cells (1×10^6/100-mm dish) were treated with agents for 24 h or transfected with siRNA for 72–96 h. All cells, including detached, were collected, washed in PBS, and