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INVITED REVIEW

Low-voltage-activated T-type Ca^{2+} channel inhibitors as new tools in the treatment of glioblastoma: the role of endostatin

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Abstract Ca^{2+} plays a key role in intracellular signaling and controls various cellular processes such as proliferation, differentiation, cell growth, death, and apoptosis. Aberrant changes in intracellular Ca^{2+} levels can promote undesired cell proliferation and migration and are therefore associated with certain tumor types. Many research groups have suggested a potential role for voltage-gated Ca^{2+} channels in the regulation of tumor growth and progression, particularly T-type channels due to their unique biophysical properties. T-type channels are expressed in normal tissues throughout the body and in different types of tumors such as breast carcinoma, retinoblastoma, neuroblastoma, and glioma. It has been demonstrated that increased functional expression of the α1 subunit of T-type channels plays a role in the abnormal proliferation of glioblastoma cells. As such, siRNA-mediated knockdown of the expression of the α1 subunit of T-type channels decreases the proliferation of these cells. Moreover, pharmacological blockade of T-type channels significantly decreases tumor growth. In this review, we focus on the use of T-type channel blockers for the potential treatment of cancers, particularly highly proliferative tumors such as glioblastoma. We conclude that T-type channel blockers such as endostatin can serve as a potential therapeutic tool for tumors whose proliferation depends on increased T-type channel expression.

Keywords T-type Ca^{2+} channels · Endostatin · Glioblastoma · Proliferation

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Introduction

Control of intracellular Ca^{2+} $[Ca^{2+}]_i$ levels is crucial for orderly cell cycle progression, and this control plays a vital role in the regulation of cell proliferation and growth [13, 37]. Voltage-gated Ca^{2+} channels (VGCCs) are found in the plasma membrane of many excitable and non-excitable cells [6]. When VGCCs are open, they permit the influx of Ca^{2+} into the cytoplasm, and these Ca^{2+} ions act as a secondary messenger and initiates diverse physiological cellular processes [68]. Ten unique α1-subunit genes are categorized into three families (Cav1, CaV2, and CaV3), and they encode low-voltage-activated (LVA) T-type and high-voltage-activated L-, N-, P/Q-, and R-type Ca^{2+} channels, with distinct biophysical and pharmacological properties [7, 63]. Among these channels, T-type Ca^{2+} channels (T-type channels) open after small depolarization of the membrane. They are found in neurons where they generate low-threshold Ca^{2+} spikes and influence action potential firing patterns [4] in endocrine cells where they regulate hormone secretion [5], in heart cells where they influence impulse conduction and pacemaking [15, 69], in smooth muscle cells where they regulate proliferation and myogenic tone [14], and in cancerous cells whereas their functional role is under investigation [18].

There is increasing evidence suggesting that T-type channels play a direct role in regulating $[Ca^{2+}]_i$, especially in non-excitable tissues, including some cancerous cells [34, 50].

Thus, far, three different T-type channel subunits have been identified, including CACNA1G, CACNA1H, and CACA N1I, which encode α1G (Cav3.1), α1H (Cav3.2), and α1I (Cav3.3) isoforms [42, 45, 53], respectively. The α1 subunit refers to the channel's primary ion-conducting protein, which consists of four domains (I–IV) each containing six transmembrane helices (S1 through S6). The S4 segment in each domain contains positively charged amino acid residues at every third or fourth position and forms part of the voltage sensor driving the channel to open and close in response to membrane potential changes (Fig. 1a). The four major domains are linked by cytoplasmic regions of different sizes and the N- and C-termini are localized on the cytoplasmic side [52] (Fig. 1b). Although there are other auxiliary channel subunits, the T-type channel α1 subunits can function as a standalone transmembrane protein. Each T-type channel isoform exhibits distinct cellular and subcellular distributions [52]. The Cav3.1 and Cav3.2 currents reflect the prototypical LVA T-type channel currents recorded in native cells, whereas Cav3.3 currents show markedly slower inactivation kinetics [29, 44, 45]. In addition, alternative splicing of Cav3 notably enhances the potential diversity of T-type channel isoforms [47], and there is growing evidence of significant differences in the tissue expression [28] and biophysical properties of the various splice variants [8, 54, 80]. The unique low-voltage-dependent activation/inactivation and slow deactivation of T-type channels indicate that these channels may play a physiological role in regulating $[Ca^{2+}]_i$ at low voltages. At appropriate membrane potentials, T-type channels produce the "window current" that results in a sustained inward Ca^{2+} current through the portion of channels that are not completely inactivated. The window current allows T-type channels to regulate Ca^{2+} homeostasis under resting membrane conditions [53]. This modulation of Ca^{2+} homeostasis allows T-type channels to control cell proliferation and differentiation; the loss of control of the T-type channel may lead to aberrant cell growth and tumor progression [34, 50]. It has been hypothesized that a link exists between abnormal T-type channel expression and cancer progression [34]. For example, silencing of Cav3.1 channels by methylation of CpG islands (GC-rich regions of DNA) was observed in primary tumors [34], indicating that Cav3.1 may be a putative tumor suppressor gene [66]. In addition, Cav3.2 T-type channels may also be involved in cancer growth, as the neuroendocrine differentiation of

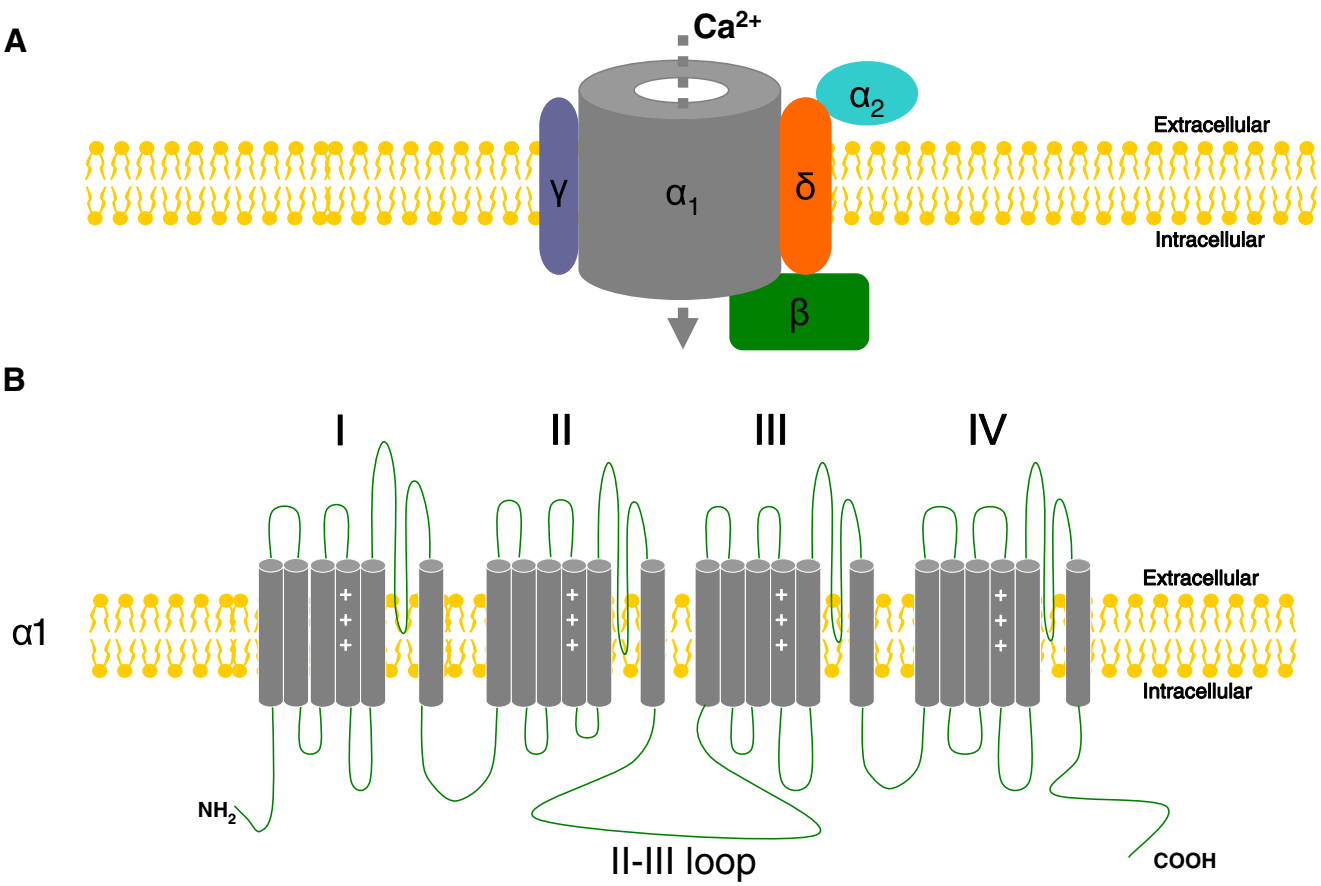

Fig. 1 Structure of the VGCCs. **a** These channels are heteromultimers comprised of a pore forming α1 subunit plus ancillary β and α2-δ subunits, which co-assemble with the α1 subunit to form a functional Ca^{2+} channel protein. **b** Transmembrane topology of the T-type channel α1 subunit

cancer epithelial cancer cells was associated with an increase in their functional expression [38]. Furthermore, the impairment of T-type channels displayed an inhibitory effect on cancer development and progression [34]. Our recent data that were consistent with these results suggested that blockade of T-type channels inhibited U87 cell proliferation and migration, whereas inhibition of L-type channels elicited no such effects. Although recent studies identified T-type channel expression in a number of cancerous cells [18], their functional role is under investigation. Therefore, T-type channel blockers may provide novel strategies for treating certain types of cancer. This review focuses on recent advances in our understating of T-type channel expression and their role in tumors, particularly the blockade of T-type channels by endostatin (ES) for the potential treatment of glioblastoma.

T-type channels are broadly expressed in tumor cells

T-type channels are expressed in normal tissue throughout the body, including the brain, kidneys, heart, and smooth muscle [65]. However, they are also expressed in several different types of tumor including human glioma [28], esophageal tumor [35], breast cancer [65], and ovarian cancer tissues [33]. A recent study revealed the presence of T-type channel mRNA in biopsies of human breast tumors [65]. It has been demonstrated that T-type channel α1 subunit genes, either α_{1H} alone or together with α_{1G} and α_{1I}, are overexpressed in human esophageal tumors compared to adjacent normal tissues which display lower α1 expression [35]. Furthermore, expression of these channels in tumor cell lines has been widely reported in prostate tumors [38], breast tumors [19, 65], fibrosarcomas [22], leukemia cells [66, 77], pheochromocytoma [16], retinoblastomas [2], neuroblastomas [29, 50], and gliomas [28], as summarized in Table 1. For example, human breast adenocarcinoma MDA-231 and MCF-7 cell lines exhibit transient expression of α_{1G} and α_{1H} T-type channel mRNA and T-type channel currents [65]. Human prostate cancer epithelial cells have also been observed to express increased T-type channel currents and α_{1H} mRNA [38]. Although these tumors may express different α1 subunit of T-type channels at different amounts, it has been reported that a tumor-specific splice variant of the α_{1G} isoform is expressed in some gliomas such as the U87 MG and U251 tumor cell lines [28]. Development of antibodies that recognize such tumor-specific α_{1G} variants may offer an antibody-mediated tumor cell-specific killing for the potential treatment [28]. As described in our recent studies, human glioblastoma U87 cells endogenously express all three α1 subunits of Cav3 [79]. However, conflicting data are available regarding the expression of α1 subunits of the T-type channels Cav3.1, $Ca_v3.2$, and $Ca_v3.3$ in U87MG cells. While Lu et al. failed to detect T-type channel mRNA and T-type channel currents by using real-time PCR and electrophysiological methods [36], Panner et al. identified a significant decrease in the expression of the α_{1G} and α_{1H} subunits, which was correlated to dramatically decreased proliferation [49].

Furthermore, changes in expression of T-type channels depend on the status of the tumor. For example, when prostate tumors become aggressive, some epithelial cells differentiate into neuroendocrine cells that express T-type channels [38, 65]. Although these neuroendocrine cells do not proliferate, their LVA T-type channel activity stimulates the release of growth factors that strongly stimulate the aggressive proliferation of neighboring cells [38]. Similarly, in pheochromocytomas, T-type channels mediate the release of growth factors, such as nerve growth factor [50].

The role of T-type channels in tumor cells, particularly in glioma cells

It is well established that the pathology of a tumor mainly consists of proliferation and migration [26, 46]. Proliferation

Table 1 Summary of cancerous cell lines that express T-type channel isoforms

Tumor type	Cell lines	T-type channel	Reference
Glioblastoma	U87; U251	Cav3.1, Cav3.2	[28, 50, 58, 79]
Neuroblastoma	NG 108-15, SK-N-MC, SK-N-SH	Cav3.1	[49]
Retinoblastoma	Y-79, WERI-Rb1	Cav3.1, Cav 3.2, Cav 3.3	[2]
Breast carcinoma	MCF-1, MDA-435 MDA-231; MB-468, BT-20; SKBR-3	Cav3.1, Cav3.2 Cav3.1	[18, 19]
Prostate carcinoma	LNCaP	Cav3.2	[38]
Esophageal carcinoma	KYSE-150, KYSE-180; TE-1,TE-10,TE-12 SKGT4; TE-3, TE-7; KYSE-70	Cav3.2 Cav3.1, Cav3.2	[35]
Pheochromocytoma	PC-12; MPC 9/3L	Cav3.2	[16]
Leukemogenesis	ATL-2; ATL-55T	Cav3.2	[66, 77]
Fibrosarcoma	HT-1080	Cav3.1	[22]

is considered a key factor in tumor development [26]. A number of previous studies suggested a potential role of T-type channels in controlling cell proliferation and migration. T-type channels play a key role in the regulation of $[Ca^{2+}]_i$ during tumor development [18, 26, 50], which is supported by the fact that T-type channel blockers inhibit tumor growth [72]. A role of these channels in promoting cell cycle progression has been previously reported in hepatic, breast, prostate, and brain tumors and leukemia, retinoblastoma, and pheochromocytoma cells [34, 50]. For example, cells in the proliferative phase (G2/M) have been reported to express T-type channels, whereas those in the nonproliferative phases (G0/G1) primarily express L-type channels [56]. In line with this, the upregulation of T-type channels in the proliferative phase has also been identified in a variety of cancer cell lines [75]. The increased T-type channel expression stimulates the proliferation of esophageal cancer cells and the blockade of T-type channels diminishes the cell proliferation [35]. The expression of T-type channels has been genetically manipulated to reveal the contribution of T-type channels for proliferation. Human embryonic kidney cells (HEK293) cells were transfected with the α_{1H} subunit of T-type channels resulting in significantly increased cell proliferation [70]. However, following overexpression of both α_{1G} and α_{1H} subunits in HEK293 cells, Chemin et al. observed increased $[Ca^{2+}]_i$ levels but found no evidence of an increased proliferation rate [9].

In gliomas, Panner and colleagues varied the expression of the α_{1G} and α_{1H} subunits of T-type channels in U87MG cells to determine the cell proliferation [49, 50]. The responses to overexpression were studied in an N1E-115 neuroblastoma cell line with known expression of T-type channels (positive control) and COS-7 primate renal tumor cells, which did not express endogenous T-type channels (negative control) [50]. The overexpression of the α_{1H} subunit significantly increased the proliferation of all three cell lines. Conversely, α_{1G} subunit antisense oligonucleotides significantly inhibited the proliferation of the human glioma cell line U87MG and the mouse neuroblastoma cell line N1E-115 (positive control), but it had no effect on the COS-7 cell line that did not express T-type channels [49, 50]. In our recent study, we demonstrated that siRNA-mediated knockdown of the expression of the $\alpha_{1G/H}$ subunit in U87 cells significantly inhibited cell proliferation, whereas the proliferation of the control siRNA-transfected cells was not significantly different from that of the untransfected cells [79]. Although these studies demonstrate the involvement of T-type channels in cell cycle regulation including proliferation, further studies are necessary to understand the underlying mechanism by which specific T-type channel subtypes regulate the proliferation of tumor cells.

Pharmacological blockade of T-type channels in tumor cells

The aforementioned observations suggest that T-type channels might be a target for the treatment of a number of tumors. Notably, the available agents that can affect T-type channels have varying degrees of specificity. Mibefradil was the first mixed T/L channel blocker to be marketed for its ability to block T-type channel currents. Mibefradil is approximately tenfold more selective for the blockade of T-type channels than the blockade of L-type channels [2]. In nude mice implanted with MCF-7 breast cancer cells, the injection of mibefradil at tumor sites resulted in marked tumor degeneration and necrosis [65]. In addition, mibefradil has been recognized as an effective inhibitor of proliferation in many other different cell types including smooth muscle cells [60], endothelial cells [48], liver cells [74], and NG1-8-15 cells [10]. Submicromolar concentrations of mibefradil suppressed the proliferation of U87MG and N1E-115 cells with a 50 % effective concentration (EC50) of 710 and 410 nM, respectively, and its specific effects on the α_{1G} subunit were clarified using siRNA [49]. Bertolesi et al. reported that the EC50 [2] of mibefradil required to block proliferation was higher than that reported by Panner et al. in retinoblastoma and C6 glioma cell lines. The authors did not observe any significant changes in cell death with mibefradil at the EC50 concentration used above [49]. Although mibefradil has been recognized as a T/L-type channel blocker, it does interfere with additional cellular functions/ion channels, particularly at supramicromolar concentrations (30 μM) [48]. Nilius et al. noted that mibefradil can inhibit Ca2+-activated Cl^- currents, volume-sensitive Cl^- currents, and store-dependent Ca^{2+} currents in endothelial cells [48]. Among other effects, the resultant cell swelling induced by mibefradil was considered a possible mechanism by which proliferation might be decreased in the cells studied. Similar results were obtained by other groups [57, 74]. In addition, mibefradil can also be metabolized by cytochromes P450 3A4 and 2D6, leading to drug-drug interactions [50].

Novel T-type channel blockers based on a 3, 4-dihydroquinazoline backbone have been recently synthesized [31, 51]. These compounds exhibited blocking potencies for transfected human Cav3.1 and Cav3.2 T-type channels in the micromolar range. However, the biological activity of these compounds in intact cells rather than transfected systems is yet to be reported. Using the structure-activity relationship of known blockers, Gray et al. synthesized two inhibitors of Ca^{2+} entry, TH-1177 and TH-1211 [20]. These two compounds with similar potencies and identical stereoselectivity blocked voltage-gated and capacitative Ca^{2+} entry. They had many properties similar to mibefradil such as the ability to decrease the proliferation of breast and prostate cancer cell lines [20]. TH-1177 was observed to extend the lifespan of mice inoculated with human prostate cancer cells. Using a

similar approach of studying the molecular structure of L-type channel blockers, McCalmont et al. tested a new series of agents. They observed a direct correlation between the dose-responses for blocking T-type channels and for blocking proliferation in several different cell lines [40]. In addition, Singh et al. recently reported that SKF96365, originally identified as a blocker of receptor-mediated calcium entry, had notable overlapping physiological and pathophysiological associations between TRPC channels and LVA T-type channels. The authors reported that SKF96365 was actually a potent blocker of LVA T-type channels, which suggests caution in interpreting the results of the use of SKF96365 alone as a diagnostic agent for TRPC activity in native tissues [61]. Other chemicals that can inhibit the T-type channel activity and probably the proliferation include dihydropyridines, benzodiazepines, diphenylbutylpiperidine derivatives, succinimide derivatives, and anesthetics [27], as well as several potential physiological moderators of channel activity that include channel protein phosphorylation by cyclic AMP or G-proteins [78], arachidonic acid derivatives including anandamide [11, 12, 64] and nordihydroguaiaretic acid [12, 71]. Although nordihydroguaiaretic acid inhibits cyclooxygenase, the addition of prostaglandins did not reverse its blocking ability.

The antipsychotic drug pimozide has been reported to inhibit the proliferation of glioma cells [30]. The effects of pimozide have been related to its ability to block T-type channels, which is similar to that of mibefradil. In previous studies, Bertolesli et al. compared the effects of mibefradil and pimozide on retinoblastoma cell lines that express mRNA for the α_{1G} and α_{1H} subunits of T-type channels. They noted a significant increase in cell apoptosis following incubation with pimozide [2]. When retinoblastoma cell lines were treated with α_{1G} subunit antisense, the effects of mibefradil were greatly attenuated, whereas those of pimozide remained unchanged. These investigators concluded that the decreased proliferation observed with pimozide involved a mechanism that was independent of T-type channel blockade but could involve the calmodulin-dependent regulation of T-type channels.

ES as a new inhibitor of T-type channels in glioblastoma

The mechanisms of in vivo antitumor action of ES, the carboxyl-terminal proteolytic fragment of collagen XVIII, are less understood and remain controversial. However, ES was previously considered to interact with several endothelial cell surface receptors that are involved in angiogenesis including caveolin-1, tropomyosin, and vascular endothelial growth factor type 1 receptor [24, 62, 73]. For instance, Peroulis et al. reported that the endogenous expression of ES by C6 glioma cells resulted in a reduced tumor growth rate [55]. Furthermore, Schmidt et al. demonstrated that local intracerebral application of ES improved treatment efficiency and survival in an orthotopic human glioblastoma model [59]. These results suggested that the antiangiogenic effect of ES was essential for the in vivo growth and expansion of tumors including glioblastoma. However, a later study failed to determine any antitumor effects of ES [39]. Growing evidence has recently revealed that ES could play a direct role in inhibiting tumor/cancerous cells [58, 76]. It has been reported recently that peptide 30 derived from ES suppresses the proliferation of HepG2 cells in vitro [32]. In our recent work, we revealed that, in addition to its antiangiogenic effects, ES also directly inhibited in vitro U87 human glioblastoma cell proliferation and migration by targeting T-type channels. We also observed that inhibition of T-type channel currents induced by ES was highly dependent on the inactivation state of the channel [79]. Although it is unclear whether the hyperpolarizing shift of the steady state inactivation curve would produce a significant modification in T-type channel "window currents," it is conceivable that it could depend on an increased number of channels remaining in the inactivated state after activation. Further studies are required to address how making less T-type Ca^{2+} channels available for opening mechanistically contributes to the inhibitory effect of ES on glioma cellular responses. In this study, we evaluated the mibefradil data obtained by the siRNA-mediated knockdown approach and by using NNC 55–0396, a mibefradil nonhydrolyzable analog without L-type channel efficacy, to avoid the nonspecific antiproliferative effects of mibefradil [50]. ES or mibefradil inhibits the fibronectin-induced migration of U87 glioma cells. Similarly, mibefradil suppressed T-type-mediated Ca^{2+} spikes, waves, cell motility, and invasive properties in fibrosarcoma cells [21]. The magnitude of the inhibitory effect of mibefradil on the motility of U87 cells was higher than that induced by ES, further supporting that mibefradil displays broader T/L-type-independent effects as described previously.

ES has been reported to interact with vascular endothelial growth factor type 1 receptor, which associates with a family of receptor protein tyrosine kinases [24]. We identified in a previous study that the effects of ES on the proliferation and migration of U87 human glioma cells is not mediated by tyrosine kinase or G-protein-coupled signaling pathways. T-channel activity can be modulated by neurotransmitters and hormones through protein kinase (PK)-dependent signaling molecules such as PKA, PKC, and PKG; calmodulin-dependent protein kinase II; tyrosine kinases; and protein kinase-independent mechanisms including zinc, redox agents, and lipid derivatives such as arachidonic acid [23, 78]. Therefore, crucial questions about the nature of the signaling pathways involved in the ES-mediated inhibition of T-type channels, and the mechanism by which they regulate Cav3 activity remain unanswered. At present, it is not completely

understood whether ES directly phosphorylates/blocks Cav3 channels or represents an indirect consequence of the phosphorylation of associated targeting, anchoring, or signaling proteins. Further studies are necessary to elucidate this issue. In addition, recent reports also suggest a role for extracellular signal-regulated kinase (ERK) pathways in T-type channel activation. For example, ciliary neurotrophic factor increases the expression and currents of T-type channels by triggering the Janus kinase/signal transducers and activators of transcription (JAK/STAT) and ERK signaling pathways [67]. However, this was not the case in this study because the inhibitory effect of ES on Cav3 T-type channel currents is rapid (within 10 min). In conclusion, in addition to the role of ES-induced angiogenesis in tumor progression, our study highlighted the therapeutic potential of ES via targeting T-type channels for the treatment of tumors with the endogenous expression of all three subunits of Cav3, including human glioblastoma multiforme (GBM). We revealed that ES directly inhibited the channel currents of only Cav3.1 and Cav3.2, but not that of Cav3.3 or Cav1.2. Our results highlight the novel mechanism and therapeutic potential of ES via targeting T-type channels for the treatment of human glioblastoma.

Perspectives

Determination of the expression and activity of T-type channels in specific tumors and the development of new, specific T-type channel blockers can potentially offer new approaches for the treatment of some cancers such as GBM that are no longer responsive to conventional treatment strategies. The search for subtype-specific T-type channel blockers has been of considerable interest from the clinical perspective. Although a number of classes of T-type blockers have been described (benzodiazepines, succinimide derivatives, diphenylbutylpiperidine derivatives, dihydropyridines, and anesthetics), their action is not sufficiently selective for T-type channels [17, 41]. T-type channels have isoform-specific properties, and therefore, the need to develop selective drugs for a given T-channel subtype has emerged. The subsequent challenge will be to determine whether specific T-channel isoforms can be selectively targeted for therapeutic intervention. Although a new treatment strategy has been identified with the utilization of genetic approaches recently, such as gene knockout or gene knockdown [3, 25], it does not represent a viable therapeutic approach at this time. Nevertheless, the search for new generations of selective T-type channel blockers is ongoing [1, 43]. In addition, it is also important to understand the regulatory mechanisms of the T-type calcium channel expression, which may lead to the identification of novel strategies of regulating T-type channel activity for therapeutic purposes, especially for controlling tumor growth.

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Conflict of interest The authors declare that they have no conflict of interests.

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