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doi:10.3748/wjg.14.4984

World J Gastroenterol 2008 August 28; 14(32): 4984-4991
World Journal of Gastroenterology ISSN 1007-9327

EDITORIAL

Calcium signaling and T-type calcium channels in cancer cell cycling

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Received: February 27, 2008 Revised: May 1, 2008
Accepted: May 8, 2008
Published online: August 28, 2008

Abstract

Regulation of intracellular calcium is an important signaling mechanism for cell proliferation in both normal and cancerous cells. In normal epithelial cells, free calcium concentration is essential for cells to enter and accomplish the S phase and the M phase of the cell cycle. In contrast, cancerous cells can pass these phases of the cell cycle with much lower cytoplasmic free calcium concentrations, indicating an alternative mechanism has developed for fulfilling the intracellular calcium requirement for an increased rate of DNA synthesis and mitosis of fast replicating cancerous cells. The detailed mechanism underlying the altered calcium loading pathway remains unclear; however, there is a growing body of evidence that suggests the T-type Ca^{2+} channel is abnormally expressed in cancerous cells and that blockade of these channels may reduce cell proliferation in addition to inducing apoptosis. Recent studies also show that the expression of T-type Ca^{2+} channels in breast cancer cells is proliferation state dependent, i.e. the channels are expressed at higher levels during the fast-replication period, and once the cells are in a non-proliferation state, expression of this channel is minimal. Therefore, selectively blocking calcium entry into cancerous cells may be a valuable approach for preventing tumor growth. Since T-type Ca^{2+} channels are not expressed in epithelial cells, selective T-type Ca^{2+} channel blockers may be useful in the treatment of certain types of cancers.

Key words: T-type calcium channels; Cancer; Cell cycle; Calcium

Peer reviewers: Anna S Gukovskaya, Professor, VA Greater Los Angeles Health Care System, University of California, Los Angeles, 11301 Wilshire Blvd, Los Angeles 91301, United States; Zong-Jie Cui, PhD, Professor, Institute of Cell Biology, Beijing Normal University, 19 Xinjiekou Waidajie, Beijing 100875, China

Taylor JT, Zeng XB, Pottle JE, Lee K, Wang AR, Yi SG, Scruggs JAS, Sikka SS, Li M. Calcium signaling and T-type calcium channels in cancer cell cycling. *World J Gastroenterol* 2008; 14(32): 4984-4991 Available from: URL: http://www.wjgnet.com/1007-9327/14/4984.asp DOI: http://dx.doi.org/10.3748/wjg.14.4984

INTRODUCTION

Calcium is an essential signal transduction element involved in the regulation of many eukaryotic cellular functions including cell cycle progression[1]. Control of intracellular Ca^{2+} ($[Ca^{2+}]_i$) is crucial for the orderly progression of the cell cycle and plays a vital role in the regulation of cell proliferation and growth[2]; however, excessive calcium or loss of control in calcium signaling can lead to cell death[3]. Therefore, careful control of calcium signaling is required for cell survival. Upon stimulation, the intracellular calcium concentration can increase dramatically, often reaching micromolar amounts. This increase in cytoplasmic calcium can occur *via* release from intracellular stores or influx through a variety of plasma membrane ion channels. Voltage-gated and ligand-gated Ca^{2+} channels in the plasma membrane, along with ryanodine receptors (RynR) and inositol triphosphate receptors (InsP3R) at the intracellular calcium stores, provide fluxes of Ca^{2+} to the cytoplasm.

The driving force for calcium entry is the result of an electrochemical gradient between the extracellular concentration (1.3×10^{-3}-2×10^{-3} mol/L) of calcium and the intracellular concentration ($< 10^{-8}$ mol/L).

In general, non-excitable tissues, including the epithelium, do not express voltage gated Ca^{2+} channels. This is partly because the ranges of membrane potential changes in these cells are too small to activate these channels. However, recent studies show that T-type Ca^{2+} channels are expressed in cancerous cells, although their functional role has only begun to be investigated. Furthermore, there is a growing body of evidence suggesting that tumor cell proliferation can be halted by the use of ion channel blockers. T-type calcium channels are a class of calcium permeable low voltage activated (LVA) ion channels which open after small depolarizations of the membrane. Molecular biology has revealed the existence of three different T-type calcium channel subunits, the α_{1G}, α_{1H} and α_{1I}. The α_1 designation refers to the channels primary ion conducting protein, which consists of four domains each containing six transmembrane segments. There are other auxiliary calcium channel subunits; however, the LVA alpha1 subunits can function as stand alone complexes. The unique low voltage dependent activation/inactivation and slow deactivation of T-type Ca^{2+} channels indicate that these channels may play a physiological role in carrying depolarizing current at low membrane potentials. Therefore, these channels may play a direct role in regulating $[Ca^{2+}]_i$, especially in non-excitable tissues, including some cancerous cells. At low voltages, T-type Ca^{2+} channels are known to mediate a phenomenon known as "window current"[4-6]. The term "window" refers to the voltage overlap between the activation and steady state inactivation at low or resting membrane potentials. As a result, there is a sustained inward calcium current carried by a small portion of channels that are not completely inactivated. Window current allows T-type Ca^{2+} channels to regulate Ca^{2+} homeostasis under non-stimulated or resting membrane conditions[7]. The most direct evidence of T-type Ca^{2+} channel mediated Ca^{2+} window current is from a study conducted in HEK-293 cells expressing the T-type isoform α_{1G}[8], which demonstrated window current peaked at -48 mV. Membrane potentials around this voltage can occur in un-stimulated non-excitable cells.

CALCIUM SIGNALING AND CELL CYCLING

As shown in Figure 1, the cell cycle is divided into four stages: G1, S, G2 and M. DNA replication occurs in the S phase and mitosis occurs in the M phase. Cells must pass through a restriction point between the G1 and S phases before continuing proliferation; otherwise, they exit the cell cycle to G0 and differentiate or terminate. Another checkpoint in the cell cycle is between phases G2 and M. For cells to pass through these various points, one of the most prominent messengers is Ca^{2+}, as demonstrated by the induction of mitotic events by injection of exogenous Ca^{2+} in a fertilized egg model[9]. Steinhardt *et al*[10] observed transient increases in cytosolic Ca^{2+} during late G1, prior to the initiation of the S phase and during G2 before entry into the M phase that were dependent upon external physiological Ca^{2+} concentration. In the transition from G1 to S phase, cells require external Ca^{2+} in addition to functional calcium channels in order to directly or indirectly trigger a myriad of critical downstream enzymes such as thymidine kinase, thymidylate synthase, ribonucleotide reductase and DNA polymerase and begin DNA replication. In the transition from G2 to M phase, Ca^{2+} flashes activate enzymes that are critical for microtubule rearrangement and microfilament contraction. In order to confirm the significant role that Ca^{2+} plays in the cell cycle, researchers have blocked the progression of the cell cycle *via* injection of Ca^{2+} chelators into the same fertilized eggs[11]. The influence of Ca^{2+} channels on cell growth is clearly demonstrated in pharmacological studies using Ca^{2+} channel antagonists. In a study by Zeitler *et al*[12], various Ca^{2+} channel blockers, including verapamil, nifedipine, diltiazem and isradipine, caused G0/G1 cell-cycle arrest in growth factor induced human umbilical arterial endothelial cells (HUAEC) during proliferation. The Ca^{2+} signal has also been linked to activation of immediate early genes (e.g. *c-fos*) that are responsible for inducing resting cells in G0 to re-enter the cell cycle, an attribute most frequently up-regulated in rapidly proliferating cells[10].

At the end of the cycle, cells can undergo suicide through a process known as apoptosis or active cell death, which is a genetic program specifically designed to shape organs during development and adjust cell population levels to appropriate values. The key players of apoptosis are a killer Ca^{2+} surge and the nuclear membrane Ca^{2+} activated endonuclease, which terminates the cell by cutting chromatin into fragments (Figure 1). Underlying mechanisms for Ca^{2+} mediated effects in cell proliferation may involve a wide variety of other intracellular signal transduction pathways such as G-proteins, protein kinase C (PKC), calmodulin, m-calpain, MAP kinase, phospholipase A2 and others[13,14]. Although the details of each pathway is beyond the scope of this discussion, there are several notable mechanisms that act to amplify $[Ca^{2+}]_i$ for activation of gene transcription or cell migration. One mechanism is the hydrolysis of inositol lipids by the enzyme phospholipase C, the activation of which is itself dependent on an initial rise in $[Ca^{2+}]_i$, producing diacylglycerol (DAG) and InsP3. Resulting from the activation of G protein-linked or tyrosine-kinase linked receptors[15], InsP3 thus causes a form of Ca^{2+} dependent Ca^{2+} release from intracellular stores. Described as "calcium puffs", which propagate into a local or global Ca^{2+} signal, this Ca^{2+} release is important for converting the cytoplasm into an excitable medium that can support repetitive Ca^{2+} oscillations[16]. The resulting amplification of $[Ca^{2+}]_i$ contributes to the signal for mitosis and DNA synthesis.

In addition to InsP3, sensory proteins also play

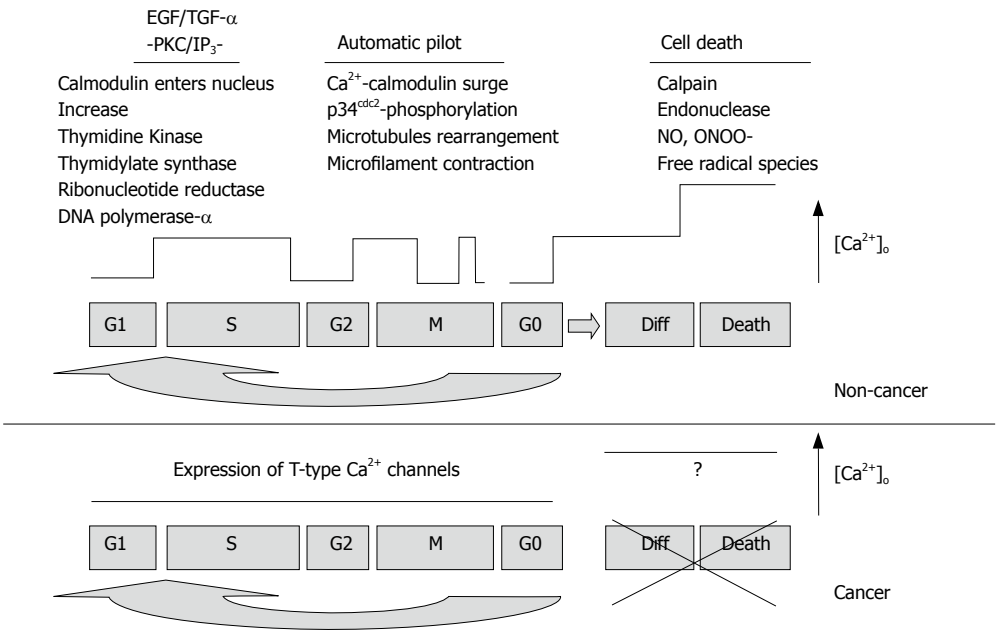

Figure 1 Ca^{2+} signaling pathways differ between cancerous and non-cancerous cells (adapted from [23]).

a role in maintaining the calcium signaling system. Calmodulin is a Ca^{2+} binding protein that acts as a Ca^{2+} sensor in the cell cycle. High expression of calmodulin has been observed during the S phase and mitosis, while inhibition of its activity by administration of calmodulin monoclonal antibodies is shown to block DNA synthesis[17]. Another sensory mechanism occurs through an extracellular calcium ion concentration sensing receptor (CaR) and calbindin, a high affinity Ca^{2+}-binding regulatory protein belonging to the same family as calmodulin. Parkash *et al* observed that CaR plays a role in sensing and responding to changes in extracellular Ca^{2+} ($[Ca^{2+}]_o$). Upon activation by increased $[Ca^{2+}]_o$, CaR interacts with phospholipase C (PLC) *via* G proteins to produce DAG and InsP3[18]. The subsequent increase in $[Ca^{2+}]_i$ is regulated by calbindin, which was previously found to bind to L-type HVA Ca^{2+} channels in pancreatic islets-cells[19]. It has also been shown that calbindin/CaR co-localization occurs in the estrogen receptor positive breast cancer cell line MCF-7[20]. Activated CaR also increases parathyroid hormone related protein (PTHrP), which appears to exacerbate cell metastasis in MCF-7 cells[21]. A study by Lewalle *et al*[22] shows that an increase in $[Ca^{2+}]_i$ mediates tumor cell transendothelial migration *in vitro*. By associating the upregulation of these mechanisms in cancerous cells, increases in $[Ca^{2+}]_i$ are shown to provide an important and pronounced signal for cell growth.

Calcium signaling in cancerous cells, however, uses an altered pathway during cell cycling[23]. Whitfield has shown that colon carcinomas undergoing carcinogenesis, which have lost their tumor-suppressing genes, have dramatically altered calcium signal mechanisms, and have ignored normal calcium-dependent restrictions by overproducing calcium-binding signal proteins (Figure 1)[18]. Attempts to terminate the mutant cells with Ca^{2+} signal surges are futile, as the cells are no longer responsive to Ca^{2+} signals: instead, they produce and respond to their own renegade growth factors. Ca^{2+} and the signaling enzymes that are directly activated by Ca^{2+} or by Ca^{2+}-binding proteins play crucial roles in most cell signals and programs and must be understood and implemented in any future differentiation therapies. Since cancerous cells express T-type Ca^{2+} channels, it is possible that these channels provide an altered Ca^{2+} influx pathway in responding to the increasing demand of Ca^{2+} during rapid cell proliferation.

T-TYPE CALCIUM CHANNELS IN CANCEROUS CELL PROLIFERATION

T-type Ca^{2+} channels and non-cancerous cell cycling

The function of regulating Ca^{2+} homeostasis may allow T-type Ca^{2+} channels to play an important role in controlling cell proliferation and differentiation in many tissues. In primary cultured rat aortic smooth muscle cells, a T-type Ca^{2+} current was found to be present in cells during the G1 and S phases but decreased or absent in all other phases of the cell cycle[24-26]. It was shown that cultured smooth muscle cells exhibited an increased T-type Ca^{2+} current during stages of proliferation and this current decreased as the cells became confluent or when they came into contact with one another[27]. T-type Ca^{2+} currents are also present in freshly dissociated or 1-2 d cultured neonatal rat ventricular myocytes when they are still able to proliferate, but are not observed in cells cultured greater than 3 d[28]. Likewise, older tissues under pathological conditions, such as cardiomyopathic hamster heart[29], hypertrophied adult feline left ventricular myocytes[30], and rat neointimal formation after vascular injury[31] have increased T-type Ca^{2+} current activity. These studies suggest that T-type calcium

Table 1 Cancerous cells that expresses T-type Ca^{2+} channels

Cell type	Cell line	T-type isoform	Reference
Breast carcinoma	MCF-7, MDA-435	α_{1G}, α_{1H}	[33-36]
	MDA-231, MDA-361 MB-468, MB-474, BT-20, CAMA1, SKBR-3	α_{1G}	[34-36]
Neuroblastoma	SK-N-SH,	α_{1G}	[34,37-40]
	NG 108-15, SK-N-MC		
	N1E-115	α_{1G}, α_{1H}	[37]
Retinoblastoma	Y-79, WERI-Rb1	α_{1G}, α_{1H}, α_{1I}	[41,42]
Glioma	Primary (biopsy)	α_{1G}	[36]
	U87-MG	α_{1G}, α_{1H}	[37]
Prostate carcinoma	TSU-PRL, DUPRO	α_{1G}	[35,43]
	LNCaP	α_{1H}	[1,34,35]
	PC-3, DU-145	α_{1G}, α_{1H}	[34,35]
Esophageal carcinoma	TE1, TE10, TE12, KYSE150, KYSE180, KYSE450	α_{1H}	[44]
	SKGT4, TE3, TE7, KYSE70	α_{1G}, α_{1H}	[44]
	COLO-680N, SEG1, TE8, TE11, KYSE30, KYSE410, KYSE510	α_{1G}, α_{1H}, α_{1I}	[44]
Fibrosarcoma	HT1080	α_{1G}	[45]
Colorectal carcinoma	Caco2, DLD-1, Lovo, SW837	α_{1G}	[35]
Pheochromocytoma	MPC 9/3L	α_{1G}	[46]
	PC-12	α_{1H}	[47]
Adenocarcinoma	H295R	α_{1H}	[48]
Insulinoma	INS-1	α_{1G}	[49]

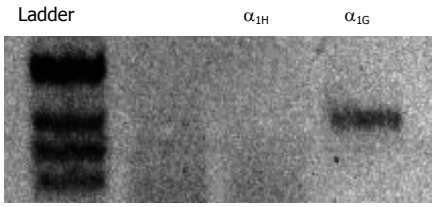

Figure 2 T-type Ca^{2+} channel expression in human malignant breast cancer tissue.

Table 2 Q-RT-PCR detected T-type Ca^{2+} channels expression in non-confluent cultures of breast cancer cell lines

Cell types	T-channels	Non-confluent Δct	Confluent Δct
MDA-MB-231	α_{1H}	14.28 ± 0.16	NA, ct > 40
MDA-MB-231	α_{1G}	9.45 ± 0.87	NA, ct > 40
MCF-7	α_{1H}	13.43 ± 0.24	NA, ct > 40
MCF-7	α_{1G}	7.32 ± 0.3	NA, ct > 40

NA: Not applicable.

channels may play a vital role in regulating proliferation under specialized conditions.

T-type Ca^{2+} channels are broadly expressed in tumor cells

If these channels do participate in proliferation under abnormal conditions, cells must first maintain control of the expression of α_{1G} T-type Ca^{2+} channel messenger RNA in order to prevent functional expression of the protein. Otherwise, loss of this control may lead to aberrant cell growth and tumor progression. A recent study revealed the presence of T-type calcium channel mRNA expressed in breast tumor tissue that was removed from human biopsies (Figure 2)[32]. In this case, the tumor was later diagnosed as malignant and estrogen receptor positive by pathological examination. Expression of these channels in tumor cells has been reported broadly, as shown in Table 1[1,33-49]. For example, MCF-7 cells, a cell line derived from a human breast adenocarcinoma that has been shown to express α_{1G} and α_{1H} T-type Ca^{2+} channel mRNA and current transiently (Table 2)[33]. T-type Ca^{2+} channels have also been suggested as a potential therapeutic target for intracranial tumor and prostate cancer. Mibefradil was found to inhibit human astrocytoma (U87-MG) and neuroblastoma (N1E-115) proliferation and that over-expression of T-type Ca^{2+} channel protein doubled the proliferation rate while antisense treatment reduced the proliferation rate of these cells[37]. Human prostate cancer epithelial cells (LNCap) have also been shown to express increased T-type Ca^{2+} channel (α_{1H}) current and mRNA. Similarly, increased T-type Ca^{2+} channel protein doubled proliferation while antisense treatment reduced the proliferation rate of these cells[1,43]. It was also shown that these channels were found to regulate intracellular calcium in LNCap cells. Another study examined the role of T-type Ca^{2+} channels in esophageal carcinoma cell proliferation; these data suggested that T-type Ca^{2+} channels may have a functional role in proliferation that can be reduced by inhibition of T-type Ca^{2+} channels[44]. Given the role that T-type calcium channels play in cell cycle progression and the relatively recent findings that show the functional expression of these channels in many different cancerous cell types, researchers have now been given the opportunity to investigate the potential of an entirely new target in the fight against cancer. Developing new compounds that target these proteins may hold the key to controlling certain types of cancer.

EFFECT OF T-TYPE Ca^{2+} CHANNEL BLOCKERS ON BREAST CANCER CELL PROLIFERATION

The function of T-type Ca^{2+} channels with regards to

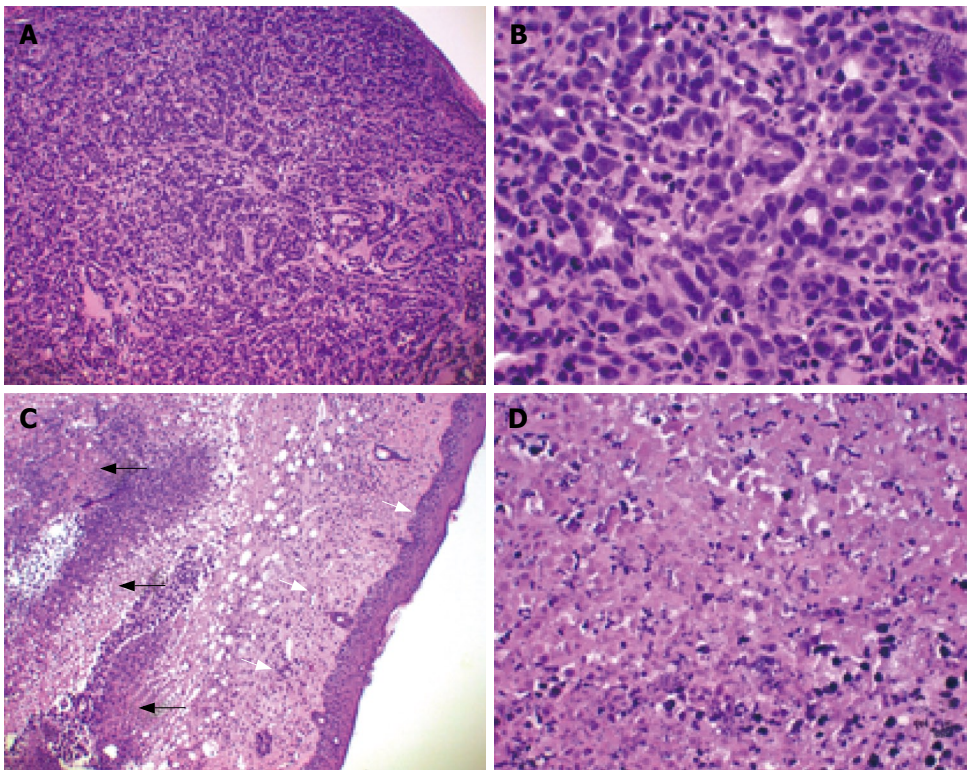

Figure 3 Mibefradil destroys tumor cells implanted in a nude mouse. HE stain was applied to show the nuclei and cytoplasm of the cells (**A** and **C**, x 100; **B** and **D**, x 400).

tumor cell proliferation was also reviewed[50,51]. One study found the T-type Ca^{2+} channel to be particularly effective in controlling oscillations in intracellular Ca^{2+} as the result of the channels unique activation/inactivation properties. It was concluded that new selective antagonists may become helpful as a therapeutic approach against tumors in which proliferation depends on T-type Ca^{2+} channel expression[51]. A study performed in knockout animals found that selective inhibition of T-type Ca^{2+} channels may have impact upon the treatment of cancer[52].

Studies have shown an inhibition in breast cancer proliferation by the channel blockers pimozide, thioridazine[53] and mibefradil[42]. The endogenous cannabinoid anandamide has also been shown to block T-type Ca^{2+} channels[54], in addition to inhibition of breast cancer cell proliferation[55], an effect that may be due to blockage of T-type Ca^{2+} channels.

The anti-cancer effect of a T-type Ca^{2+} channel antagonists on tumor cells *in vivo* has been investigated[32]. MCF-7 cells were implanted into nude mice, athymic nude BSLB/c, and then either mibefradil (0.5 mg/100 μL) or saline (0.5 mg/100 μL) was injected locally at the tumor sites (s.c) twice a week. After 30 d of the treatment, mice were sacrificed and the tumors were removed for histochemistry examination.

As shown in Figure 3A and B, in the saline injected tissue the proliferation of the malignant tumor cells formed nodules in subcutis. The tumor cells were malignant as indicated by hyperchromatic nuclei with enlarged nuclei, irregular nuclear membrane, prominent nucleoli, and many mitotic features. No signs of degeneration and necrosis were detected. In contrast, the mibefradil injected tissue showed large areas of tumor degeneration and necrosis (Figure 3C and D). The tumor necrosis was accompanied by prominent edema. Furthermore, as shown in Figure 3C, mibefradil more potently destroyed breast cancer cells (indicated by the black arrows) than non-cancerous cells at adjacent areas (indicated by the white arrows), including fibroblasts, endothelial cells and keratinocytes. These results indicate that a local injection of mibefradil induces necrosis of human breast carcinoma cells implanted into subcutaneous adipose tissue in mice.

More recently, the antiproliferative effect of the T-type calcium channel inhibitor NNC 55-0396[56] has been examined in cell lines derived from breast epithelial tissue, MCF-7, MDA-MB-231(ER-α), and an adriamycin resistant cell line ADR. All three of these cell types express α_{1G} and α_{1H} Ca^{2+} channel mRNA and their proliferation was suppressed by NNC 55-0396, with IC_{50} of about 1-2 μmol/L[32,33]. The specificity of NNC 55-0396 antagonism on cancerous cell proliferation was investigated in a prostate epithelial cell line (RWPE-1) that does not express T-type Ca^{2+} channels[32]. As shown in Figure 4, NNC 55-0396 exhibited neither dose-dependent (up to 20 μmol/L Figure 4A) nor time-dependent (up to 60 h, Figure 4B) inhibitory effects on RWPE-1 cell growth, suggesting that the anti-proliferation effect of NNC 55-0396 most likely resulted from blocking T-type Ca^{2+} channels of breast cancer cells. It also suggested that the general toxicity of NNC 55-0396 is minimal at the concentration that induces suppression of proliferation. T-type Ca^{2+} channel antisense treatment in these cells reduced the proliferation rate by 45% and antisense had no effect on proliferation on tumor cells not expressing T-type Ca^{2+} channels.

The role of T-type Ca^{2+} channels in cancerous cell proliferation has also been examined with specific siRNA

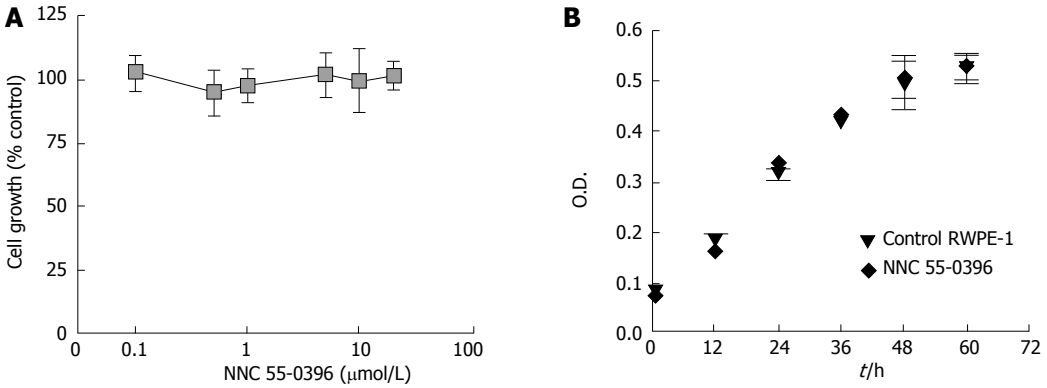

Figure 4 Effect of NNC 55-0396 on RWPE-1 cell proliferation (Error bars represent SE; $n = 3$).

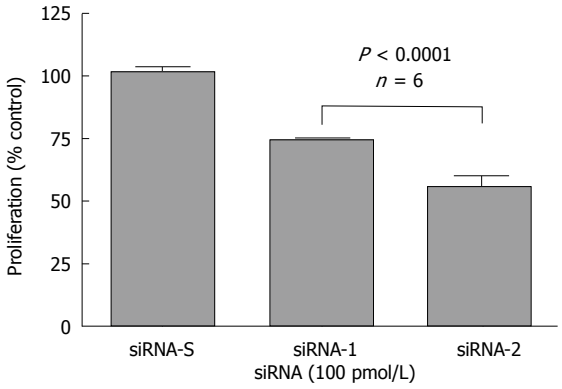

Figure 5 Effect of siRNAs on MCF-7 cell proliferation.

antagonism[33]. Specifically, MCF-7 cells were treated with siRNA targeting both α_{1G} and α_{1H} ($\alpha_{1G/H}$). The cells were treated with scrambled (siRNA-S, 100 pmol/L), $\alpha_{1G/H}$-1 (siRNA-1) or $\alpha_{1G/H}$-2 (siRNA-2) for 48 h and subjected to MTT assay. The effects of siRNAs on cell proliferation were shown as percent (%) of vehicle control. As shown in Figure 5, scrambled siRNA was not significantly different than the control. However, both siRNA-1 and siRNA-2 treated cells had significantly lower proliferation rates compared to the scrambled and vehicle control siRNAs. These results strongly support the role of T-type Ca^{2+} channels in breast cancer cell proliferation and indicate that the effect of NNC 55-0396 on the breast cancer cell proliferation is due to the blockade of these channels.

CONCERNS

Since T-type Ca^{2+} channels are normally expressed in the brain, heart and endocrine tissues of the human body, the potential side-effects of T-type Ca^{2+} channel blockers to these systems are of concern for therapeutic applications. Although T-type Ca^{2+} channel blockers have been used clinically for the treatment of neurological disorders (e.g. ethosuximide for absence seizures), the adverse effects of these drugs on the cardiovascular and central nervous systems are still unclear. Specifically, it is important to determine the possible arrhythmic and sedative effects of these drugs.

Human blood cells do not express T-type Ca^{2+} channels; therefore, it is advantageous to apply T-type Ca^{2+} channel blockers in the hemopoietic system, since current chemotherapeutic drugs have displayed severe side effects on this system. If we can locally deliver T-type Ca^{2+} channel blocker into the hemopoietic system, the compound should be very selective in eliminating the breast cancer cells in the blood stream. Thus, T-type Ca^{2+} channel blockers can be potential anti-metastasis drugs for adjuvant therapy of breast cancer.

PERSPECTIVES

The function of T-type Ca^{2+} channels may not be restricted to cancerous cell proliferation. These channels may also play roles in cancerous cell colonization, invasion, secretion and angiogenesis. The growing number of proliferating cells need to attract blood vessels (angiogenesis) in order to receive nutrients, O_2, *etc.* to sustain themselves. The transformed cells are able to enter the blood stream and survive there, and colonize (metastasize) other tissues. Invasive growth or cell migration is a highly regulated process in which the migrating cells must secrete matrix proteases that disrupt the extracellular matrix (ECM) and permit easier transit through the surrounding environment. In addition, they must also profoundly reshape their structure, which involves massive cytoskeletal rearrangement. Precise regulation of intracellular calcium concentration is crucial for all of these processes. It is very possible that T-type Ca^{2+} channels also play significant roles in these processes[45].

An expansion of the list of ion channels implicated in cancer development is expected, and the tools needed to investigate this issue are more readily available. As is the case with other protein families, it will be probably difficult to ascribe tumor development to the malfunction of a single ion channel. Rather, defects in T-type Ca^{2+} channels probably contribute to the neoplastic phenotype through complex interactions with other ion channels, most of which have not been properly identified. For instance, regulation of K^+ channels can affect the membrane potential, which in turn regulates the window currents mediated by T-type Ca^{2+} channels. However, since in many cases there are already known pharmacological modulators (blockers

and activators) of ion channels, identification of a single defective ion channel in a particular cancer could provide a ready-to-go therapeutic approach.

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