ACTIVITY OF VOLTAGE-GATED K\(^+\) CHANNELS IS ASSOCIATED WITH CELL PROLIFERATION AND Ca\(^{2+}\) INFLUX IN CARCINOMA CELLS OF COLON CANCER

Xiaoqiang Yao, Hiu-Yee Kwan

Department of Physiology, Faculty of Medicine, Chinese University of Hong Kong, Shatin, Hong Kong

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Summary

Cell proliferation of carcinoma cells DLD-1 derived from colon cancer as measured by \([3H]\) thymidine incorporation was drastically reduced in the presence of 4-aminopyridine, an inhibitors of voltage-gated K\(^+\) channel. A number of nonspecific K\(^+\) channel inhibitors including TPeA, TEA, verapamil and diltiazem also inhibited \([3H]\) incorporation at the concentration reported to inhibit voltage-gated K\(^+\) channels. The presence of voltage-gated K\(^+\) channels was confirmed by reverse transcription-PCR and cDNA sequencing. Charybdotoxin and iberiotoxin, inhibitors for Ca\(^{2+}\)-sensitive K\(^+\) channel, and glibenclamide, a specific inhibitor for ATP-sensitive K\(^+\) channel, did not have effect on cell proliferation. These experiments suggested a critical role of voltage-gated K\(^+\) channels in proliferation of colon cancer cells. Mechanism of action of K\(^+\) channel activity in cell proliferation was explored by studying the relationship between the K\(^+\) channel activity and Ca\(^{2+}\) entry. The results from experiments indicated that K\(^+\) channel inhibitors blocked \([Ca^{2+}]_i\) influx. Therefore, it is likely that K\(^+\) channel activity may modulate Ca\(^{2+}\) influx into colon cancer cells, and subsequently modulate the proliferation of these cells.

Key Words: K\(^+\) channels, Ca\(^{2+}\) influx, cell proliferation, colon cancer

K\(^+\) channels have been reported to be involved in the proliferation of many types of cells, including tumor cell lines. Increased potassium channel activity is associated with increased proliferation rates. Mitogenic stimulation increases the level of expression of potassium channels in T lymphocytes and epithelial cells (1-3). In addition, drugs or toxins that block potassium channels inhibit the proliferation of mitogen-stimulated normal human T lymphocytes (1), malignant rat lymphoma cells (4), human melanoma cells (5) and human breast cancer cells (6). Of particular interest to cancer cell biology is the increase in potassium channel activity following oncogenic transformation of cells by ras/raf (7) or SV-40-virus (8).

A variety of different K\(^+\) channels may be involved in the proliferation of different tumor cells. ATP-sensitive K\(^+\) channel (K\(_{ATP}\)) and Ca\(^{2+}\)-sensitive K\(^+\) channel (K\(_{Ca}\)) channel are critical for the proliferation of breast cancer cell line MCF-7 cell (6). K\(_{ATP}\) is critical for human brain tumor cells (9) and voltage-dependent K\(^+\) channel (K\(_v\)) is essential for malignant T lymphocytes (4). Although the mechanism of action of these K\(^+\) channels is unknown, the potassium-dependent changes in membrane potential might be a likely candidate. An alteration of membrane potential by K\(^+\) channel modulators may interfere with cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) which
could then influence the transition from G1 to S during mitosis and affect the proliferation of cancer cells (6). However, there is no direct evidence linking K⁺ channel activity to Ca²⁺ entry. The goal of this study is to determine if K⁺ channel activity is required for the proliferation of carcinoma cells derived from colorectal cancer. If so, what is the mechanism underlying it? Blockers against different types of K⁺ channels were used in order to differentiate the type of K⁺ channel critically involved in the proliferation of colorectal adenocarcinoma cell line DLD-1. We also explored the mechanism of action of K⁺ channel in cell proliferation by studying the relationship between the K⁺ channel activity and Ca²⁺ entry with the use of confocal Ca²⁺ imaging system.

Materials and Methods

Materials
Fluo3/acetoxymethyl ester (Fluo3/AM) and Pluronic F127 were obtained from Molecular Probes, Inc., OR, USA. Tissue culture media and materials were from Gibco/BRL, USA. Culture flasks and culture plates were from Becton Dickinson, NJ, USA. Guanidinium thiocyanate, 4-aminopyridine (4-AP), tetraethylammonium (TEA) and tetrapentylammonium (TPeA), iberiotoxin, charybdotoxin, glibenclamide, verapamil, diltiazem, 2-nitro-4-carboxyphenyl,N,N-diphenylcarbamate (NCDC), Hepes, EGTA, EDTA and [³H] thymidine (50 mCi/mmol) were purchased from Sigma, MO, USA. Oligo-dT primers, dATP, dCTP, dGTP, dTTP and M-MLV reverse transcriptase were obtained from Gibco-BRL, USA.

Cell culture
Colorectal adenocarcinoma cell line DLD-1 was obtained from the American Type Culture Collection (ATCC). Cells were cultured in 90% RPMI 1640 and 10% fetal bovine serum (FBS) containing 100 U/ml penicillin and 100 µg/ml streptomycin and incubated in T-75 tissue culture flasks in an atmosphere of 37°C and 5% CO₂ in air. Confluent cell monolayers were passaged using trypsin containing EDTA.

³H thymidine uptake and cell growth
10⁵ cells per well were cultured in flat-bottomed 96-well culture plates in 0.25 ml of 90% RPMI 1640 supplemented with 10% FBS containing 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were incubated for 24 hours with or without K⁺ channel inhibitors in an atmosphere of 37°C and 5% CO₂ in air. Cultures were pulsed with 1 µCi/well of [³H] thymidine, and uptake was measured 6 hours later by harvesting the cells with a multiple cell harvester (Cambridge Technology, Inc. USA). The amount of [³H] thymidine incorporated into cellular DNA was measured by a liquid scintillation counter (Beckman LS1801). Some cells without [³H] thymidine pulse were harvested directly from the monolayer and counted under phase contrast in a haemocytometer. The drug concentration which blocks one half of the current (Kᵢ) was determined from the least-squares fit of the experimental points by the equation Iₓ = I/(1 + (X/Kᵢ)), where Iₓ is the normalized [³H] incorporation at concentration X.

RT-PCR
Total RNA was isolated from cultured cells by the acid guanidinium thiocyanate method (10). mRNA was transcribed into first strand cDNA using oligo-dT primers and M-MLV reverse transcriptase. Degenerate primers K,(+) (GCCATCCTG/CAGG/AG/ATC/GAT) and K,(−) (GCCCACCAG/ATAG/AGCATC) were used to detect mRNA of voltage-dependent K⁺ channels. The primers were designed based on a conserved region spanning S4 and pore region of voltage-gated potassium channels K⁺I1, K⁺I3 and K⁺I5. Primers LCa(+) (GGGATCCGCACATCGCTCTG) and LCa(−) (GGAAGGCACAGAGCATGAAG) were used to detect mRNA of L-type calcium channel. The primers were designed according to highly conserved sequence that included the putative membrane spanning segment IVS5 and IVS6 of L-type calcium channel ø1 subunit (11). PCR reactions of 50 µL contained 1 µL first strand cDNA, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, dATP, dCTP, dGTP, dTTP 0.2 mM each, 1.0 µM primers and 2.5 Unit Taq DNA polymerase. Fifty cycles (94°C for 1 min, 50°C for 1 min, 72°C for 1 min) were performed with a Robocycler (Stratagene). Control reactions without first strand cDNA were included for each PCR amplification experiment. PCR fragments
were resolved on agarose gel. Amplified PCR products were sequenced with ABI 310 Autosequencer.

**[Ca^{2+}]_i** measurement
DLD-1 cells were grown overnight in 90% RPMI 1640 supplemented with 10% FBS containing 100 U/ml penicillin and 100 μg/ml streptomycin on circular discs (Fisher brand 25 CIR-1) at 37°C and 5% CO₂ in air. Cells were loaded with Fluo3/AM for 1 hour in the dark at room temperature by incubation with 10 μM membrane-permeant Fluo3/AM and 0.02% Pluronic F127 in Ca^{2+}-free physiological saline solution (0Ca-PSS) containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM glucose, 5 mM Hepes (pH 7.4), 0.2 mM EGTA. To test the effect of external K⁺ on resting Ca^{2+} level, cells were loaded with Fluo3/AM for 1 hour in normal physiological saline solution (N-PSS) containing 140 mM NaCl, 1 mM KCl, 1 mM MgCl₂, 10 mM glucose, 5 mM Hepes (pH 7.4), 1 mM CaCl₂. After Fluo3/AM loading, cells were transferred to 0Ca^{2+}-PSS or N-PSS to remove excessive external Fluo3/AM. The circular discs containing the DLD-1 cells were then pinned in a specially designed chamber. The chamber was placed on the stage of an inverted microscope (Nikon Diaphot 200) and fluorescence signal is recorded by MRC-1000 Laser Scanning Confocal Imaging System with MRC-1000 software (Bio-Rad). The basal cytoplasmic free Ca^{2+} is not drastically higher for cells treated with normal physiological saline solution (N-PSS) compared to those treated with Ca-free solution, probably because the duration of the treatment lasts only for 1 hour. Solution 80K-PSS contains 60 mM NaCl, 80 mM KCl, 1 mM MgCl₂, 10 mM glucose, 5 mM Hepes (pH 7.4), and 1 mM CaCl₂. Solution OCa-80K-PSS contains 60 mM NaCl, 80 mM KCl, 1 mM MgCl₂, 10 mM glucose, 5 mM Hepes (pH 7.4), and 0.2 mM EGTA. Data analysis was performed with Confocal Assistant and MetaFlour (Bio-Rad).

**Results**

The effect of a variety of K⁺ channel blockers on cell proliferation
DLD-1 is colorectal carcinoma cell line of epithelial origin. Many different types of K⁺ channels have been reported in nonexcitable epithelial cells, including Kᵥ (12), Kca (13), and K_ATP (14). To differentiate the types of K⁺ channels critically involved in the proliferation of DLD-1 cell, we examined whether [³H]thymidine incorporation can be influenced by a variety of different K⁺ channel inhibitors. Among these inhibitors, glibenclamide is a well-known inhibitor of K_ATP (15) whereas iberiotoxin and charybdotoxin are specific inhibitors of Kca (16). Table 1 shows that [³H] thymidine incorporation is not influenced by 100 μM glibenclamide or 10 nM charybdotoxin or 10 nM iberiotoxin, indicating that Kca and K_ATP are not essential for the proliferation of DLD-1 cells. [³H] thymidine incorporation is inhibited by 84% in the presence of 5 mM 4-aminopyridine, a known inhibitor for Kᵥ, suggesting a possible role of Kᵥ in cell proliferation. Tetraethylammonium (TEA) and tetrapentylammonium (TPEA) are nonspecific K⁺ channel blockers which inhibit K_Ca, K_ATP, and Kᵥ (17-19). We found that 50 μM TPEA almost

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<td>Effect of K⁺ channel blockers on [³H] thymidine incorporation in cultured carcinoma cell lines.</td>
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<tr>
<td>Control</td>
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<tr>
<td>Glibenclamide (100 μM)</td>
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<td>Charybdotoxin (10 nM)</td>
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<td>Iberiotoxin (10 nM)</td>
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<td>4-AP (5 mM)</td>
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<td>TPEA (50 μM)</td>
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<td>TEA (20 mM)</td>
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Data are presented as means ± S.E in percentage. (n=5-20). Control with no inhibitors treatment is normalized to 100%.
completely blocked cell proliferation while 20 mM TEA inhibited cell proliferation to 66% of its original value. Table 1 shows that [³H]thymidine incorporation is also significantly reduced in the presence of 100 μM verapamil or 100 μM diltiazem. The effect of K⁺ channel inhibitors on cell proliferation was further confirmed by counting the cell number after 30 hours of inhibitor treatment; the cell growth was inhibited to a percentage close to that in [³H]thymidine incorporation study (data not shown). The cultured cells appeared to be healthy after treating with channel inhibitors since cells retained their normal morphology and more than 95% of cells were viable as indicated by 0.2% trypan blue test.

The sensitivity of cell proliferation to channel blockers

To test the sensitivity of cell proliferation to different channel inhibitors, we used different concentrations of inhibitors to examine their effects on [³H] thymidine incorporation. 4-AP, TPeA, diltiazem, and verapamil inhibited [³H] thymidine incorporation in a dose dependent manner with same potency sequences as voltage-gated K⁺ channel blockers (Figure 1) (17-21). 4-AP inhibited [³H] thymidine incorporation in DLD-1 cells with Kᵢ of 3.8 mM while TPeA was a more potent inhibitor with Kᵢ of 5 μM. Kᵢ values of verapamil and diltiazem together with those of 4-AP and TPeA are given in figure legend of Figure 1.

RT-PCR

L-type voltage gated Ca²⁺ channel has been reported in some epithelial cells (21) while it may not exist in other types of epithelial cells (22). Since a number of blockers we used, such as verapamil and diltiazem, could inhibit both L-type calcium channel and voltage-dependent K⁺ channels (11,20,23), we examined the presence of L-type calcium channel with the use of RT-PCR. RT-PCR was performed with RNA isolated from cultured carcinoma cells. A 50 cycles PCR did not yield PCR product in DLD-1 cells while the same procedure amplified a product of expected size (350 bp) in lung squamous carcinoma cells NCI-H520 (Figure 2a). DNA sequencing confirmed that the amplified product represented authentic L-type voltage-gated calcium channel. These results suggest that L-type calcium channel may not be present in DLD-1, while it may exist in other carcinoma cell lines. We used similar strategy to examine the presence of voltage-gated potassium channels. PCR reactions of 50 cycles amplified a 280-bp product from total RNA isolated from DLD-1 cells (Figure 2b). We sequenced the amplified PCR products with ABI 310 Autosequencer. DNA sequencing data confirmed the existence of voltage-gated K⁺ channels.

The effect of external K⁺ on [Ca²⁺]ᵢ

To further examine voltage-gated Ca²⁺ channels in DLD-1 cell, we monitored the change of [Ca²⁺]ᵢ in the presence or absence of high extracellular K⁺ (80 K⁺-PSS). Depolarization with extracellular K⁺ slightly decreased the resting Ca²⁺ level (Figure 3). It also inhibited the increase of [Ca²⁺]ᵢ evoked by extracellular Ca²⁺ (Figure 4). These results further demonstrated that voltage-gated Ca²⁺ channels did not contribute to the [Ca²⁺]ᵢ regulation in DLD-1.
RT-PCR-based detection of mRNA in DLD-1 cells. a: detection of L-type voltage-gated calcium channel in ethidium bromide-stained agarose gel. b: detection of voltage-gated potassium channel in ethidium bromide-stained agarose gel.

Fig. 2

Depolarization with 80 mM K⁺ on resting [Ca²⁺], [Ca²⁺] was monitored in Fluo3/AM loaded DLD-1 external cells. Cells were placed in N-PSS. At time indicated by the arrow, the medium was changed to 80K-PSS. The points represent the mean ± SEM (n=35).

Fig. 3

Depolarization with 80mM K⁺ on the increase of [Ca²⁺], [Ca²⁺] was monitored in Fluo 3/AM loaded DLD-1. Cells were placed in 0Ca-PSS (control) or 0Ca-80K-PSS. At time indicated by the arrow, the media were changed to respective media containing 500uM Ca²⁺. The points represent the mean ± SEM (n =35).

Fig. 4

The effect of K⁺ channel inhibitors on [Ca²⁺]

To explore the mechanism of K⁺ channel involvement in carcinoma cell proliferation, we
examined the effect of K⁺ channel inhibitors on Ca²⁺ influx. After one hour pre-incubation of cultured cells in Ca²⁺-free medium, Ca²⁺ influx was evoked by replacing Ca²⁺-free extracellular medium with that containing 500 μM Ca²⁺. We use TPeA, a potent K⁺ channel blocker, and verapamil, a different class of inhibitor, to examine the effect of K⁺ channel inhibitors on Ca²⁺ influx. We did not use 4-AP since preliminary study revealed that 4-AP itself could cause a transient increase in [Ca²⁺]i presumably through a transient Ca²⁺ release from internal Ca²⁺ store (24). Figure 5a and 5b illustrates that 50 μM TPeA and 100 μM verapamil almost completely abolishes the increase of [Ca²⁺]i evoked by external Ca²⁺. To confirm that the change of [Ca²⁺]i is caused by change in Ca²⁺ influx, we used Ni²⁺, a potent blocker for Ca²⁺ entry that competes for Ca²⁺ binding sites, and NCDC, a blocker for receptor-operated cation channels. 3 mM Ni²⁺ completely eliminated the [Ca²⁺]i increase elicited by external Ca²⁺ (Figure 6a). 3 mM NCDC also blocked the [Ca²⁺]i increase evoked by external Ca²⁺ (Figure 6b).

**Fig. 5**
Effect of K⁺ channel blockers on the increase of [Ca²⁺]i evoked by external Ca²⁺. (a). Effect of TPeA on [Ca²⁺]i. (b). Effect of verapamil on [Ca²⁺]i. [Ca²⁺]i was monitored in Fluo3/AM loaded DLD-1 cells. Cells were placed in OCa-PSS with or without inhibitors. At time indicated by the arrow, the media were changed to respective media containing 500 μM Ca²⁺. The points represent the mean ± SEM (n = 28-32).

**Fig. 6**
Effect of blockers of Ca²⁺ influx on the increase of [Ca²⁺]i evoked by external Ca²⁺. (a). Effect of Ni²⁺ on [Ca²⁺]i. (b). Effect of NCDC on [Ca²⁺]i. [Ca²⁺]i was monitored in Fluo3/AM loaded DLD-1 cells. Cells were placed in OCa-PSS with or without inhibitors. At time indicated by the arrow, the media were changed to respective media containing 500 μM Ca²⁺. The points represent the mean ± SEM. (n = 25-35).

**Discussion**
Colorectal cancer is one of the most frequent cancers. We chose colorectal adenocarcinoma cell line DLD-1 because they were widely used as in vitro model for the study of human colon (25).
This cell line has been proven useful in cell proliferation studies and antitumor drug test (25). We studies the relationship between K\(^+\) channel activity and proliferation of the cells. The cell proliferation was not affected by charybdotoxin or iberiotoxin, at concentrations which were sufficient to completely block K\(_{\text{Ca}}\) (10 nM charybdotoxin or 10 nM iberiotoxin) (Table 1) (16). Therefore, unlike the situation in breast cancer cell line MCF-7, K\(_{\text{ATP}}\) may not be important for the proliferation of colorectal carcinoma cells. K\(_{\text{ATP}}\) was proven to be critical in the proliferation of breast cancer and brain tumor cells, however, treatment by inhibitor of K\(_{\text{ATP}}\) channel glibenclamide up to 100 \(\mu\)M did not reduce the \([^{3}\text{H}]\) thymidine incorporation (Table 1), suggesting that K\(_{\text{ATP}}\) channel was not involved either. In contrast, the cell proliferation was drastically reduced by 4-AP, an inhibitor for voltage-gated K\(^+\) channel, and by TEA and TPeA, nonspecific K\(^+\) channel blockers which inhibit K\(_{\text{Ca}}\), K\(_{\text{ATP}}\) and K\(_{\text{v}}\). As has been noted, K\(_{\text{ATP}}\) and K\(_{\text{Ca}}\) may not be involved in the proliferation of DLD-1 cells, TEA or TPeA probably exert their effect through inhibition of voltage-gated K\(^+\) channel. The presence of voltage-gated K\(^+\) channel is evident by RT-PCR study which is shown in Figure 2b.

\([^{3}\text{H}]\) thymidine incorporation was also reduced in the presence of verapamil and diltiazem (Table 1, Fig.1). Although these chemicals have been widely used as blockers for L-type calcium channel (11), they inhibit voltage-gated potassium channel in a variety of tissues and cells (20, 23). RT-PCR using primers specific to L-type calcium channel did not yield any noticeable PCR product in DLD-1 cell (Fig. 2a). In addition, confocal Ca\(^{2+}\) imaging studies revealed that membrane depolarization by 80 mM K\(^+\) reduced the resting Ca\(^{2+}\) level (Figure 3). Depolarization also blocked the increase of [Ca\(^{2+}\)]\(_i\) evoked by extracellular Ca\(^{2+}\) (Figure 4). These evidences do not support the presence of L-type voltage-gated Ca\(^{2+}\) channel since depolarization could presumably open this channel and cause Ca\(^{2+}\) influx. While these experiments can not exclude the presence of L-type calcium channel, it does suggest that, even if L-type calcium channel exists, the number of functional channel must be very small. It does not contribute to regulation of intracellular Ca\(^{2+}\). Therefore, the decreased proliferation of carcinoma cell DLD-1 in the presence of verapamil and diltiazem is probably caused by the inhibitory effect of these compounds on K\(^+\) channel. The blockage effect of verapamil on the [Ca\(^{2+}\)]\(_i\) increase evoked by extracellular Ca\(^{2+}\) (Figure 5b) may also result from its inhibitory action on voltage-gated K\(^+\) channels. Blockage of K\(^+\) channels could depolarize the membrane and reduce the driving force for [Ca\(^{2+}\)]\(_i\) entry.

One hypothesis for the role of K\(^+\) channels in cell proliferation is that K\(^+\) channels keep the resting membrane potential sufficiently polarized to allow the influx of Ca\(^{2+}\) via membrane ion channels (6). Another possibility is that K\(^+\) current per se is an essential event for cell proliferation (4). The first hypothesis implies that blockage of K\(^+\) channels will directly modulate Ca\(^{2+}\) entry in malignant cells. Nevertheless, no direct evidence has ever been presented to support the relationship between K\(^+\) channel activity and Ca\(^{2+}\) ion entry. Figure 5a and 5b illustrated that inhibition of K\(^+\) channel activities by TPeA and verapamil almost completely blocked the increase of [Ca\(^{2+}\)]\(_i\) evoked by elevated extracellular Ca\(^{2+}\). The change in [Ca\(^{2+}\)]\(_i\) could be due to a change in Ca\(^{2+}\) influx from extracellular medium or an alteration in Ca\(^{2+}\) release from internal store. Two lines of evidence suggest that the inhibitors of K\(^+\) channels modulate Ca\(^{2+}\) influx process: 1) these blockers abolish the change in [Ca\(^{2+}\)]\(_i\) which is evoked by elevated external Ca\(^{2+}\); 2) the change in [Ca\(^{2+}\)]\(_i\) evoked by elevated external Ca\(^{2+}\) is resulted from Ca\(^{2+}\) influx since it can be blocked by membrane depolarization induced by 80 mM external K\(^+\) and it can also be blocked by 3 mM Ni\(^{2+}\), a potent blocker for Ca\(^{2+}\) entry (Figure 6a), and by 3 mM NCDC, a blocker for receptor-operated cation channels (Figure 6b) (26). Through these experiments, a solid linkage has been established between K\(^+\) channel activity and Ca\(^{2+}\) influx. Modulation of Ca\(^{2+}\) influx could then influence the transition from G1 to S during mitosis and affect the cell proliferation (6).

In conclusion, we have demonstrated that voltage-gated K\(^+\) channel is critically involved in the proliferation of colorectal carcinoma cell line DLD-1. It is likely that K\(^+\) channel activity may modulate Ca\(^{2+}\) influx into these cells, and therefore affect the proliferation of these cells.
Acknowledgments

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