



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

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(Received in final form March 12 1999)

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 inhibitor 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_v 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

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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 (K_i) was determined from the least-squares fit of the experimental points by the equation $I_x = I/(1 + X/K_i)$, where I_x 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_v(+) (GCCATCCTG/CAGG/AG/ATC/GAT) and K_v(-) (GCCACCAGA/TAG/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_{v1.1}, K_{v1.3} and K_{v1.5}. Primers LCa(+) (GGGATCCGCACATTGCTCTG) 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 $\alpha 1$ 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.

[Ca²⁺]_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 Ca²⁺-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 Ca²⁺ 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 0Ca²⁺-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 Ca²⁺ 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 0Ca-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 K_v (12), K_{Ca} (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 K_{Ca} (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 K_{Ca} 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 K_v, suggesting a possible role of K_v in cell proliferation. Tetraethylammonium (TEA) and tetrapentylammonium (TPeA) are nonspecific K⁺ channel blockers which inhibit K_{Ca}, K_{ATP}, and K_v (17-19). We found that 50 µM TPeA almost

TABLE 1
Effect of K⁺ channel blockers on [³H] thymidine incorporation in cultured carcinoma cell lines.

	DLD-1
Control	100 ± 7
Glibenclamide (100 µM)	135 ± 37
Charybdotoxin (10 nM)	100 ± 3
Iberiotoxin (10 nM)	105 ± 3
4-AP (5 mM)	16 ± 2
TPeA (50 µM)	4.6 ± 0.1
TEA (20 mM)	66 ± 3
Verapamil	13 ± 2
Diltiazem (24 ± 1

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.

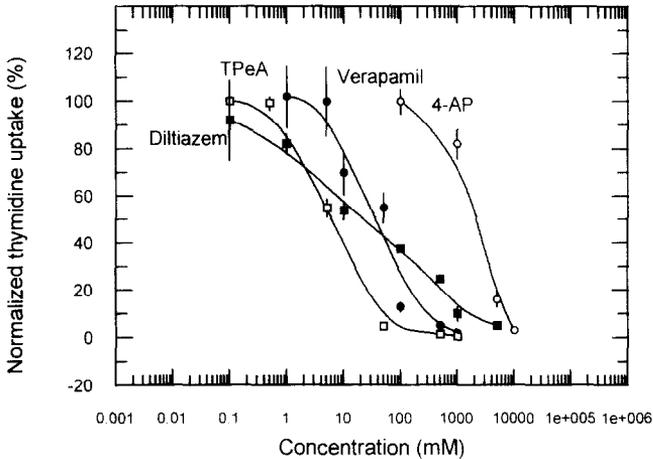


Fig. 1

Ability of different channel blockers to inhibit cell proliferation. The amount of [³H] thymidine incorporation in the absence of blockers was normalized to 100%. Each point represents the mean \pm SEM ($n = 5$). (●), 4-AP; (○), verapamil; (♥), TPeA; (∇), diltiazem. K_i of 3.8 mM for 4-AP; K_i of 5 μM for TPeA; K_i of 60 μM for verapamil; and K_i of 30 μM for diltiazem.

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 K_i of 3.8 mM while TPeA was a more potent inhibitor with K_i of 5 μM. K_i values of verapamil and diltiazem together with those of 4-AP and TPeA are given in figure legends 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.

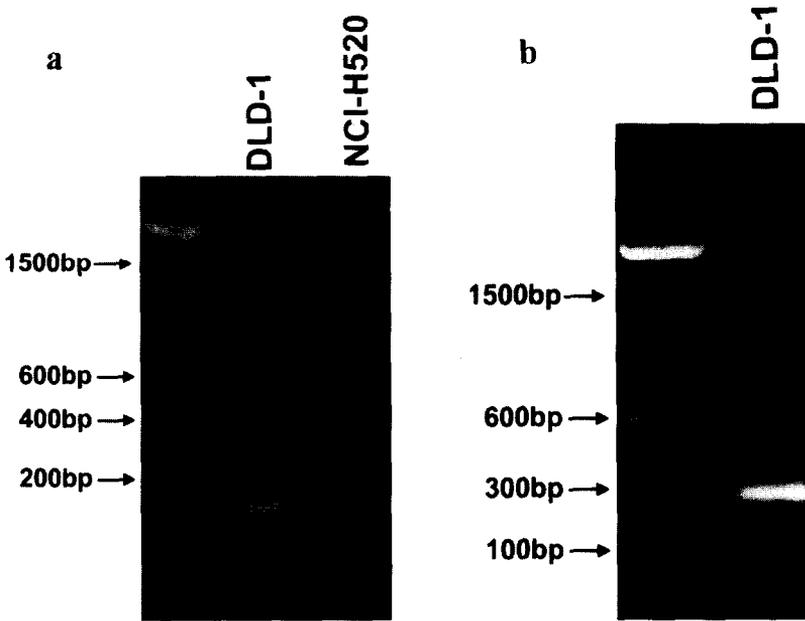


Fig. 2

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.

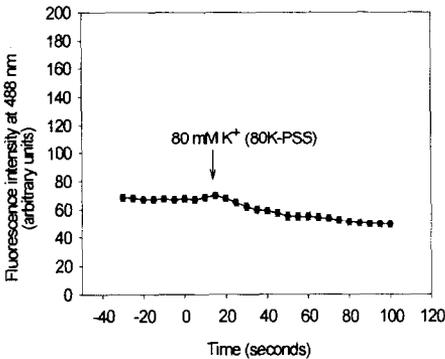


Fig. 3

Depolarization with 80 mM K⁺ on resting [Ca²⁺]_i. [Ca²⁺]_i 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).

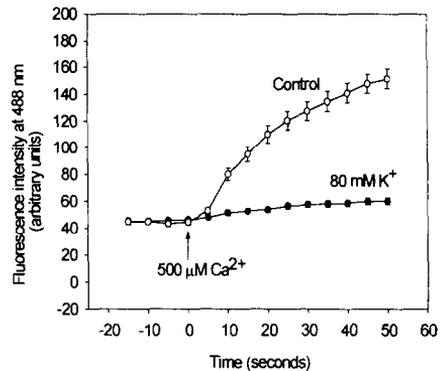


Fig. 4

Depolarization with 80mM K⁺ on the increase of [Ca²⁺]_i evoked by external Ca²⁺. [Ca²⁺]_i was monitored in Fluo 3/AM loaded DLD-1. Cell 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).

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

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).

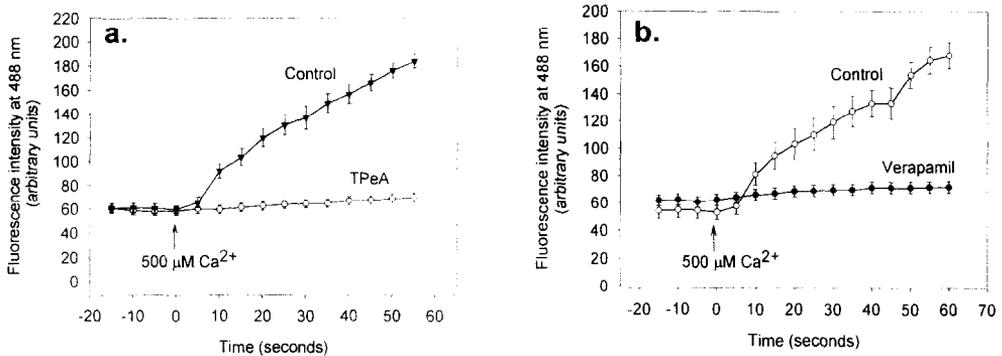


Fig. 5

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 0Ca-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).

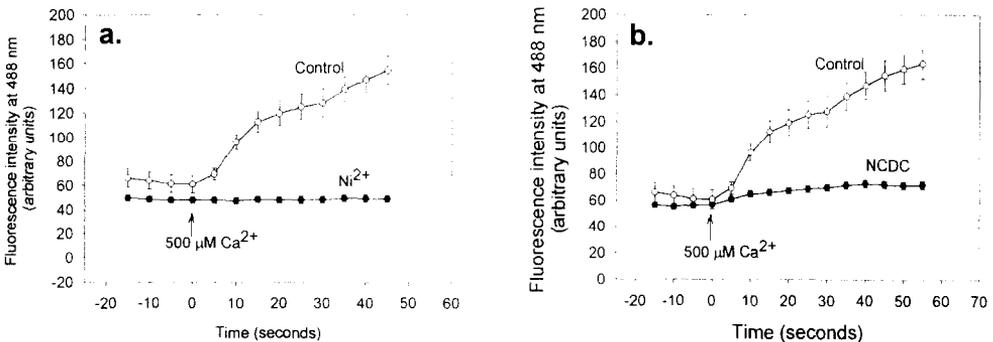


Fig. 6

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 0Ca-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 studied 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_{Ca} (10 nM charybdotoxin or 10 nM iberiotoxin) (Table 1) (16). Therefore, unlike the situation in breast cancer cell line MCF-7, K_{Ca} may not be important for the proliferation of colorectal carcinoma cells. K_{ATP} was proven to be critical in the proliferation of breast cancer and brain tumor cells, however, treatment by inhibitor of K_{ATP} channel glibenclamide up to 100 μM did not reduce the [³H] thymidine incorporation (Table 1), suggesting that K_{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 TPcA, nonspecific K⁺ channel blockers which inhibit K_{Ca}, K_{ATP} and K_v. As has been noted, K_{ATP} and K_{Ca} may not be involved in the proliferation of DLD-1 cells, TEA or TPcA 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.

[³H] 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 Ca²⁺ imaging studies revealed that membrane depolarization by 80 mM K⁺ reduced the resting Ca²⁺ level (Figure 3). Depolarization also blocked the increase of [Ca²⁺]_i evoked by extracellular Ca²⁺ (Figure 4). These evidences do not support the presence of L-type voltage-gated Ca²⁺ channel since depolarization could presumably open this channel and cause Ca²⁺ 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 Ca²⁺. 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_v channel. The blockage effect of verapamil on the [Ca²⁺]_i increase evoked by extracellular Ca²⁺ (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 [Ca²⁺]_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 Ca²⁺ 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 Ca²⁺ entry in malignant cells. Nevertheless, no direct evidence has ever been presented to support the relationship between K⁺ channel activity and Ca²⁺ ion entry. Figure 5a and 5b illustrated that inhibition of K⁺ channel activities by TPcA and verapamil almost completely blocked the increase of [Ca²⁺]_i evoked by elevated extracellular Ca²⁺. The change in [Ca²⁺]_i could be due to a change in Ca²⁺ influx from extracellular medium or an alteration in Ca²⁺ release from internal store. Two lines of evidence suggest that the inhibitors of K⁺ channels modulate Ca²⁺ influx process: 1) these blockers abolish the change in [Ca²⁺]_i which is evoked by elevated external Ca²⁺; 2) the change in [Ca²⁺]_i evoked by elevated external Ca²⁺ is resulted from Ca²⁺ influx since it can be blocked by membrane depolarization induced by 80 mM external K⁺ and it can also be blocked by 3 mM Ni²⁺, a potent blocker for Ca²⁺ 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 Ca²⁺ influx. Modulation of Ca²⁺ 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 Ca²⁺ influx into these cells, and therefore affect the proliferation of these cells.

Acknowledgments

This study was supported by Grant for Research from Chinese University of Hong Kong Research Committee Funding. We would like to acknowledge Dr. Yu Huang for his numerous suggestions, Dr. Michael Tam and Mr. Ho Xian-Hui for their help in [³H] thymidine uptake study.

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