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Research Paper

Effects of Tween 80 on volume-regulated chloride channel and cell proliferation in rat basilar artery smooth muscle cell

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Abstract

Objectives We have previously found that volume-regulated chloride current (VRCC) is involved in cell cycle progression and cell proliferation. This study was to examine the effect of Tween 80, a nonionic surfactant, on VRCC and cell proliferation in rat basilar artery smooth muscle cells (BASMCs).

Methods VRCC was recorded using a whole-cell patch clamp. Cell proliferation and cell cycle were determined by CCK-8, cell count and flow cytometry.

Key findings The results showed that endothelin-1 promotes cell cycle transition from the G0/G1 phase to the S phase and significantly increases VRCC in BASMCs. The effect of Tween 80 on VRCC is reversible and concentration dependent. However, this chemical has no effect on the calcium-activated chloride channel. Tween 80 also concentration-dependently inhibits BASMCs proliferation and arrests cells in the G1/S checkpoint. The antiproliferative effect is paralleled with the inhibitory effect on VRCC.

Conclutision Our study demonstrates that the inhibitory effect of Tween 80 on VRCC contributes importantly to arrest of the cell cycle and prevention of cell proliferation.

Keywords basilar artery; cell proliferation; smooth muscle cells; Tween 80; volume regulated chloride current

Introduction

Tween 80 is an amphipathic nonionic surfactant, composed of fatty acid esters of polyoxyethylene sorbitan,[1] which has been used as a solubilizer and stabilizer. However, the pharmacological effects of this chemical have been essentially ignored for a long time. Recent studies have revealed that Tween 80 may influence ion channel activity. For example, Tween 80 inhibits hERG potassium tail currents in a concentration-dependent manner[2] and lengthens action potential duration (APD) in rat atrial cells.[3] Many recent studies have paid close attention to the roles of Tween 80 in cell growth. It has been shown that Tween 80 decreases concanavalin A-induced proliferative response in human lymphocytes.[4] This effect also occurrs in different tumor cells, including lung carcinoma, colon tumor and stomach tumor cells *in vitro* [5,6] or *in vivo*.[7,8] It is noteworthy that the inhibition effects of Tween 80 on human tumor cell growth can be attained through the induction of apoptosis.[9] Furthermore, Tween 80 can fuse and accumulate in tumor cell membranes, resulting in apoptosis through activation of mitochondria or caspases.[10,11] In addition, Tween 80 can induce EL4 lymphoma cell apoptosis via a 'non-classical' caspase-independent pathway.[12] These results indicate that Tween 80 displays a significant antiproliferative effect, but the detailed mechanism still needs to be elucidated.

Volume-regulated chloride channel (VRCC) is widely expressed in mammalian cells.[13–16] Our previous studies have shown that 4,4-Diisothiocyano-2,2-stilbenedisulfonic acid, the blocker of Cl^- channels, can prevent VSMC proliferation.[17] Furthermore, many recent studies have demonstrated that VRCC is involved in the control of cell cycle and cell proliferation in many cell types, such as rat C6 glioma,[18] T-lymphocytes,[19] endothelial cells [20] and mouse liver cells.[21] The results show that VRCC mediates the process of cell proliferation in a variety of cell types.

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We originally planned to investigate the effects of another drug on VRCC. It was a surprise for us to find that the solvent Tween 80 directly produces an inhibitory effect on VRCC. Because of an important role of VRCC in cell proliferation and the cell cycle, this study therefore was intended to further investigate the effect of Tween 80 on VRCC and cell proliferation in rat basilar artery smooth muscle cells (BASMCs). We found that Tween 80 significantly decreases VRCC activity, arrests cells in the G0/G1 phase and prevents cell proliferation in BASMCs. There is a significantly positive correlation between the inhibitory effects of Tween 80 on VRCC and proliferation. We conclude that Tween 80 inhibits cell proliferation via inhibition of VRCC in BASMCs.

Materials and Methods

All animals were supplied by the Experimental Animal Center of Sun Yat-Sen University in Guangzhou, China. All experimental procedures were performed in accordance with the policies of the Sun Yat-Sen University Animal Care and Use Committee and comformed to the 'Guide for the Care and Use of Laboratory Animals' of the National Institute of Health in China.

Cell culture

Male Sprague–Dawley rats (100–120 g) were anaesthetized with chloral hydrate and decapitated. Basilar arteries were harvested rapidly,[22] and immersed in Kreb's solution containing (in mM): NaCl 137, KCl 5.4, $CaCl_2$ 2.0, $MgCl_2{\cdot}6H_2O$ 1.1, $NaH_2PO_4{\cdot}2H_2O$ 0.4, glucose·H_2O 5.6, $NaHCO_3$ 11.9, penicillin 105 U/l and streptomycin 100 mg/l. Kreb's solution was continuously perfused with 95% $O_2$for 30 min before the experiment. The artery was carefully cleaned of connective tissue and fat tissue. After that, it was cut into small pieces about 2 mm long and placed in Dulbecco's Modified Eagle Medium (DMEM/F12) containing 20% fetal calf serum (FCS). BASMCs were cultured in a humidified atmosphere of 5% CO_2 and 95% O_2 at 37°C and allowed to grow out from the tissues for 1 week. BASMCs in the eighth to twelfth passages were used. BASMCs were confirmed by the positive responses to antibody against smooth muscle α-actin.

Electrophysiological experiments

Cells in a chamber of 500 μl volume were continuously superfused at the rate of 2 ml/min. The Cl^- currents were recorded with an Axopatch 200B amplifier (Axon Instrument, Foster City, CA) using the conventional whole-cell recording technique. Patch pipettes were made from borosilicate glass using a two-stage puller (pp-83, Narishige, Tokyo, Japan) and had resistances of 3–5 Ω when the pipettes were filled with the solution. A 3 mM KCl–agar salt bridge between the bath and the Ag–AgCl reference electrode was used to minimize the changes in liquid-junction potentials. To determine the whole-cell current–voltage curve, the cell was held at −40 mV and test potentials from −100 mV to +120 mV were applied for 400 ms in +20 mV increments at intervals of 5 s. Currents were filtered at a frequency of 2 kHz and digitized at 5 kHz using pCLAMP8.0 software (Axon Instruments). The data were directly entered into the hard drive of a PC-compatible computer. All experiments were performed at room temperature (25°C). In the VRCC recording experiments, the hypotonic bath solution contained (in mM): 107 *N*-methyl-D-glucamine chloride (NMDG-Cl), 1.5 $MgCl_2$, 2.5 $MnCl_2$, 0.5 $CdCl_2$, 0.05 $GdCl_3$, 10 glucose, and 10 Hepes, pH 7.4 adjusted with NMDG. The solution's osmolarity was measured with a freezing point depression osmometer (OSMOMAT030, Germany) and was 230 mosmol/kg·H_2O. A 300 mosmol/kg·H_2O isotonic bath solution was made by adding 70 mM D-mannitol to the hypotonic solution. The pipette solution contained (in mM): 95 CsCl, 20 TEACl, 5 ATP-Mg, 5 EGTA, 5 Hepes, and 80 D-mannitol, pH 7.2 adjusted with CsOH. The osmolarity of this solution was 300 mosmol/kg.H_2O. In the recording CaCC experiments, the bath solution contained (in mM): NMDG·Cl 125, KCl 5, $CaCl_2$ 1.5, $MgSO_4$ 1, HEPES 10, glucose 10, pH 7.4. The pipette solution contained (in mM): CsCl 130, Mg·ATP 1, $MgCl_2$ 1.2, HEPES 10, EGTA 2, pH 7.3 adjusted with CsOH or HCl.

Cell proliferation

CCK-8 assays

The proliferation of BASMCs was estimated by CCK-8 assay according to the manufacturer's guidelines. The 8–12 passage cells were plated in 96-well plates at a density of 4×10^4 cells per well for 24 h and cells were rendered quiescent at the G_0/G_1 phase by addition of 0.5% FCS for 24 h. Quiescent cells were then incubated with or without various concentrations of treating agents for another 48 h. Tween 80 was added 0.5 h prior to endothelin-1 (ET-1). At the end of each time point, 10 μl cck-8 was added to each well, and the plates were incubated for an additional 2 h at 37°C. The absorbance of each plate was measured at 450 nm (absorbance) and 600 nm (background) with a spectrophotometer. The absorbance, $OD_{450\,nm}$–$OD_{600\,nm}$, has a direct correlation with the number of cells in the well. BASMCs alone were used as the control group (cell viability of the control group was taken as 100%). The inhibition of ET-1-induced BAVSMC proliferation by Tween 80 was expressed as the ratio of absorbance of the Tween 80 treated groups and that of the ET-1 treated group.

Cell counts assays

The 8–12 passage cells were incubated in six-well plates at a density of 2×10^5 cells per well for 24 h and cells were rendered quiescent at the G_0/G_1 phase by 0.5% FCS for 24 h. Quiescent cells were then incubated with or without various concentrations of treating agents for another 48 h. Tween 80 was added 0.5 h prior to ET-1. After treatment for 48 h the cells were harvested and counted by haemacytometer.

Flow cytometry for cell cycle analysis

Cell cycle status was evaluated by flow cytometry as previously described.[23] Cultured BASMCs were rendered quiescent and synchronized at G_0/G_1 phase by 0.5% FCS for 24 h. Then the drugs were added to the medium and Tween 80 was added 30 min prior to ET-1. After 48 h, BASMCs were collected from the medium by centrifugation at 3000 rpm for 5 min at 4°C. Pellets were rinsed with ice-cold PBS three times and fixed with 70% ethanol for 24 h. Samples were stained with staining buffer (PBS containing 50 μg/ml of propidium iodide, 10 μg/ml RNase A, 0.1% sodium citrate and 0.1% TritonX-100) for 30 min at room temperature and then filtered through 40-μm diameter mesh. DNA content was

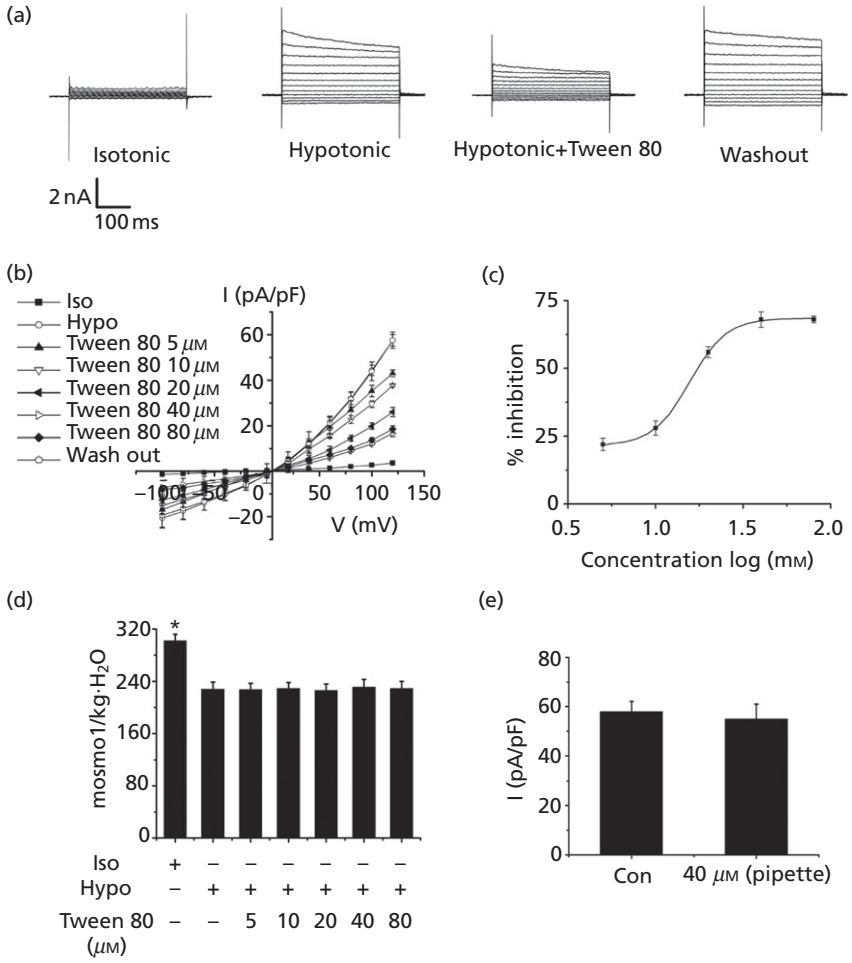

Figure 1 Effect of Tween 80 on VRCC in BASMCs. (a) Representative traces of VRCC currents induced by isotonic (isotonic), hypotonic (hypotonic), hypotonic + 20 μM Tween 80 (hypotonic + Tween 80) solutions and washing out Tween 80 (washout). (b) I-V curves for VRCC currents under isotonic solutions (iso), hypotonic solutions (hypo), the treatment of 5, 10, 20, 40, 80 μM Tween 80 ($n = 6$, $P < 0.05$ vs hypo) and washing out Tween 80 (washout). (c) Concentration response curve for inhibition of VRCC by Tween 80. The value of IC50 for inhibition of VRCC by Tween 80 was 22.5 μM (each point value from six different experiments). (d) Osmotic pressure in presence and absence of Tween 80 at different concentrations tested. ($n = 6$, $^*P < 0.01$ vs hypo). (e) Intracellular (pipette) effect of Tween 80 on VRCC ($n = 6$).

analysed by a flow cytometer in order to characterize the population fractions in each phase of the cell cycle.

Statistical analysis

All data were expressed as mean ± SEM. Statistical analyses were performed using Student's *t*-test, Dunnett's test or ANOVA. $P < 0.05$ was considered significant.

Reagents

Tween 80, *N*-methyl-D-glucamine was obtained from Sigma (St Louis, MO). FCS and DMEM/F12 were obtained from Gibco.

Results

Effect of Tween 80 on VRCC in BASMCs

Hypotonic solution evoked a large outward rectifying VRCC current in BASMCs, which is consistent with our previous report in A10 vascular smooth muscle cells.[24] First, we observed the effect of Tween 80 on VRCC current at different concentrations. It was found that the effective concentration range was from 5 to 80 μM. At a concentration of 80 μM, Tween 80 produced its maximum inhibitory effect on the current, therefore we used this concentration range to investigate Tween 80's effect on VRCC current. Figure 1 shows that Tween 80 inhibits VRCC current in a concentration-dependent manner. This effect is completely reversed after washing out the Tween 80 (Figure 1a and b). At concentrations of 5, 10, 20, 40 and 80 μM, Tween 80 reduces the VRCC current densities from −20.6 ± 4.4 pA/pF to −16.9 ± 1.9, −14.1 ± 4.0, −12.0 ± 2.3, −7.2 ± 3.2 and −8.0 ± 1.0 pA/pF, respectively, at −100 mV ($n = 6$, $P < 0.05$ vs VRCC in hypotonic solutions), and from 44.4 ± 2.5 pA/pF to 35.3 ± 2.1, 29.5 ± 1.4, 19.8 ± 1.4, 12.0 ± 1.0 and 13.7 ± 1.1 pA/pF, respectively, at +100 mV ($n = 6$, $P < 0.05$ vs VRCC in hypotonic solutions). The evaluative value of IC50 for the inhibition by Tween 80 was 22.5 uM (Figure 1c).

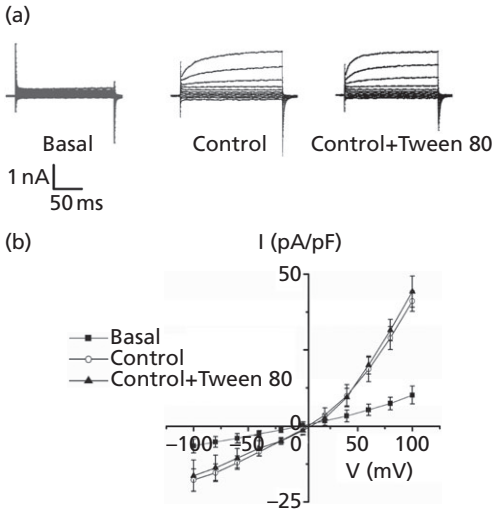

Figure 2 Effect of Tween 80 on CACC. (a) Representative current traces in normal solution (basal), the current is activated by 10 μM ATP (control) and the treatment of 80 μM Tween 80 (control + Tween 80). (b) I-V curves for CACC in A (each point value from six different experiments).

Effect of Tween 80 on CaCC current in BASMCs

To certify the specificity of Tween 80 on VRCC, we tested the effect of Tween 80 on CaCC, which is another chloride channel with different current characteristics from VRCC. The result showed that Tween 80 does not significantly alter CaCC (Figure 2). At +100 mV, the current densities of CaCC evoked by ATP were 44.2 ± 5.1 and 41.1 ± 3.3 pA/pF with or without Tween 80, respectively ($n = 6$, $P > 0.05$).

Effects of Tween 80 on ET-1 induced cell proliferation

Because, as in the above result, Tween 80 reduces the VRCC current, and VRCC is closely associated with ET-1-induced VSMC proliferation,[17,25] we investigated whether Tween 80 affects ET-1-induced proliferation in BASMCs. The results show that 10 nM ET-1 increases cell viability by 36% and the cell number by 34%, respectively. The treatment of Tween 80 with 10, 20, 40, 80 μM for 48 h reduced cell viability (percentage of control) from 136 ± 8% to 125 ± 4%, 117 ± 4%, 114 ± 5% and 111 ± 5%, respectively ($n = 7$, $P < 0.01$; Figure 3a), and the cell number (percentage of control) from 134 ± 7% to 122 ± 6%, 116 ± 5%, 113 ± 5% and 109 ± 4%, respectively ($n = 7$, $P < 0.01$; Figure 3b). However, Tween 80 did not affect cell viability and cell number of BASMCs in the absence of ET-1 ($n = 7$, $P > 0.05$; Figure 3c and d). Tween 80 produced an inhibition effect on BASMCs proliferation in a concentration-dependent manner with an evaluative IC50 value of 26.2 uM. The anti-proliferative effect was paralleled by the inhibition effect on VRCC. There was a significantly positive correlation between the effects of Tween 80 on VRCC and cell proliferation ($r = 0.97$, $P < 0.01$; Figure 3e).

Tween 80 prevented ET-1 induced cell cycle progression in BASMCs

Recent studies have implied that VRCC is critical to cell-cycle progression,[26], and our results revealed that Tween reduces VRCC current and cell proliferation, therefore we used flow cytometry to examine whether cell-cycle progression is affected by Tween 80. It was found that Tween 80 concentration-dependently suppresses the cell-cycle progression induced by ET-1. We therefore chose the maximum concentration of 80 μM to determine the effect of Tween 80 on cell-cycle progression. As shown in Figure 4, ET-1 promotes the cell cycle transition from G0/G1 phase to S phase ($n = 6$, $P < 0.05$ vs control). Treatment with 80 μM Tween 80 significantly increases the percentage of the cells at the G0/G1 phase from 67 ± 5% to 80 ± 5% ($n = 6$, $P < 0.05$ vs ET-1), and significantly diminishes the percentages of the cells at S phase and G2/M phase from 22 ± 2% to 14 ± 3% and from 12 ± 3% to 6 ± 2%, respectively ($n = 6$, $P < 0.05$ vs ET-1).

Comparison of VRCC in non-proliferative and proliferative group

To further confirm the role of VRCC in the process of cell proliferation, we compared VRCC current in non-proliferative (without treatment of ET-1) and proliferative (treatment with ET-1) cells. As shown in Figure 5, the VRCC current densities were 45 ± 4.9, 68 ± 5.8 ($n = 6$, $P < 0.05$ vs non-proliferative cells) and 46 ± 6.1 pA/pF ($n = 6$, $P < 0.05$ vs proliferative cells) at +100 mV in non-proliferative cells. These results suggest that VRCC activity is high in the cell proliferative state.

Discussion

In this study, we found that Tween 80 produces a reversible and concentration-dependent inhibitory effect on VRCC in BASMCs. This effect is due to extracellular action, not intracellular action, because addition of 40 μM Tween 80 in pipette solution did not influence the VRCC current (Figure 1d). It is well known that VRCC is regulated by osmotic pressure, therefore we measured whether addition of Tween 80 changed the osmotic pressure in the bath solution. The results showed that Tween 80 does not change the osmolarity of the solution (Figure 1e), indicating that the inhibition effect on VRCC is not related to the alteration of the solution's osmotic pressure. We also excluded the possibility that Tween 80's effect on VRCC is attributable to damage of the cell membrane because the inhibitory effect on VRCC was completely reversed through washout of the Tween 80. Furthermore, Tween 80 did not alter the activity of CACC, another kind of Cl^- channel, in the same concentration range. It appears that Tween 80's effect on VRCC is selective. Our earlier study indicated that the nature of VRCC is ClC-3, in vascular smooth muscle.[24] The high affinity block of Tween 80 on VRCC may be due to specific direct hydrophobic interactions with the ClC-3 within the membrane bilayer and changes to the conformation of the ClC-3 channel protein, which is associated with channel activity.[27–29] Although the exact mechanism is unclear, we have found that Tween 80 can inhibit VRCC, therefore Tween

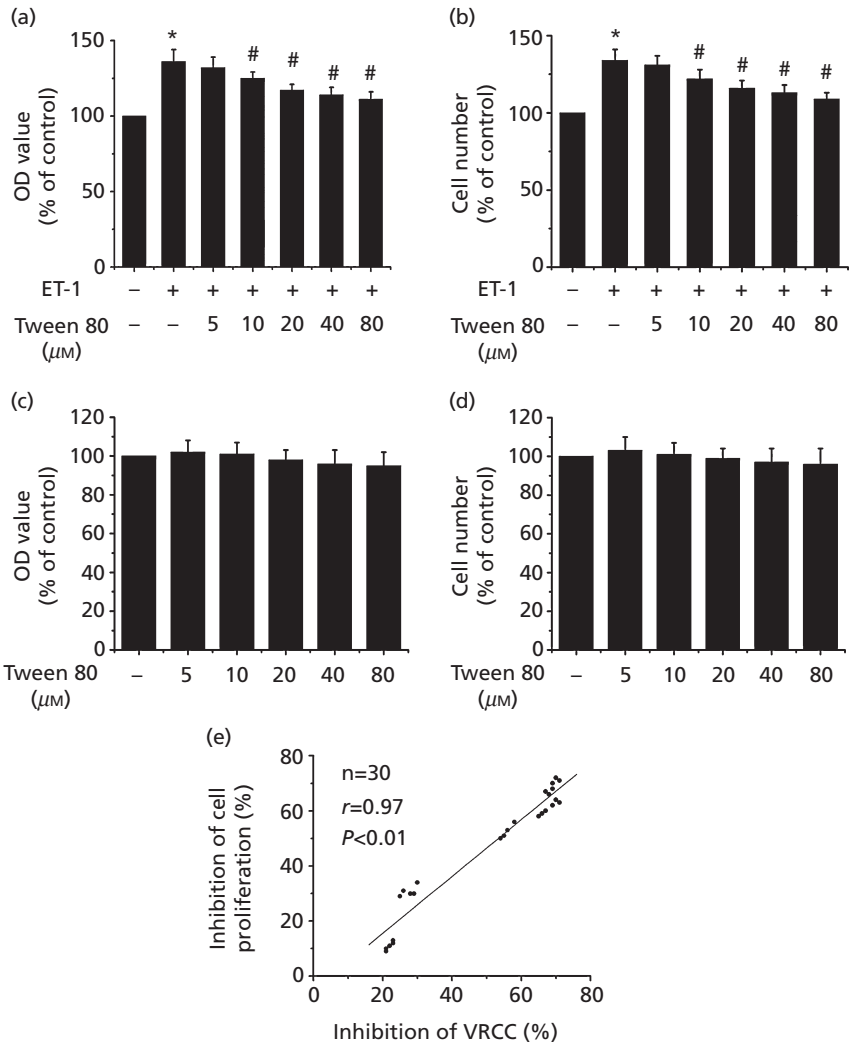

Figure 3 Inhibition of Tween 80 on ET-1 induced cultured BASMCs proliferation. (a, c) Effect of Tween 80 on the cell viability in presence (a) and absence of ET-1 (c). (b, d) Effect of Tween 80 on the cell number in presence (b) and absence of ET-1(d). The cell viability and the cell number in the control group were considered to be 100% ($n = 7$, $^{*}P < 0.01$ vs control group, $^{\#}P < 0.01$ vs ET-1 group). (e) Correlation between inhibition of VRCC and inhibition of cell proliferation by Tween 80 in BASMCs ($n = 6$, $r = 0.97$, $P < 0.01$).

80 should be avoided when conducting electrophysiological in-vitro assessments of drug effects.

During the early phase of cell proliferation, most cells swell and cell volume increases in the cell cycle.[30, 31] This usually initiates the regulatory volume decrease process through activation of ion channels and transporters, including chloride and potassium channels, which evokes effluxes of Cl^-, K^+ and H_2O, and the cell volume returns to normal.[21, 25] Activation of VRCC therefore plays an essential role in cell proliferation. Our present results show that Tween 80 concentration-dependently inhibits VRCC activity and cell proliferation in BASMCs. The antiproliferative effect of Tween 80 is paralleled by the inhibition of VRCC. The evaluative value of IC50 for inhibition of VRCC was 22.5 μM, which is close to the IC50 value of 26.2 μM for inhibition of cell proliferation. There was a significant positive correlation between the inhibitions of both cell proliferation and VRCC. These results strongly suggest that the inhibition of the cell proliferation by Tween 80 is related to the blockage of VRCC.

BASMCs proliferation is governed by passage through cell cycle. Proliferating cells pass through several cell-cycle checkpoints, mainly the G1 to S and G2 to M transitions.[32] The two checkpoints are considered to be important in the replication of DNA.[33] Recent studies have found that VRCC varies in different cell cycle phases, and Cl^- channel blockers suppress cell proliferation and arrest cells in the G0/G1 phase.[34–36] These data indicate that VRCC also plays a critical role in regulation of cell cycle and cell proliferation.[18, 37, 38] In this study, we found that ET-1 promotes the cell cycle transition from the G0/G1 phase to the S phase, resulting in BASMCs proliferation. VRCC was significantly increased in ET-1-induced rapid proliferative BASMCs. The results suggest that the VRCC may ensure the concentration of critical factors needed for controlling progress through the restriction point in the course of cell proliferation. More recent

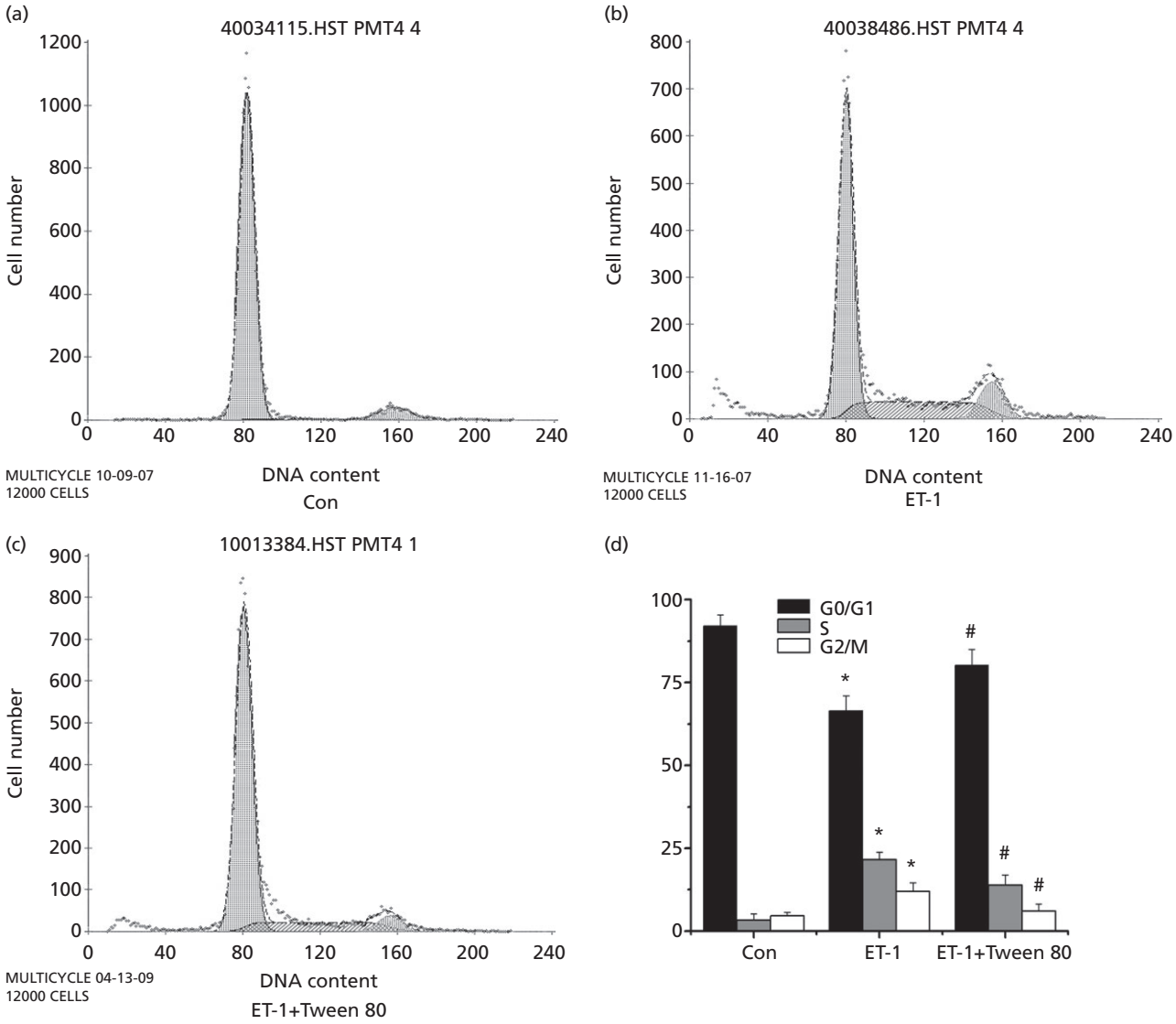

Figure 4 Tween 80 caused cell cycle arrest in BASMCs analysed by flow cytometer. (a, b, c) Representative images of cell cycle analysis in control group (a), ET-1 induced proliferative group (b) and in the presence of Tween 80 (c). (d) Statistical analysis shows the percentage distribution of cells in G0/G1, S, G2/M stages of the cell cycle in control group (con), ET-1 induced proliferative group (ET-1) and in the presence of Tween 80 (ET-1 + Tween 80) ($n = 6$, $^{*}P < 0.05$ vs con; $^{\#}P < 0.05$ vs ET-1).

evidence indicates that the activity of VRCC is critical for G1/S checkpoint progression. Thus, we speculated that Tween 80 may affect BASMC proliferation through alterations in the cell cycle progression. Indeed, our data from flow cytometry show that Tween 80 blocks cell cycle progression at the G1/S transition and stops BASMCs entering the S phase from G0/G1, consequently inhibiting the replication of DNA and the proliferation of BASMCs. It is also clear that pharmacological inhibition of Cl^- channels causes arrest of human cervical cancer cells in the G0/G1 stage, thus preventing G1/S checkpoint progression,[39] which in turn disturbs cell proliferation. These results support the hypothesis that Tween 80 inhibits VRCC, which is required for G1/S checkpoint progression, preventing cells from progressing into the S phase and resulting in the blockage of cell proliferation.

Conclusions

Our data show that Tween 80 can inhibit VRCC and arrest the cell cycle at the G1/S checkpoint, which, in turn, prevents cell proliferation in BASMCs. We therefore conclude that the inhibiting effect of Tween 80 on VRCC contributes significantly to the inhibition of cell proliferation.

Declarations

Competing interests

The Author(s) declare(s) that they have no conflicts of interest to disclose.

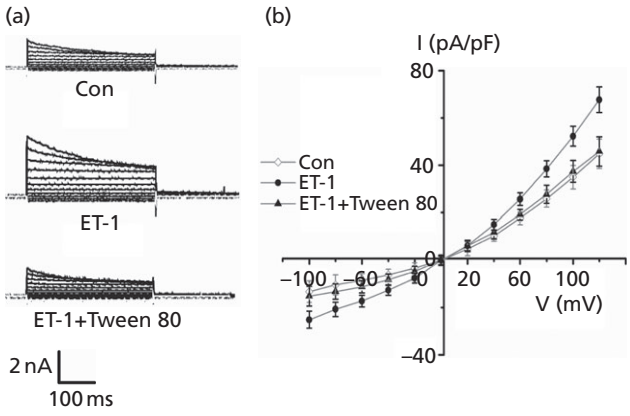

Figure 5 Comparison of VRCC in non-proliferative and proliferative group. (a) Representative current traces from control group (Con), ET-1 induced proliferative group (ET-1) and treated by 80 μM Tween 80 group (ET-1 + Tween 80). (b) I-V curves for VRCC in control group (Con), ET-1 induced proliferative group (ET-1) ($n = 6$, $P < 0.05$ vs con) and treated by 80 μM Tween 80 group (ET-1 + Tween 80) ($n = 6$, $P < 0.05$ vs ET-1).

Funding

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