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Cell cycle-dependent expression of potassium channels and cell proliferation in rat mesenchymal stem cells from bone marrow

X. L. Deng*,‡, C. P. Lau*, K. Lai*, K. F. Cheung*, G. K. Lau* and G. R. Li*,†

*Department of Medicine, and Research Centre of Heart, Brain, Hormone and Healthy Ageing, †Department of Physiology, Li Ka Shing Faculty of Medicine, University of Hong Kong, Pokfulam, Hong Kong and ‡Department of Physiology and Pathophysiology, Medical School of Xi'an Jiaotong University, Xi'an, China

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Abstract. *Objective*: Recently, our team has demonstrated that voltage-gated delayed rectifier K^+ current (IK_{DR}) and Ca^{2+}-activated K^+ current (I_{KCa}) are present in rat bone marrow-derived mesenchymal stem cells; however, little is known of their physiological roles. The present study was designed to investigate whether functional expression of IK_{DR} and I_{KCa} would change with cell cycle progression, and whether they could regulate proliferation in undifferentiated rat mesenchymal stem cells (MSCs). *Materials and Methods*: Membrane potentials and ionic currents were recorded using whole-cell patch clamp technique, cell cycling was analysed by flow cytometry, cell proliferation was assayed with DNA incorporation method and the related genes were down-regulated by RNA interference (RNAi) and examined using RT-PCR. *Results*: It was found that membrane potential hyperpolarized, and cell size increased during the cell cycle. In addition, IK_{DR} decreased, while I_{KCa} increased during progress from G_1 to S phase. RT-PCR revealed that the mRNA levels of Kv1.2 and Kv2.1 (likely responsible for IK_{DR}) reduced, whereas the mRNA level of KCa3.1 (responsible for intermediate-conductance I_{KCa}) increased with the cell cycle progression. Down-regulation of Kv1.2, Kv2.1 or KCa3.1 with the specific RNAi, targeted to corresponding gene inhibited proliferation of rat MSCs. *Conclusion*: These results demonstrate that membrane potential, IK_{DR} and I_{KCa} channels change with cell cycle progression and corresponding alteration of gene expression. IK_{DR} and intermediate-conductance I_{KCa} play an important role in maintaining membrane potential and they participate in modulation of proliferation in rat MSCs.

INTRODUCTION

Ion channels play important roles in maintaining physiological homeostasis. In proliferative cells, ion channels have been shown to participate in cell proliferation (see review, Wonderlin & Strobl 1996). Recent studies have demonstrated that ion channels modulate the progression of the cells

Correspondence: Gui-Rong Li, L8-01, Laboratory Block, Faculty of Medicine Building, The University of Hong Kong, 21 Sassoon Road, Pokfulam, Hong Kong SAR, China. Tel.: +852-2819-9513; Fax: +852-2855-9730; E-mail: grli@hkucc.hku.hk

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through the cell cycle, and that K^+ channel expression changes with different stages in spinal cord astrocyte cycling (MacFarlane & Sontheimer 2000). Blockade of K^+ channels has been shown to be antiproliferative for numerous types of cells including T-lymphocytes (DeCoursey *et al*. 1984), vascular smooth muscle cells (Grgic *et al*. 2005) and cancer cells (Ouadid-Ahidouch *et al*. 2004b), etc.

Mesenchymal stem cells (MSCs) from bone marrow of various species (e.g. mice, rats and humans) exhibit multilineage potential to incorporate into a variety of tissues (Caplan & Bruder 2001; Jiang *et al*. 2002; Reyes *et al*. 2002; Pittenger & Martin 2004), including bone, cartilage, muscle and lung by *in vivo* transplantation (Pittenger *et al*. 1999; Deans & Moseley 2000; Jiang *et al*. 2002; Zhao *et al*. 2002), and to form a variety of cell types *in vitro*, for example, hepatocytes, cardiomyocytes and neuronal cells (Caplan & Bruder 2001; Reyes *et al*. 2001; Jiang *et al*. 2002). In addition, transplantation of MSCs into infarcted myocardium has been found to improve heart function significantly in experimental studies (Tomita *et al*. 1999; Orlic *et al*. 2001; Sussman 2001). Thus, it is believed that MSCs are an ideal cell source for regeneration of the myocardium (Caplan & Bruder 2001; Reyes *et al*. 2001; Cahill *et al*. 2003; Pittenger & Martin 2004). Recent studies from ours and other groups have demonstrated that multifunctional ion channels were heterogeneously expressed in undifferentiated human (Kawano *et al*. 2003; Heubach *et al*. 2004; Li *et al*. 2005), rat (Li *et al*. 2006) and rabbit (Deng *et al*. 2006) MSCs. We found that the delayed rectifier K^+ current (IK_{DR}) (likely to be encoded by Kv1.2 and Kv2.1) and Ca^{2+}-activated K^+ current (I_{KCa}) (likely to be encoded by KCa3.1 and KCa1.1) were major ion channel currents in rat MSCs. IK_{DR} was present in almost all rat MSCs, while I_{KCa} were observed in one-third of rat MSCs (Li *et al*. 2006). However, little is known regarding the mechanism underlying heterogeneous expression of ion channels and the biological and physiological roles of these ion channels in rat MSCs. The present study was therefore designed to investigate whether IK_{DR} and I_{KCa} would change during cell cycle progression, and whether they could regulate cell proliferation in undifferentiated rat MSCs.

MATERIALS AND METHODS

Isolation and culture of rat MSCs

Rat MSCs were isolated from the bone marrow of Sprague–Dawley rats (150–200 g, either sex) using a modified procedure described previously (Li *et al*. 2006). Guidelines for animal care and use from the Committee on the Use of Animals in Teaching and Research, University of Hong Kong, were followed. Briefly, rat MSCs that adhered to the flask bottom gradually proliferated to form colonies in Iscove's modified Dulbecco's medium (IMDM; Sigma-Aldrich Chemicals, St Louis, MO, USA), 10% foetal bovine serum (FBS, Invitrogen, Hong Kong, China), antibiotics (100 U/mL penicillin G, 100 μg/mL streptomycin sulphate, 0.25 μg/mL amphotericin B; Invitrogen) and 10 ng/mL leukaemia inhibition factor (Invitrogen) until they reached 80–90% confluence. The cells were then detached from the flasks by trypsinization, were centrifuged at 170 **g** for 8 min and were suspended in the medium for continuous culture or ionic current recording. For ion current studies, detached cells were transferred to a cell chamber for 15–20 min, and were allowed to attach to the bottom of the cell chamber. Subsequently, these cells were superfused with normal Tyrode solution (1.5 mL/min).

Synchronization of rat MSCs and flow cytometric analysis

Rat MSCs (passages from 2 to 5) were synchronized in the cell cycle using a procedure described previously by Ouadid-Ahidouch *et al*. (2004b). Briefly, cells were plated initially in

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25 cm^2 flasks in IMDM containing 10% FBS for 24 h, and then were synchronized to early G_1 (i.e. to G_0/G_1 phase) by starving them for 24 h using IMDM medium containing 0.5% FBS. By returning to 10% FBS in the medium for 8–10 h, cells were progressed to G_1 phase. To synchronize the cells at the end of G_1 phase, 2 mM thymidine (Sigma-Aldrich Chemicals) added to the culture medium containing 10% FBS for 24 h and finally the cells were allowed to progress to S phase by removing the thymidine for 8–10 h.

The flow cytometric analysis was performed on the MSCs from different cycling phases. Cells were harvested by trypsinization at the end of each treatment, washed with PBS and were fixed in ice-cold 70% ethanol at 4 °C for 4 h, followed by centrifuging cell pelleting at 200 ***g*** for 5 min and washing with PBS to remove the fixative. Cells were then suspended in 1 mL propidium iodide/Triton X-100 staining solution with RNase A (final concentration 20 μg/mL propidium iodide) and were incubated for 30 min at room temperature. Stained cells were then analysed using a flow cytometer (Cytomics FC 500, Beckman, Fullerton, CA, USA) as described previously (Collecchi *et al*. 2000; Tadi *et al*. 2005) to measure cellular DNA content. The data were stored on a compatible IBM PC computer, and were analysed using ModFit software for cell cycle distribution patterns (G_0/G_1, S and G_2/M phases).

Electrophysiology

Mesenchymal stem cells from passages 2–5 or from different cell cycle phases were used for ion current studies with the whole-cell patch-clamp technique, as previously described (Li *et al*. 2005, 2006). Tyrode solution contained: 136 mM NaCl, 5.4 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, 0.33 mM NaH_2PO_4, 10 mM glucose and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; pH adjusted to 7.4 with NaOH. The pipette solution contained (mM): 20 mM KCl, 110 mM K-aspartate, 1.0 mM $MgCl_2$, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.05 mM ethyleneglycoltetraacetic acid, 0.1 mM GTP, 5.0 mM Na_2-phosphocreatine, 5.0 mM Mg_2-ATP; pH adjusted to 7.2 with KOH. The experiments were conducted at room temperature (21–22 °C).

Reverse transcriptase-polymerase chain reaction

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed by the procedure as described previously (Li *et al*. 2005, 2006). Briefly, total RNA was isolated using the TRIzol method (Invitrogen) from cell cycle phases of the MSCs or from RNAi-treated MSCs. Reverse transcription was performed using the RT system (Promega, Madison, WI, USA) protocol in a 20-μL reaction mixture. Then, the polymerase chain reaction was conducted with primers of Kv1.2 (accession no. NM_012970, sense: GAGATGTTTCGGGAGGATGA; antisense: CTCTGTCCCCAGGGTGATAA), Kv2.1 (accession no. NM_013186, sense: GCTGCAGAGCCTAGACGAGT; antisense: TGCTTTTGAACTTGGTGTCG), KCa1.1 (accession no. AF135265, sense: TGTGGGCTCCATCGAGTA; antisense: GCTTAGCGAGTTCCGTGA) and KCa3.1 (accession no. NM_023021, sense: CACGCTGAGATGTTGTGGTT; antisense: CGATGCTGCGGTAAGACG) to amplify corresponding cDNA. PCR was performed using the Promega PCR system with Taq polymerase and accompanying buffers. The cDNA at 3 μL aliquots was amplified by a DNA thermal cycler (Mycycler; Bio-Rad, Hercules, CA, USA) in a 25-μL reaction mixture. PCR products were electrophoresed through a 1.5% agarose gel, and amplified cDNA bands were visualized by ethidium bromide staining. The bands, imaged by Chemi-Genius Bio Imaging System (Syngene, Cambridge, UK), were analysed using GeneTools software (Syngene). Amplified cDNA levels of the genes were expressed as relative values to house keeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (accession no. NM_017008, sense: GTGCTGAGTATGTCGTGGAG; antisense: GTCTTCTGAGTGGCAGTGAT). When the cDNA was replaced by the RNA sample, no significant bands were detected (Li *et al*. 2006).

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RNA interference

Stealth^{TM} RNAi molecules targeted to specific K^+ channels were purchased from Invitrogen Life Technology (Invitrogen) (Wu *et al*. 2006). Sense RNA sequences of Stealth^{TM} RNAi molecules were as follows: 5′-AAAUAGACAGCACUAGAGAAGAGGA-3′ for Kv1.2; 5′-UGCUAGUGCUGUGUGUUUCUCAGGG-3′ for Kv2.1; 5′-UCCCUCCCGUGUUUGUGUCUGUAUA-3′ for KCa1.1 and GCCACUGGUUCGUGGCCAAACUAUA for KCa3.1. In addition, Silencer^{R} GAPDH siRNA (Ambion, Austin, TX, USA) was used as the positive control. Stealth^{TM} RNAi molecules at 100 nM were transfected into the MSCs at 60% confluence, using Lipofectamine^{TM} 2000 reagent (Invitrogen) for 48 h according to the manufacturer's instructions. Stealth^{TM} RNAi of medium GC content (Invitrogen), which had no known target in mammalian genomes, was used as the control. Transfected cells were used for cell proliferation assay and/or RNA extraction. Transfection efficiency was monitored using fluorescent RNA duplex (Invitrogen) according to the manufacturer's instructions.

Cell proliferation assay

The cell proliferation assay was performed on the MSCs by determining incorporation level of [^{3}H]-thymidine into DNA with a modified procedure as described previously (Wu *et al*. 2006). Briefly, the MSCs were seeded in 96-well plates at a density of 2×10^4 cells/well in antibiotic-free IMDM containing 10% FBS for 24 h, and was incubated in IMDM containing 10% FBS and ion channel blockers or specific Stealth^{TM} RNAi ion channels for 24 h, and then was exposed to [^{3}H]-thymidine (0.5 μCi/well) for an additional 24 h. The level of [^{3}H]-thymidine incorporation was finally assayed with TopCount·NTX^{TM} microplate scintillation and luminescence counter (PerkinElmer Life and Analytical Sciences, Boston, MA, USA).

Statistical analysis

Results are presented as means ± SEM. Paired and/or unpaired Student's *t*-tests were used as appropriate to evaluate the statistical significance of differences between two group means, and analysis of variance (ANOVA) was performed for multiple groups. Values of $P < 0.05$ were considered to indicate statistical significance.

RESULTS

Pharmacological separation of K^+ currents in rat MSCs

Our previous studies have shown that IK_{DR} were present in almost all of rat MSCs, and I_{KCa} was seen in one-third of them (Li *et al*. 2006). IK_{DR} was sensitive to inhibition by 4-aminopyridine (4-AP) or tetraethylammonium, while I_{KCa} was mostly blocked by the intermediate-conductance K_{Ca} channel blocker clotrimazole. In addition, a small portion of iberiotoxin (a blocker of high-conductance I_{KCa})-sensitive high-conductance I_{KCa} was detected only in a small population of cells (Li *et al*. 2006). We therefore used 4-AP and clotrimazole to separate IK_{DR} and/or I_{KCa} to study changes in these two types of currents during cell cycle progression. In addition, iberiotoxin was also employed in further cases.

Figure 1a displays membrane currents recorded in an MSC, with the voltage protocol as shown in the inset. A gradually activating IK_{DR} and noisy oscillation like I_{KCa} were observed, indicating that the two components of outwards currents were copresent in this cell. IK_{DR} was significantly inhibited by 5 mM 4-AP (Sigma-Aldrich Chemicals), while the remaining I_{KCa} was suppressed by co-application of 5 mM 4-AP and 1 μM clotrimazole (Sigma-Aldrich Chemicals).

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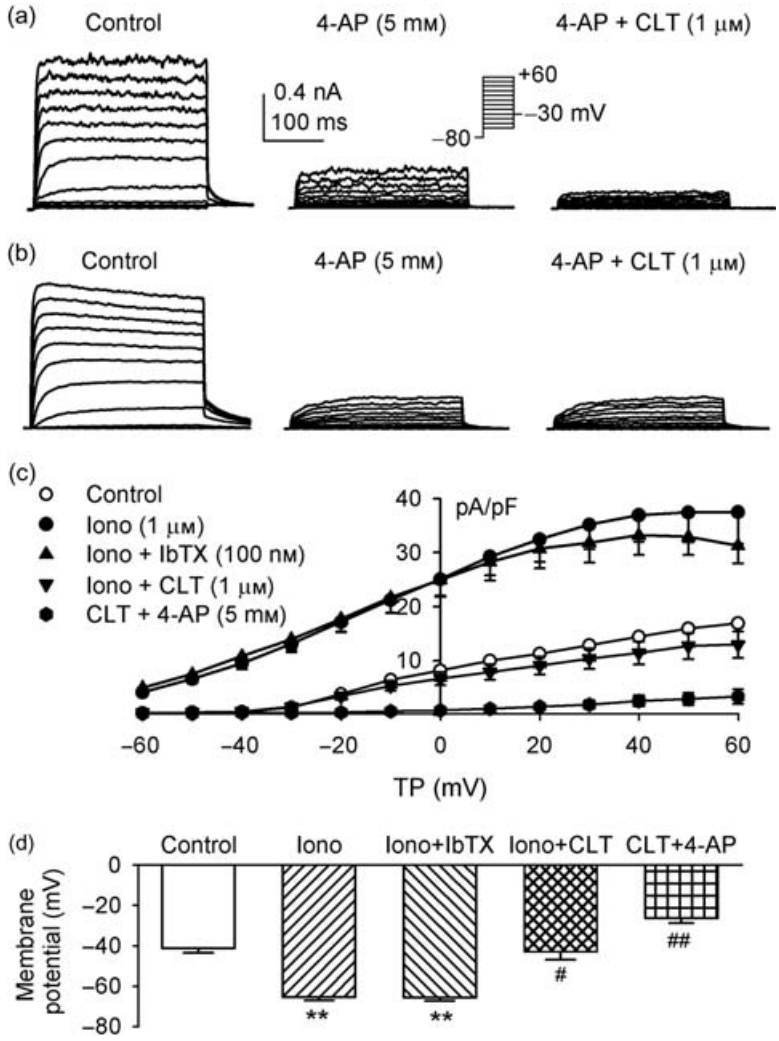

Figure 1. Pharmacological separation of K^+ channel currents in rat MSCs. (a) Membrane currents recorded in a cell with 300-ms voltage steps from –80 to between –50 and +60, and then back to –30 mV as shown in the inset (0.2 Hz). Two components of outwards currents were observed in this cell. One gradually activating current was delayed rectifier K^+ current (IK_{DR}), sensitive to inhibition by 5 mM 4-AP, and another component with noisy oscillation was Ca^{2+}-activated K^+ current (I_{KCa}) sensitive to inhibition by 1 μM clotrimazole (CLT). (b) Membrane current recorded in another cell with the same voltage protocol. Current was inhibited by 5 mM 4-AP, the remaining current not sensitive to clotrimazole, suggesting that only IK_{DR} is present in this cell. (c) *I-V* relationships of membrane currents in rat MSCs ($n = 8$) expressing both IK_{DR} and I_{KCa}. Application of 1 μM ionomycin (Iono) increased membrane current, iberiotoxin (IbTX, 100 nM) slightly decreased current at +30 to +60 mV, and clotrimazole (1 μM) reversed ionomycin-induced current and produced a further reduction of membrane current. Remaining current was suppressed by co-application of clotrimazole and 5 mM 4-AP. (d) Mean values of membrane potentials determined in current clamp mode in the same rat MSCs as in (c), control, after application of 1 μM ionomycin, co-application of ionomycin and 100 nM iberiotoxin, combination of ionomycin with 1 μM clotrimazole, and clotrimazole plus 5 mM 4-AP.
$^{**}P < 0.01$ vs control; $\#P < 0.05$, $\#\#P < 0.01$ vs ionomycin plus iberiotoxin.

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Figure 1b shows membrane currents recorded in another cell. Current was markedly suppressed by 5 mM 4-AP. No additional inhibition was observed with co-application of 4-AP and 1 μM clotrimazole, suggesting that IK_{DR} only was present in this cell.

The MSCs were found to have variable membrane potentials from –15 to –55 mV (Li *et al*. 2006). To study possible contribution of I_{KCa} and IK_{DR} to membrane potential, the membrane current amplitude and membrane potential were determined, then, the Ca^{2+} ionophore ionomycin was applied in these cells ($n = 8$). Ionomycin at 1 μM was found to increase current amplitude, and hyperpolarize membrane potential. Figure 1c illustrates the current-voltage relationships of membrane current density under control conditions, in the presence of 1 μM ionomycin, ionomycin plus 100 nM iberiotoxin and co-application of ionomycin and 1 μM clotrimazole. Ionomycin substantially increased current density. The current increased by ionomycin was slightly reduced by 100 nM iberiotoxin from +30 to +60 mV ($P > 0.05$), while it was substantially suppressed by application of 1 μM clotrimazole. These results indicate that the ionomycin-activated component is mainly contributed by intermediate-conductance I_{KCa}. The remaining current, mainly IK_{DR}, was inhibited by co-application of clotrimazole and 5 mM 4-AP.

Figure 1d illustrates membrane potential recorded in current clamp mode in the MSCs with different treatments. The membrane potential hyperpolarized to –65.5 ± 1.7 mV from –45.6 ± 2.3 mV of control ($n = 8$, $P < 0.01$) by application of 1 μM ionomycin. Increased membrane potential was not affected by 100 nM iberiotoxin (–65.6 ± 1.6 mV), but reduced to –42.8 ± 3.9 mV by 1 μM clotrimazole ($P < 0.01$ versus ionomycin) treatment. Co-application of clotrimazole and 5 mM 4-AP induced additional reduction of membrane potential (to –26.5 ± 2.3 mV, $P < 0.01$ versus clotrimazole or control). These results suggest that IK_{DR} and intermediate-conductance I_{KCa}, but not high-conductance I_{KCa}, play an important role in controlling membrane potential in rat MSCs.

Synchronization of rat MSCs

Mesenchymal stem cells from passages 2–5 were synchronized with the procedure described in the Materials and Methods section. Figure 2 illustrates representative results of flow cytometric analysis of the MSCs from controls and a variety of treatments. In controls, approximately 51% of cells were in G_0/G_1 stage, 43% were in S phase and only 6% were in G_2/M phase. MSCs in low FBS (0.5%) for 24 h (starvation), arrested in early G_1 phase, and showed 81% of cells at G_0/G_1 phase. Cells treated with 2 mM thymidine synchronized to the end of G_1 phase, and 88% were at G_0/G_1 phase, while cells with removal of thymidine for 8 h switched to S phase and showed 92% of cells in there. Average data from four experiments are summarized in Table 1.

Cell cycle-dependent changes in membrane potential, cell size, IK_{DR} and I_{KCa}

Cell cycle-dependent alterations of membrane potential and cell size (defined by membrane capacitance) were studied in the MSCs from different cycling phases. Figure 3a illustrates membrane

Table 1. Cell cycle confirmation by flow cytometry (FC)

FC cell cycle	Control	Early G_1	End G_1	S phase
G_0/G_1 (%)	52.2 ± 1.6	81.0 ± 0.9	87.8 ± 0.5	7.5 ± 3.6
S (%)	41.7 ± 2.3	11.4 ± 1.4	6.5 ± 1.3	90.2 ± 2.7
G_2/M (%)	6.1 ± 0.7	7.6 ± 2.3	5.8 ± 1.8	2.3 ± 0.9

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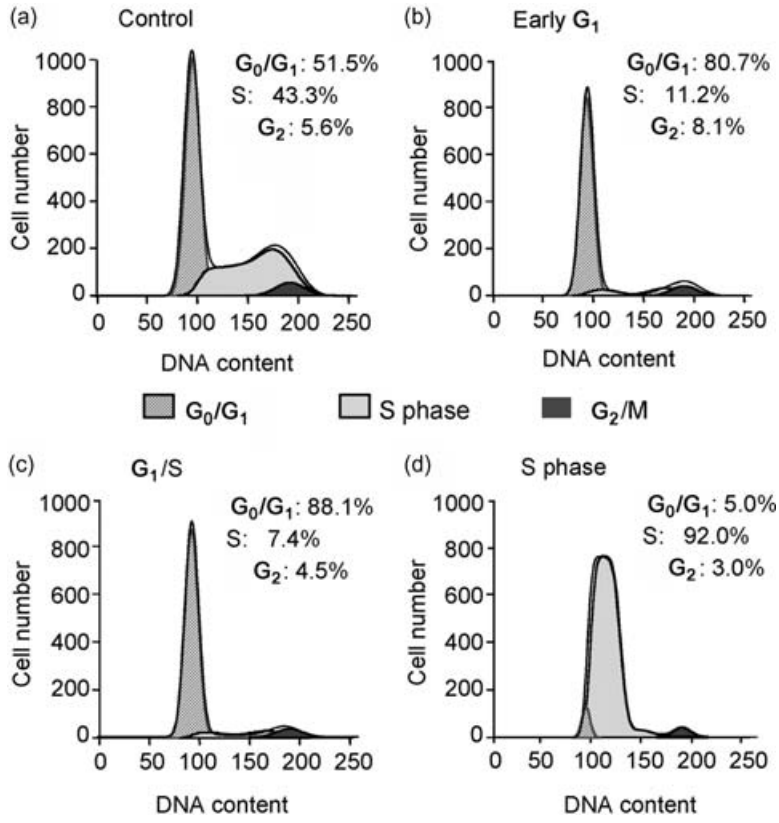

Figure 2. Cell cycle distribution in rat MSCs determined by flow cytometry. (a) Untreated control rat MSCs. (b) Cells from early G_1, treated with starvation medium (0.5% FBS) for 24 h. (c) Cells from the end of G_1, treated with regular culture medium (10% FBS) containing 2 mM thymidine for 24 h. (d) Cells from S phase, cultured with normal culture medium (10% FBS) for 8–10 h after 24 h of thymidine treatment.

potential and membrane capacitance determined in a total of 298 MSCs from the different cycle phases. The membrane potential was –41.3 ± 1.6 mV in early G_1 rat MSCs ($n = 74$), increased to –48.6 ± 1.4 mV ($n = 68$, $P < 0.05$) in cells progressing to G_1 phase, to –52.2 ± 2.5 mV ($n = 62$, $P < 0.01$) in cells at the end of G_1 phase and to –52.3 ± 1.3 mV ($n = 94$, $P < 0.01$) in cells of S phase. These results suggest that the membrane potential of rat MSCs hyperpolarizes during cycle progression from early G_1 to S phase. In addition, cell size significantly increased as the cells developed from early G_1 to end of G_1, and to S phase ($P < 0.01$ versus early G_1 phase).

Cell cycle-dependent changes of I_{KCa} (defined by 1 μM clotrimazole) and IK_{DR} (defined by 5 mM 4-AP) were determined in the MSCs ($n = 298$) from different cell cycle phases. It was found that cell numbers expressing I_{KCa} increased with cell cycle progression. Noise-like I_{KCa} was copresent with IK_{DR} in only 9% (7 out of 74) of cells from early G_1 phase, 63% (43 out of 68) of cells progressing in G_1 phase, 76% (47 out of 62), cells from the end of G_1 phase and in 82% (77 out of 94) of cells from S phase.

Figure 3(b,c) display the 4-AP-sensitive IK_{DR} and clotrimazole-sensitive I_{KCa} determined in the MSCs from the different cell cycle phases, with a 300-ms voltage step from –80 to +50 mV. IK_{DR} decreased from 37.3 ± 1.5 pA/pF in cells from early G_1 phase to –25.7 ± 1.4, 23.1 ± 2.8,

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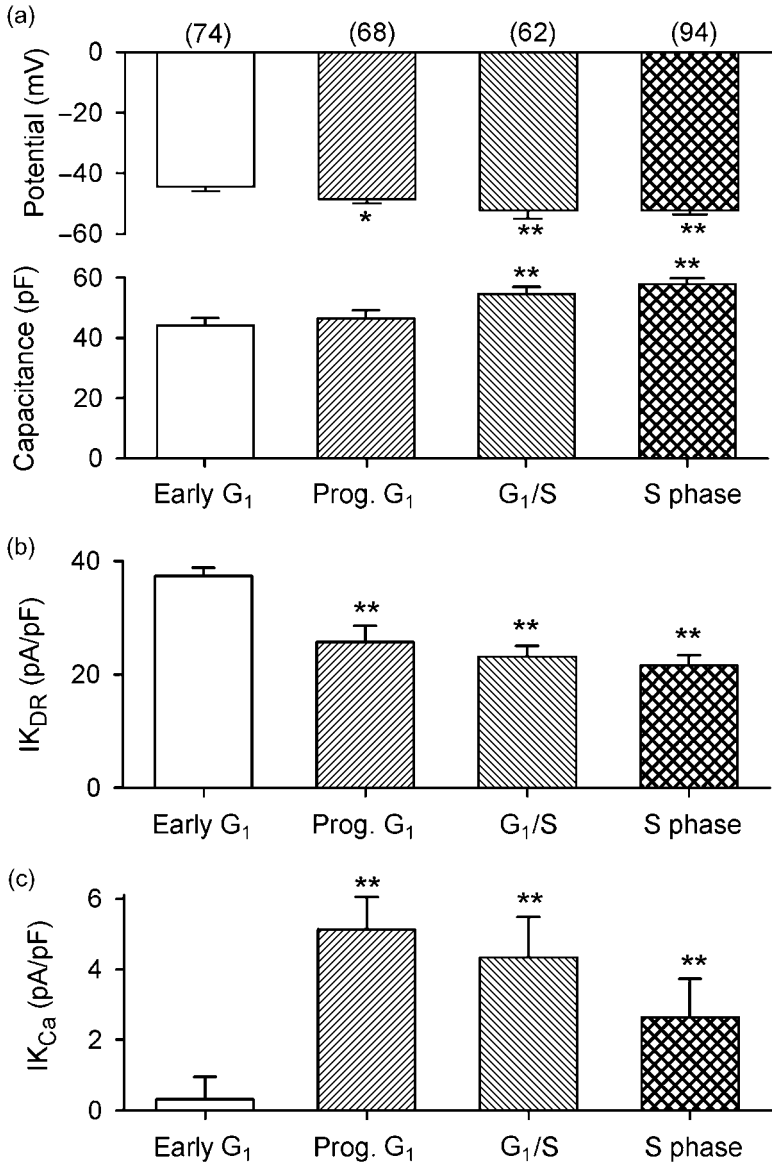

Figure 3. Cell cycle-dependent changes in membrane potential, cell size, IK_{DR} and I_{KCa} in rat MSCs. (a) Membrane potential (upper panel) hyperpolarized in rat MSCs from progressing (Prog) G_1 to S phase, and cell size (lower panel, defined by membrane capacitance, C_m) increased in cells from progressing G_1 to S phase. (b) IK_{DR} density reduced in cells from progressing G_1 to S phase. (c) I_{KCa} density increased in cells progressing from G_1 to S phase.

21.6 ± 1.8 pA/pF in cells from progressing G_1, end G_1 and in S phase, respectively. However, I_{KCa} density increased from 0.3 ± 0.6 pA/pF in cells from early G_1 phase to 5.1 ± 0.9 pA/pF in cells from progressing G_1 phase, 4.3 ± 1.2 pA/pF in cells from the end of G_1 phase and 2.6 ± 1.1 pA/pF in cells from S phase. These results suggest that membrane hyperpolarization observed in cells from progressing G_1 to S phase is most likely related to the increase of I_{KCa}.

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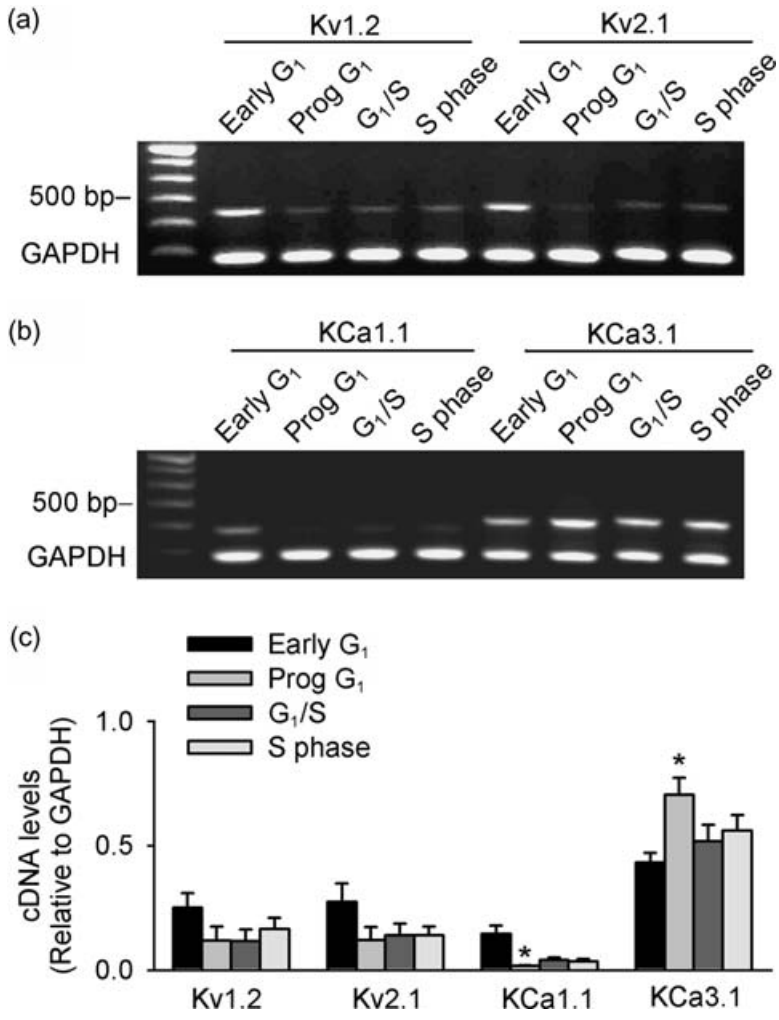

Figure 4. Cell cycle-dependent changes of mRNA levels of K^+ channel α-subunits in rat MSCs. (a,b) Original gels showing expression of Kv1.2, Kv2.1, KCa1.1 and KCa3.1 mRNA from different cell cycle phases (Prog: progressing). (c) Mean values of cDNA levels (relative to GAPDH) of Kv1.2, Kv2.1, KCa1.1 and KCa3.1 from different cycling phases. $*P < 0.05$ versus early G_1 ($n = 4$ different treatments).

Cell cycle-dependent changes in mRNA levels of IK_{DR} and I_{KCa}

Messenger RNA levels of α-subunits of K^+ channels responsible for IK_{DR} and I_{KCa} were determined in the cells from different cycling phases using RT-PCR. Our recent study showed that IK_{DR} was likely to be encoded by Kv1.2 and Kv2.1, while I_{KCa} was likely to be encoded by KCa3.1 and KCa1.1 (Li *et al*. 2006). Thus, mRNA levels of α-subunits for Kv1.2, Kv2.1, KCa3.1 and KCa1.1 were investigated in cells from different cycling phases. Figure 4(a,b) display the original gels of RT-PCR. Kv1.2, Kv2.1 and KCa1.1 mRNA levels reduced, while

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KCa3.1 increased in MSCs from early G_1 to progressing G_1, to the end of G_1, and S phase. No bands were seen (data not shown) when RNA was used directly for PCR (which is without a reverse transcription product), as previously reported (Li *et al*. 2006). Average cDNA levels relative to the housekeeping gene for GAPDH are summarized in Fig. 4c. Statistically significant changes were observed for KCa1.1 and KCa3.1 from progressing G_1 ($P < 0.05$). These results provide molecular evidence for the cell cycle-dependent alteration of functional ion channels observed with patch clamp experiments (Fig. 3).

Effects of IK_{DR} and I_{KCa} on cell proliferation

The cell proliferation assay was initially performed by determining [^{3}H]-thymidine incorporation level in the MSCs, in the absence or in the presence of varied concentrations of K^+ channel blockers, although there were no specific blockers available for IK_{DR} and intermediate-conductance I_{KCa}. We determined effects of 4-AP (0.1–3 mM, for inhibiting IK_{DR}), clotrimazole (0.1–1 μM, for inhibiting intermediate-conductance I_{KCa}) and the specific high-conductance I_{KCa} inhibitor iberiotoxin (10–100 nM), on cell proliferation, by incubation for 48 h in culture medium. We found that [^{3}H]-thymidine incorporation levels were reduced by 0.3, 1 and 3 mM 4-AP (15.6 ± 1.9, 26.4 ± 1.6 and $34.4 \pm 1.8\%$, $P < 0.01$ versus control), 0.3 and 1 μM clotrimazole (10.5 ± 1.5 and $16.4 \pm 2.2\%$, $P < 0.05$ or $P < 0.01$ versus control), but not by 100 nM iberiotoxin ($3.5 \pm 2.9\%$, P = NS). These results suggest that IK_{DR} and intermediate-conductance I_{KCa}, but not high-conductance I_{KCa}, participate in regulation of cell proliferation.

Results from flow cytometric analysis showed that $54.9 \pm 1.9\%$ of the cells were in G_0/G_1 phase in controls, which increased to $67.1 \pm 0.6\%$ of cells treated with 3 mM 4-AP ($n = 4$ experiments, $P < 0.01$), and to $68.9 \pm 2.5\%$ of cells treated with 1 μM clotrimazole ($n = 4$ experiments, $P < 0.01$), suggesting that blockade of IK_{DR} or intermediate-conductance I_{KCa} may interfere with cell cycle progression.

Recent studies have shown that specific RNAi was an effective tool to examine the effects of ion channels on cell proliferation (Lan *et al*. 2005; Weber *et al*. 2006; Wu *et al*. 2006). To rule out possible non-specific effects of ion channel blockers on proliferation, we used specific RNAi targeted to IK_{DR} (i.e. Kv1.2 and Kv2.1) and I_{KCa} (i.e. KCal.1 and KCa3.1). Figure 5 illustrates the effects of the specific RNAi (100 nM) of Kv1.2, Kv2.1, KCa1.1 or KCa3.1 on related gene expression and on cell proliferation. Transfection efficiency of RNAi reached 80–90% (Fig. 5a, right panel). By the use of GAPDH RNAi as positive control, we found that GAPDH RNAi remarkably down-regulated its mRNA level by 49% ($n = 5$ experiments). Figure 5b shows that the mRNA level of Kv1.2, Kv2.1, KCa1.1 or KCa3.1 was substantially reduced by specific RNAi targeted to the corresponding gene. In four experiments, cDNA from RT-PCR was reduced by 51%, 57%, 67% and 64% for Kv1.2, Kv2.1, KCa1.1 and KCa3.1 RNAi, respectively.

Flow cytometric analysis showed that $58.4 \pm 2.6\%$ of the cells were in G_0/G_1 phase in cells treated with control RNAi, which increased to $91.2 \pm 2.1\%$, $83.6 \pm 2.6\%$ and $85.4 \pm 3.5\%$ in cells ($n = 3$, $P < 0.01$ versus control) treated, respectively, with Kv1.2, Kv2.1 and KCa3.1 siRNAs, while no change ($59.3 \pm 2.6\%$, P = NS) was observed in cells treated with KCa1.1 siRNA, suggesting that down-regulation of IK_{DR} or intermediate-conductance I_{KCa} interfered with cell cycle progression.

Figure 5c shows [^{3}H]-thymidine incorporation levels in the MSCs treated by RNAi targeted to different genes. Cell proliferation was reduced by 70%, 58% and 42.0% with the specific RNAi targeted to Kv1.2, Kv2.1 and KCa3.1, respectively. However, cell proliferation was not affected by knockdown of KCa1.1. These results further suggest that IK_{DR} and intermediate-conductance I_{KCa}, but not high-conductance I_{KCa}, regulate cell proliferation in these cells.

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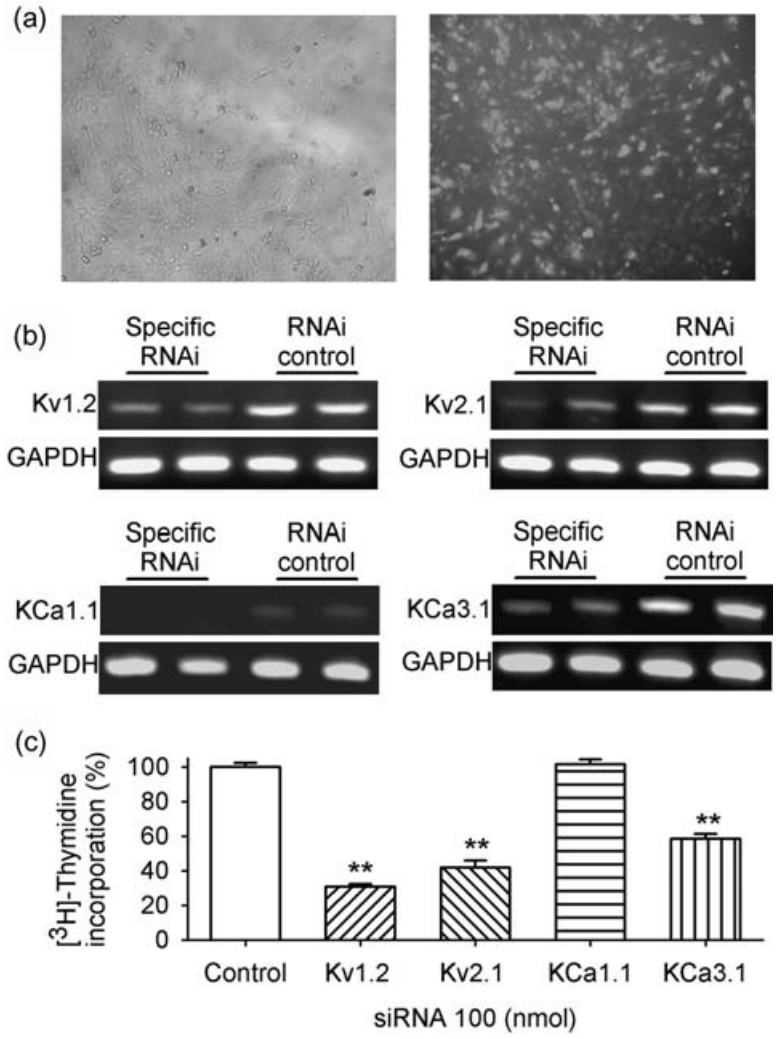

Figure 5. Effects of down-regulation of K^+ channels with specific RNAi on cell proliferation in rat MSCs. (a) Images showing an example of transfecting efficiency with fluorescent RNA duplex, phase contrast (left panel) and fluorescence (right panel). (b) Original gels showing reduced messenger RNA levels of Kv1.2, Kv2.1, KCa1.1 and KCa3.1 with the corresponding specific RNAi, compared to RNAi control. (c) Cell proliferation was reduced by the down-regulation of the specific RNAi of Kv1.2, Kv2.1 or KCa3.1, but not KCa1.1.
** $P < 0.01$ vs control

DISCUSSION

In the present study, we have demonstrated for the first time that membrane potential and cell size increased with cell cycle progression from early G_1 to S phase in undifferentiated rat MSCs. IK_{DR} decreased, while I_{KCa} increased with corresponding changes of mRNA levels likely responsible for these two types of K^+ channels. Pharmacological blockade of IK_{DR}, with 4-AP or I_{KCa} with clotrimazole, but not with iberiotoxin, decreased cell proliferation. Moreover, specific

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RNAi targeted to Kv1.2, Kv2.1 or KCa3.1 down-regulated the corresponding genes, and inhibited cell proliferation.

Cell proliferation is a crucial function, and is strictly controlled by a number of independent mechanisms, and one of them is ion channel activity. It is generally believed that cells require K^+ channels to proliferate (see reviews, Wonderlin & Strobl 1996; Pardo 2004). There has been increasing evidence that K^+ channels participate in regulation of cell cycle progression, because the leading study in lymphocytes by DeCoursey *et al*. (1984). Inhibition of K^+ channels causes a decrease of proliferation in a variety of types of cells under both physiological (e.g. lymphocytes) and pathological (e.g. cancer cells) conditions (see reviews, Wonderlin & Strobl 1996; Pardo 2004), and therefore K^+ channel functions have been proposed to be involved in cell cycle progression in general (Gollapudi *et al*. 1988; Wonderlin & Strobl 1996; Bruggemann *et al*. 1997; Cahalan *et al*. 2001; Parihar *et al*. 2003; Grgic *et al*. 2005).

Although MSCs have been used for a number of years in the investigation of cell therapy and differentiation (Bruder *et al*. 1997; Deans & Moseley 2000; Caplan & Bruder 2001; Janderova *et al*. 2003), ion channel expression and roles of ion channels in biological and physiological activity are not well understood. It is generally accepted that K^+ channels are key players in controlling membrane potential, and therefore they are important in controlling proliferation processes. Here, we have demonstrated that enhancement of I_{KCa} by ionomycin, hyperpolarized membrane potential in rat MSCs, while inhibition of intermediate I_{KCa} remarkably reduced I_{KCa} amplitude and membrane potential. In addition, blockade of IK_{DR} produced an additional depolarization in these cells (Fig. 1), suggesting that IK_{DR} and intermediate I_{KCa} are important in controlling membrane potential. High-conductance I_{KCa} was not as significant as that of intermediate conductance I_{KCa} (Li *et al*. 2006), and iberiotoxin-sensitive high-conductance I_{KCa} was low in these cells; therefore membrane potential was not affected by blocking the current in cells treated with ionomycin (Fig. 1).

Cell cycle-dependent changes in IK_{DR} and I_{KCa} were observed by synchronizing the cells to early G_1, progressing through G_1, end G_1, and S phase, with a modified procedure (Ouadid-Ahidouch *et al*. 2004a). Results from flow cytometry demonstrated that 81–88% of cells from early G_1 and end G_1 were in G_0/G_1 phase, and only 7.5% of those from S phase were in G_0/G_1 phase (Table 1). However, flow cytometric analysis could not differentiate the G_0 cells from G_1 cells, which is a limitation of the present study. Nevertheless, our data from electrophysiology and RT-PCR have revealed that cell size, membrane potential, IK_{DR} and I_{KCa} densities, and mRNA levels of Kv1.2, Kv2.1, KCa1.1 and KCa3.1 altered with cell cycle progression (Figs 3 and 4).

Our results have shown that membrane potential relatively depolarized in rat MSCs from early G_1, compared to that in cells from progressing G_1, end G_1 and S phase, suggesting that most cells are most likely arrested in G_0 by (24 h) starvation using low serum (0.5%) medium. The cells from early G_1 exhibited very low density of I_{KCa} in a small population of cells (9%, 7 out of 74 cells), and a higher density of IK_{DR}, which implies that membrane potential is likely controlled by IK_{DR} at this stage. In addition, cell numbers expressing I_{KCa} increased, and clotrimazole-sensitive I_{KCa} were augmented 81–159-fold in cells from early G_1 to progressing G_1, end G_1 and S phase, while density of 4-AP-sensitive IK_{DR} decreased by 31–42% (Fig. 3), suggesting that the membrane potential of rat MSCs progressing through G_1 to S phase is controlled by I_{KCa}. However, contribution of IK_{DR} to membrane potential could not be excluded, as density of IK_{DR} was always greater than that of I_{KCa} through G_1 to S phase (Fig. 3).

Importantly, RT-PCR revealed that cell cycle-dependent changes in IK_{DR} and I_{KCa} were parallel to alterations in mRNA levels of Kv1.2 and Kv2.1 (responsible for IK_{DR}) and KCa3.1 (responsible for intermediate-conductance I_{KCa}). Expression of KCa1.1 (responsible for high-conductance

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I_{KCa}) was not as great as KCa3.1, and Kv1.2 and Kv2.1. Although KCa1.1 mRNA level reduced in cells from early G_1 to S phase, it may not play a significant role in controlling membrane potential during cell cycling of rat MSCs.

It is believed that membrane hyperpolarization is required for cell cycle progression from G_0/G_1 to progressing G_1 and to S phases (see reviews, Wonderlin & Strobl 1996; Pardo 2004). Earlier reports showed that membrane hyperpolarization increased the electrochemical gradient for Ca^{2+} influx (Wang *et al*. 2000) and elevation of intracellular Ca^{2+} levels enhanced activity of Ca^{2+}-dependent kinases that regulate cyclins and cyclin-dependent kinases (Santella *et al*. 1998). For instance, Ca^{2+}-dependent calmodulin kinases are believed to increase cell cycle progression at several transition checkpoints (Kahl & Means 2003). Blockade of IK_{DR} or I_{KCa} with 4-AP or clotrimazole resulted in a depolarization of membrane potential in rat MSCs (Fig. 1). This effect is likely related to inhibition of cell proliferation by interference with cell cycle progression. Application of 4-AP or clotrimazole inhibited proliferation, although the effect was not as strong as the specific RNAi. Specific RNAi, targeted to Kv1.2, Kv2.1 or KCa3.1, but not KCa1.1, decreased the corresponding mRNA levels, substantially inhibiting cell proliferation (Fig. 5), and arrested cells at the G_0/G_1 phase. The lower effect of 4-AP and clotrimazole on cell proliferation may be due to incomplete blockade of the potassium channels. These results strongly suggest that IK_{DR} and intermediate-conductance I_{KCa} regulate proliferation of rat MSCs.

One of the limitations of this study was that it focused mainly on observation whether the dominant K^+ currents IK_{DR} and I_{KCa} changed with cell cycle progression and whether they participated in cell proliferation in rat MSCs. However, possible contributions of other ion channel currents (e.g. I_{to}, $I_{Na.TTX}$, and $I_{Ca.L}$ expressed in a small population of cells) (Li *et al*. 2006) to regulation of cell proliferation could not be excluded (see review, Wonderlin & Strobl 1996). Another limitation was that specific ion channel blockers are still unavailable to separate IK_{DR} from I_{KCa}. We used 4-AP (5 mM) to define IK_{DR}; however, high concentrations of 4-AP might affect other K^+ currents (see review, Gutman *et al*. 2005). This might underestimate intermediate-conductance I_{KCa}. Clotrimazole was used to define intermediate-conductance I_{KCa}, and this compound was reported to have Kv channel inhibition at high concentrations (see review, Wei *et al*. 2005). Maximum concentration (1 μM) we used here had no effect on I_{Kur} and I_{to} (Kv1.5 and Kv4.3) on human atrial myocytes (Tian *et al*. 2006) or IK_{DR} in rat MSCs (Fig. 1b). Thus, possible non-specific action of clotrimazole on Kv channels may not be involved in the effect on intermediate I_{KCa} observed in the present study. In addition, although a number of ion channel genes (mRNAs) were detected proteins of these have not been demonstrated, which remains to be studied in the future.

In summary, the present study provides novel information that IK_{DR} and intermediate I_{KCa} channels exhibit cell cycle-dependent expression, and play an important role in controlling membrane potential in rat MSCs. IK_{DR} and intermediate-conductance I_{KCa}, but not high-conductance I_{KCa}, participate in regulation of proliferation in rat MSCs.

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