Large T-antigen up-regulates $K_v4.3$ $K^+$ channels through Sp1, and $K_v4.3$ $K^+$ channels contribute to cell apoptosis and necrosis through activation of calcium/calmodulin-dependent protein kinase II

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Down-regulation of $K_v4.3$ $K^+$ channels commonly occurs in multiple diseases, but the understanding of the regulation of $K_v4.3$ $K^+$ channels and the role of $K_v4.3$ $K^+$ channels in pathological conditions are limited. HEK (human embryonic kidney)-293T cells are derived from HEK-293 cells which are transformed by expression of the large T-antigen. In the present study, by comparing HEK-293 and HEK-293T cells, we find that HEK-293T cells express more $K_v4.3$ $K^+$ channels and more transcription factor Sp1 (specificity protein 1) than HEK-293 cells. Inhibition of Sp1 with Sp1 decoy oligonucleotide reduces $K_v4.3$ $K^+$ channel expression in HEK-293T cells. Transfection of pN3-Sp1FL vector increases Sp1 protein expression and results in increased $K_v4.3$ $K^+$ expression in HEK-293 cells. Since the ultimate determinant of the phenotype difference between HEK-293 and HEK-293T cells is the large T-antigen, we conclude that the large T-antigen up-regulates $K_v4.3$ $K^+$ channel expression through an increase in Sp1. In both HEK-293 and HEK-293T cells, inhibition of $K_v4.3$ $K^+$ channels with 4-AP (4-aminopyridine) or $K_v4.3$ small interfering RNA induces cell apoptosis and necrosis, which are completely rescued by the specific CalMII (calcium/calmodulin-dependent protein kinase II) inhibitor KN-93, suggesting that $K_v4.3$ $K^+$ channels contribute to cell apoptosis and necrosis through CalMII activation. In summary, we establish: (i) the HEK-293 and HEK-293T cell model for $K_v4.3$ $K^+$ channel study; (ii) that large T-antigen up-regulates $K_v4.3$ $K^+$ channels through increasing Sp1 levels; and (iii) that $K_v4.3$ $K^+$ channels contribute to cell apoptosis and necrosis through activating CalMII. The present study provides deep insights into the mechanism of the regulation of $K_v4.3$ $K^+$ channels and the role of $K_v4.3$ $K^+$ channels in cell death.

Key words: apoptosis, calcium/calmodulin-dependent protein kinase II (CalMII), human embryonic kidney (HEK) cell; $K_v4.3$ potassium channel, large T-antigen, necrosis.

INTRODUCTION

Voltage-dependent $K_v4.3$ $K^+$ channels, the major component of $I_{to}$ (transient outward current) in cardiomyocytes and A-type $K^+$ currents in neurons, are associated with many diseases. In hypertrophic failing hearts or rapid pacing cardiomyocytes, $K_v4.3$ $K^+$ currents and channel expression decrease [1–4]. In dogs with ventricular tachypacing-induced CHF (congestive heart failure), $K_v4.3$ $K^+$ currents and channel expression decrease [1–4]. In a rat model of Type 1 diabetes, the protein density in $K_v4.3$ in Purkinje fibres decreases [5]. In a model of the left ventricular myocytes decreases [6]. Inversely, the decrease in $K_v4.3$ expression leads to certain consequences. The down-regulation of $K_v4.3$ $K^+$ currents and channel expression enhances sympathoexcitation in hypertrophic or failing hearts [7]. Reduction of $K_{4.2}/K_{4.3}$-based $I_{to}$ prolongs action potential duration, resulting in cardiac hypertrophy [8]. Lebeche et al. [9] found that in vivo gene transfer of $K_v4.3$ restores the down-regulation of $I_{to}$ and abrogates the hypertrophic response. On the other hand, it has been recently reported that genetically encoded KCND3-encoded $K_v4.3$ $I_{to}$ participates in the pathogenesis of Brugada syndrome [10]. In the neural field, $K_v4.3$ $K^+$ currents participate in the rhythmic activity in hippocampal interneurons [11], and nerve injury down-regulates $K_v4.3$ expression in dorsal root ganglion [12]. Although $K_v4.3$ $K^+$ channels are associated with many diseases, the understanding of the signal pathway that regulates $K_v4.3$ expression and the pathological contribution of $K_v4.3$ are still limited.

In the present study, we use electrophysiological studies of HEK (human embryonic kidney)-293 and HEK-293T cells and find that HEK-293T cells express a type of native $K^+$ current which is increased in HEK-293T cells compared with HEK-293 cells. Then we identify it as $K_v4.3$ $K^+$ channels. The HEK-293 cell is a cell line derived from HEK cells. The HEK-293T cell is derived from the HEK-293 cell line by the addition of the SV40 (simian virus 40) large T-antigen. Both HEK-293 and HEK-293T cells are commonly used as tools to exogenously express active proteins. It is of note that there is a difference in $K_v4.3$ $K^+$ channel expression between HEK-293 and HEK-293T cells, which will lead to further investigation of the potential mechanism of $K_v4.3$ $K^+$ channel regulation and the role of $K_v4.3$ $K^+$ channels in cell biological processes. In the present study, we find that the large T-antigen up-regulates $K_v4.3$ $K^+$ channel expression through increasing the levels of the transcription factor Sp1 (specificity protein 1), and $K_v4.3$ $K^+$ channels contribute to cell apoptosis and necrosis by activating CalMII (calcium/calmodulin-dependent protein kinase II). The present study puts forward new insights into the mechanism of the regulation of $K_v4.3$ $K^+$ channels and the role of $K_v4.3$ $K^+$ channels in cell death.
Table 1 Human gene-specific primers for real-time PCR

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<tr>
<th>Gene name</th>
<th>Accession number</th>
<th>Forward primer (5′–3′)</th>
<th>Reverse primer (5′–3′)</th>
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<tr>
<td>GAPDH</td>
<td>XM_001068276.2</td>
<td>AGAAGGTGTTGAACGGACGC</td>
<td>TCCACCACTCGAGTGCTGA</td>
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<tr>
<td>K,1.3</td>
<td>NM_002232</td>
<td>GACCTGCTTCCTTTCAGC</td>
<td>GGGATTATTCTGGTGGTGC</td>
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<td>NM_002233</td>
<td>CCATCAGTGAAGGAGCAAG</td>
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<td>NM_002234</td>
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<td>NM_012281</td>
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<tr>
<td>K,10.1 (EAG1)</td>
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<tr>
<td>HERG</td>
<td>NM_172056</td>
<td>CAGCGATTGACAGATC</td>
<td>GTGGGGCTGCTCCTTATC</td>
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</table>

Table 2 Sp1 decoy sequence

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Sense (5′–3′)</th>
<th>Antisense (5′–3′)</th>
</tr>
</thead>
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<tr>
<td>Sp1 decoy</td>
<td>ATTACGAGGCGGGGGGCTAC</td>
<td>GTAGCCCTATCTACCGGTAAT</td>
</tr>
<tr>
<td>Sp1 (mutant)</td>
<td>ATTACCGGTAGGAGGGCTAC</td>
<td>GTAGCCCTATCTACCGGTAAT</td>
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MATERIALS AND METHODS

Materials

Anti-K,1.4, -K,4.2 and -K,4.3 K⁺ channel antibodies were purchased from Alomone. Anti-CaMKII and -p-CaMKII antibodies were purchased from Promage. Anti-Sp1 antibody was purchased from Cell Signaling Technology. Anti-caspase-3, -Bax and -Bcl-2 antibodies were purchased from Santa Cruz Biotechnology. Anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody was purchased from Abcam. Anti-Kv1.4, -Kv4.2 and -Kv4.3 Kv channel antibodies were purchased from Invitrogen. Anti-Kv1.4, -Kv4.2 and -Kv4.3 antibodies were purchased from Cell Signaling Technology. Anti-CaMKII and -p-CaMKII antibodies were purchased from Promage.

Real-time PCR

The viability of cells cultured in the 96-well culture plates was assessed by measuring the mitochondrial dehydrogenase activity using the colorimetric MTT assay.

Western blot

The viability of cells cultured in the 96-well culture plates was assessed by measuring the mitochondrial dehydrogenase activity using the colorimetric MTT assay.
LDH (lactate dehydrogenase) measurement

Cell necrosis was determined by measuring the release of LDH from cells (LDH assay kit, Nanjing Jiancheng Bioengineering Institute). Briefly, HEK-293 cells and HEK-293T cells were seeded in a six-well plate at a density of $3.75 \times 10^4$/ml and incubated overnight at 37°C in a humidified 5% CO₂ incubator. The cells were treated with 4-AP or siRNA-Kv4.3 for 24 h. The medium was collected and the absorbance of LDH was measured at 440 nm with a microplate reader according to the manufacturer’s instructions (Tecan).

Live- and dead-cell staining

The LIVE/DEAD® Viability/Cytotoxicity Assay Kit (Invitrogen) was used to detect live and dead cells. Briefly, HEK-293 cells and HEK-293T cells were grown on coverslips at a density of $3.75 \times 10^4$/ml and incubated overnight at 37°C in a humidified 5% CO₂ incubator. The cells were treated with 4-AP or siRNA-Kv4.3 for 24 h. The cells were then washed with PBS and dyed according to the manufacturer’s instructions. The labelled cells were photographed under a fluorescence microscope. The live cells fluoresce green and dead cells fluoresce red.

Flow cytometry analysis

HEK-293 and HEK-293T cells were stained with FITC–annexin V and PI (propidium iodide) to evaluate cell apoptosis and necrosis by using flow cytometry (FACSCalibur, Becton Dickinson). The dot plots in quadrants quantified the percentage of cells.

Data analysis

Data are presented as means ± S.E.M. Significance was determined by using one-way ANOVA in SigmaStat analysis software. $P < 0.05$ was considered significant.

RESULTS

HEK-293T cells express large T-antigen and proliferate faster than HEK-293 cells

HEK-293T cells are derived from HEK-293 cells and stably express the SV40 large T-antigen. Large T-antigen binds to several proteins involved in the cell cycle, thus resulting in the faster growth of HEK-293T cells. We first examined the large T-antigen expression in HEK-293 and HEK-293T cells. As shown in Figure 1(A), HEK-293T cells expressed abundant large T-antigen, but no large T-antigen was detected in HEK-293 cells. Next, we examined the cell growth of HEK-293 and HEK-293T cells. As shown in Figure 1(B), HEK-293T cells proliferated significantly faster than HEK-293 cells at 24 and 48 h after the same density of HEK-293 and HEK-293T cells were seeded. The cell viability was measured using the MTT method and the analysed data are shown in Figure 1(C), indicating that HEK-293T cells proliferate faster than HEK-293 cells.

HEK-293T cells express more Kv4.3 K⁺ channels than HEK-293 cells

By using a patch-clamp technique with 140 mM K⁺ in the pipette (intracellular solution), we found a depolarization-induced outward current ($I_{\text{outward}}$) in HEK-293 and HEK-293T cells bathed in 5 mM K⁺ tyrode solution (Figure 2A). This kind of current was significantly larger in HEK-293T cells than in HEK-293 cells (right-hand panel of Figure 2A). In order to identify the type of current, we replaced 5 mM K⁺ with 140 mM K⁺ in extracellular solution (Figure 2B, panel a), and replaced K⁺ with an equal molar concentration of Cs⁺ in the pipette and extracellular solution (Figure 2B, panel b). Under these conditions, the depolarization-induced outward currents in both HEK-293 and HEK-293T cells were significantly inhibited, indicating that the depolarization-induced outward currents were K⁺ currents. This type of K⁺ current in HEK-293 and HEK-293T cells was inhibited by 2 mM 4-AP and restored partially after 4-AP washout (Figure 2C, panels a and b). The net 4-AP-sensitive K⁺ currents were obtained by subtracting the currents recorded in the presence of 4-AP from the control currents. As shown in Figure 2(C, panel c), compared with HEK-293 cells, HEK-293T cells had significantly larger 4-AP-sensitive K⁺ currents.

The properties of 4-AP-sensitive K⁺ currents recorded in HEK-293 and HEK-293T cells were much like the transient outward K⁺ currents encoded by Kv4.3, Kv4.2 or Kv1.4 genes. Therefore, we compared the Kv4.3, Kv4.2 and Kv1.4 mRNA expression in HEK-293 and HEK-293T cells. Meanwhile, we also compared other K⁺ channel gene expression, such as HERG (human ether-a-go-go-related gene), eag1, Kv1.3 and Kv1.5 K⁺ channels. As shown in Figure 3(A), among these K⁺ channel genes, only the Kv4.3 mRNA expression was increased in HEK-293T cells compared with HEK-293 cells ($P < 0.01$). Next, we compared the K⁺ currents recorded in HEK-293 and HEK-293T cells. There was no difference in K⁺ currents between HEK-293 and HEK-293T cells, but the Kv4.3 protein expression level was much higher in HEK-293T cells than in HEK-293 cells (Figure 3B). Furthermore, we used siRNA against Kv4.3 (siRNA-Kv4.3) to confirm the Kv4.3 K⁺ channel expression in HEK-293T cells. As shown in Figures 3(C) and 3(D), siRNA-Kv4.3 significantly down-regulated the Kv4.3 mRNA and protein
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Figure 2 HEK-293T cells expressed more voltage-dependent 4-AP-sensitive K⁺ currents than HEK-293 cells

(A) Left-hand panel, the representative recordings of depolarization-induced outward currents (Iₒ, Iₒ(outward)) in HEK-293 and HEK-293T cells. Right-hand panel, the analysed results. (B) Panel (a), current recordings under the conditions where 140 mM K⁺ was in the pipette (intracellular solution) and cells were bathed in 5 mM K⁺ tyrode solution. Panel (b), current recordings under the conditions where K⁺ in the pipette and bath solution was replaced with an equal molar concentration of Cs⁺. (C) The voltage-dependent K⁺ currents recorded in HEK-293 and HEK-293T cells were sensitive to 4-AP. The net 4-AP sensitive K⁺ currents were obtained by subtracting the currents recorded in the presence of 4-AP from the control currents (panels a and b). Compared with HEK-293 cells, HEK-293T cells had significantly larger 4-AP-sensitive K⁺ currents (c).

expression. Taken together, these data suggest that HEK-293T cells express more K⁺,4.3 K⁺ channels than HEK-293 cells.

The increase in Sp1 levels leads to more K⁺,4.3 expression in HEK-293T cells

The large T-antigen is a powerful oncoprotein capable of perturbing the retinoblastoma (pRB) and p53 tumour suppressors, and binding to several cellular factors, including Sp1, AP-1 (activator protein 1) and p300, which may contribute to large T-antigen transformation function. The transcription factor Sp1 has been found to regulate K⁺,1.5 [14], KCNQ2, KCNQ3 [15] and BK [16] K⁺ channel expression. In the human K⁺4.3 promoter (−1000 to 299 bp), the computational analysis showed that there were three Sp1-binding sites: (−413)TCCCCACCCC(−404); (−138)TCCCCGCCCC(−129) and (−122)GCGCGCGC-CCC(−113). We speculated that Sp1 might be the candidate substrate of large T-antigen and that Sp1 regulates K⁺,4.3 K⁺ channel expression. Western blot results showed that HEK-293T cells expressed more Sp1 than HEK-293 cells (Figure 4A) and the Sp1 decoy significantly decreased K⁺,4.3 expression in HEK-293T cells (Figure 4B). Overexpression of Sp1 by transfecting the pN3-Sp1FL vector increased Sp1 (Figure 4C) and K⁺,4.3 (Figure 4D) protein expression in HEK-293 cells, which had a reduced basal K⁺,4.3 level. We have proved that Sp1 was the pivotal factor in large T-antigen-induced K⁺,4.3 expression, and established that the large T-antigen up-regulated K⁺,4.3 K⁺ channel expression by increasing Sp1.

Inhibition of K⁺,4.3 K⁺ channels by 4-AP and siRNA-K⁺,4.3 induces HEK-293 and HEK-293T cell apoptosis and necrosis

Since HEK-293T cells express more K⁺,4.3 K⁺ channels and proliferate faster than HEK-293 cells, we investigated the role of K⁺,4.3 K⁺ channels in the cell biological processes further. As shown in Figures 5(A) and 5(B), 4-AP, the K⁺,4.3 K⁺ channel blocker, significantly reduced HEK-293 and HEK-293T cell viability after a 24 h treatment in a dose-dependent manner. Figure 5(C) shows the representative photographs of the live and dead HEK-293 and HEK-293T cells treated with different concentrations of 4-AP for 24 h. It is apparent that 4-AP induces HEK-293 and HEK-293T cell death. Compared with HEK-293 cells, HEK-293T cells were more vulnerable to 4-AP (Figure 5D).

Next, we investigated whether the cell death was via apoptosis or necrosis. Flow cytometric analysis showed that 4 mM 4-AP
induced both apoptosis and necrosis in HEK-293 and HEK-293T cells with annexin V/PI staining (Figure 5E). 4-AP (4 mM) treatment significantly increased cleaved caspase-3 expression (Figure 5F) and caspase-3 activity (Figure 5G) in both HEK-293 and HEK-293T cells. LDH release generally reflects an increase in cell necrosis [17]. 4-AP (4 mM) treatment increased LDH release in both HEK-293 and HEK-293T cells (Figure 5H). It was noticeable that the basal caspase-3 activity of HEK-293T cells was higher than that of HEK-293 cells (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/441/bj4410859add.htm). Moreover, the quantity of LDH release in 4-AP-treated HEK-293T cells was 1.357 ± 0.058-fold, which was higher than that in 4-AP-treated HEK-293 cells (1.15 ± 0.049-fold, *P < 0.05) (Figure 5H). These results were consistent with the results in Figures 5(C) and 5(D), which showed that 4-AP induced more HEK-293T cell death.

4-AP-induced apoptosis and necrosis in HEK-293 and HEK-293T cells were further evaluated by transmission electron microscopy. As shown in Figures 6(A) and 6(C), the normal HEK-293 and HEK-293T cells showed a clear nucleolus and smooth nuclear membrane. 4-AP-treated HEK-293 cells showed typical apoptosis characteristics, including chromatin condensation and disruption of mitochondrial cristae (Figure 6B). 4-AP-treated HEK-293T cells showed typical necrosis characteristics, including dissolved cell membrane, nuclear condensation, disruption of mitochondrial cristae, more lipid droplets and autophagosomes (Figure 6D).

We investigated further the effect of inhibition of Kv4.3 K+ channels by silencing K4.3 with siRNA (siRNA-Kv4.3) on apoptosis and necrosis of HEK-293 and HEK-293T cells. The live and dead assay results showed that siRNA-Kv4.3 induced significant HEK-293 and HEK-293T cell death, and the amount of HEK-293T cell death was increased compared with HEK-293 cells (Figure 7A, live cells are green and dead cells are red). siRNA-Kv4.3 induced increased caspase-3 activity and LDH release in both HEK-293 and HEK-293T cells (Figures 7B and 7C), indicating that the induction of cell apoptosis and necrosis by siRNA-Kv4.3 was similar to that of direct inhibition of Kv4.3 channels by 4-AP. The basal caspase-3 activity of HEK-293T cells was higher than that of HEK-293 cells when both cells were treated with scrambled siRNA (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/441/bj4410859add.htm). We used two methods, 4-AP and siRNA-Kv4.3, to inhibit Kv4.3 K+ channels. Both methods showed similar results, indicating that Kv4.3 K+ channels contribute to cell apoptosis and necrosis.

Bcl-2 and Bax, the key anti-apoptotic and pro-apoptotic proteins, influence caspase-3 activation. We investigated further whether caspase-3 activation induced by inhibition of Kv4.3 K+ channels was related to the changes of Bcl-2 and Bax expression in HEK-293 and HEK-293T cells. 4-AP had no
induced increased caspase-3 activity and LDH release in both HEK-293 and HEK-293T cells.

Kv4.3 K⁺ channels coupled with Kᵥ4.3 K⁺ channels induces cell apoptosis and necrosis [17–19]. Recent studies show that CaMKII has been shown previously to mediate cell apoptosis and necrosis [17–19]. Recent studies show that CaMKII is coupled with Kᵥ4.3 K⁺ channels [20,21], and inhibition of Kᵥ4.3 K⁺ channels leads to substantial CaMKII activation [21]. We hypothesized that inhibition of Kᵥ4.3 K⁺ channels induced apoptosis and necrosis of HEK-293 and HEK-293T cells through activating CaMKII. HEK-293 and HEK-293T cells expressed an equal amount level of CaMKII protein (Figure 8A). 4-AP (4 mM) treatment increased the CaMKII autophosphorylation in HEK-293 and HEK-293T cells, indicating that inhibition of Kᵥ4.3 K⁺ channels led to CaMKII activation (Figure 8A). Furthermore, the autophosphorylation level was higher in HEK-293T cells than HEK-293 cells after treatment of the same concentration of 4-AP (4 mM) (Figure 8A). We then used KN-93 to specifically inhibit CaMKII with KN-92 as a negative control. The cell viability was evaluated using the MTT assay, cell apoptosis was evaluated by measurement of caspase-3 activity and cell necrosis by measurement of LDH release. The results showed that KN-93 abolished 4-AP (4 mM)-induced cell death (Figure 8B), and 4-AP (4 mM)- and siRNA-Kᵥ4.3-induced cell apoptosis (Figure 8C) and necrosis (Figure 8D). Hence we established a new mechanism of the Kᵥ4.3 K⁺ channel contributing to cell apoptosis and necrosis via CaMKII (Figure 8E).

**DISCUSSION**

Kᵥ4.3 K⁺ channels play an important role in physiological and pathological conditions in cardiac and neuronal systems [1–12], but the understanding of the regulation of Kᵥ4.3 K⁺ channels is limited. The related HEK-293 and HEK-293T cells are commonly used in biological studies. Now this old acquaintance displays a new face for us. The novel findings of the present study are: (i) large T-antigen increases Kᵥ4.3 K⁺ channel expression through increasing Sp1; and (ii) inhibition of Kᵥ4.3 K⁺ channels induces cell apoptosis and necrosis through activating CaMKII. This work provides new insights into the mechanism of the regulation of Kᵥ4.3 K⁺ channels and the role of Kᵥ4.3 K⁺ channels in cell death.

**Large T-antigen and Kᵥ4.3 K⁺ channels**

The large T-antigen was encoded in the early region of the polyomaviruses BK virus, JC virus and SV40 genome. The large T-antigen is capable of transforming a variety of cell lines. The transforming activity of the large T-antigen is mainly due to its perturbation of the retinoblastoma (pRB) and p53 tumour suppressor proteins; in addition, several other cellular factors, including the transcriptional coactivators p300/CBP [CREB (cAMP-response-element-binding protein)-binding protein] and AP-2, are also involved [22,23]. HEK-293T cells are derived from HEK-293 cells which stably express the large T-antigen, and thus show an increase in proliferative activity. The unique difference between HEK-293 and HEK-293T cells is the expression of the large T-antigen, which is the ultimate determinant of the phenotype difference between these two types of cells.

We have identified the different K⁺ currents between HEK-293 and HEK-293T cells and further identify them as Kᵥ4.3 K⁺ currents. Since the ultimate difference between HEK-293 and HEK-293T cells is the large T-antigen, it must be the large T-antigen that leads to the increased Kᵥ4.3 K⁺ channel expression. We aimed to find the intermediate link between the large T-antigen and Kᵥ4.3 K⁺ channels and focused on the transcription factor Sp1. SV40 has been shown to stimulate Sp1 expression [24]. Sp1 has been reported to regulate some kinds of K⁺ channels, such as Kᵥ1.5 [14]. We found that Sp1 and Kᵥ4.3 K⁺ channel protein expression was significantly higher in HEK-293T cells than HEK-293 cells. The Sp1 decoy significantly decreased the Kᵥ4.3 expression in HEK-293T cells. Overexpression of Sp1 increased Kᵥ4.3 protein expression in HEK-293 cells, which have a decreased basal Kᵥ4.3 level. We take advantage of the
Large T-antigen up-regulates Kv4.3 and Kv4.3 and contributes to cell death through CaMKII

Figure 8 Inhibition of Kv4.3 K⁺ channels induced cell apoptosis and necrosis through activating CaMKII

(A) Western blot results showing that both HEK-293 and HEK-293T cells expressed CaMKII, and 4-AP (4 mM) treatment increased CaMKII phosphorylation in HEK-293 and HEK-293T cells. There was no difference of CaMKII protein expression between HEK-293 and HEK-293T cells. 4-AP (4 mM) treatment caused a larger increase in CaMKII phosphorylation in HEK-293T cells compared with HEK-293 cells. The protein level was normalized to those of HEK-293 cells. Molecular masses in kDa are shown to the right-hand side of the Western blot. (B) 4-AP (4 mM)-induced cell death was completely prevented by the CaMKII-specific inhibitor KN-93 but not by negative control KN-92 in HEK-293 and HEK-293T cells. *P < 0.01 compared with control. **P < 0.01 compared with 4-AP. (D) The increased caspase-3 activity induced by 4-AP (4 mM) and siRNA-Kv4.3 (20 nM) was inhibited by the CaMKII-specific inhibitor KN-93, but not by negative control KN-92 in HEK-293 and HEK-293T cells. **P < 0.01 compared with control. #P < 0.05 compared with 4-AP or siRNA-Kv4.3. (E) A schematic diagram showing that inhibition of Kv4.3 K⁺ channels induced cell apoptosis and necrosis through activation of CaMKII.

characterization difference between HEK-293 and HEK-293T cells and utilize another elaborate way to prove that the large T-antigen increases Kv4.3 K⁺ channel expression through Sp1.

We have established the correlation between the large T-antigen with Sp1 and Kv4.3 expression. However, it is noteworthy that, as the large T-antigen stimulates or represses multiple gene or gene products [25], it is difficult to conclude unequivocally that the large T-antigen is directly responsible for the increases in Sp1 and Kv4.3 expression, and there might be other intermediate gene products involved in the process.

Kv4.3 K⁺ channels are down-regulated in many diseases such as heart hypertrophy, heart failure, cardiac infarction, atrial fibrillation and nerve injury, but the understanding of the signal pathway that increases Kv4.3 expression is limited. The present study provides a new pathway that up-regulates the decreased Kv4.3 K⁺ channels.

Role of Kv4.3 K⁺ channels in cell apoptosis and necrosis

The contribution of voltage-gated potassium channels to the regulation of apoptosis has been well reviewed [26–28]. It is generally thought that K⁺ efflux leads to reduced intracellular K⁺ concentration, which promotes cytochrome c release, proteolytic cleavage of procaspase-3 and cell fragmentation. However, if it is the K⁺ channel activity that leads to cell apoptosis, the prerequisite of the contribution of K⁺ channels to cell apoptosis should be the channel activation. It has been reported that the membrane potential through the cell cycle ranges from −9 to −40 mV in MCF-7 cells [29], and the mean resting membrane potentials of bovine pulmonary artery endothelial cells are approximately −26 mV [30]. It is apparent that the resting membrane potential of cells is generally less than −10 mV, and Kv4.3 K⁺ channels cannot be significantly activated under these membrane potentials (as shown in Figure 2). Therefore there might be another mechanism for the contribution of Kv4.3 K⁺ channels to cell apoptosis. Recently, Keskanokwong et al. [21] showed that blockade of Kv4.3 with 4-AP or Ad-Kv4.3 antisense induced CaMKII dissociation from the Kv4.3–CaMKII complex and subsequent activation of the dissociated CaMKII; in addition, these effects were independent of Kv4.3 K⁺ channel activation and action potential. Since CaMKII mediates cell apoptosis and necrosis, we speculated that inhibition of Kv4.3 K⁺ channels induced HEK-293 and HEK-293T cell death through CaMKII activation. Results from the present study showed that the CaMKII-specific inhibitor KN-93 abolished cell apoptosis and necrosis induced by Kv4.3 K⁺ channel inhibition (Figure 8), confirming our hypothesis.

As mentioned above, the down-regulation of Kv4.3 K⁺ channels occur in many diseases, but it is not clear whether the
down-regulation of \( K_{v4.3} \) \( K^+ \) channels is only a consequence of disease or whether it can be an initiator to promote the disease process. We found that the CaMKII-specific inhibitor KN-93 abolished \( K_{v4.3} \) inhibition-induced cell apoptosis and necrosis, indicating that inhibition of \( K_{v4.3} \)-induced cell death occurred through CaMKII activation, thus the down-regulation of \( K_{v4.3} \) \( K^+ \) channels could be regarded as a promoter for certain disease processes through activating CaMKII. Keskanokwong et al. [21] showed that blockade of \( K_{v4.3} \) with 4-AP or Ad-\( K_{v4.3} \) antisense induced CaMKII dissociation from the \( K_{v4.3} \)-CaMKII complex and subsequent activation of the dissociated CaMKII, which strongly supported our results from the present study. In cardiac hypertrophy, heart failure or cardiac infarction, \( K_{v4.3} \) \( K^+ \) channels generally decreased. We have put forward a new explanation for the pathological role of \( K_{v4.3} \) \( K^+ \) channels, namely, the down-regulation of \( K_{v4.3} \) \( K^+ \) channels worsens these disease processes through activating CaMKII, resulting in more cardiomyocyte hypertrophy and apoptosis/necrosis. A combination of the present study and that by Keskanokwong et al. [21] suggests that the activation of CaMKII by \( K_{v4.3} \) \( K^+ \) channel inhibition is a common cellular process. Although we have discovered the \( K_{v4.3} \)/CaMKII/cell death pathway in HEK-293 and HEK-293T cells, the relevance of the finding to physiology or pathophysiology of cardiomyocytes needs to be identified further.

Our results from the present study showed that the extent of apoptosis and necrosis in HEK-293 and HEK-293T cells was different. HEK-293T cells expressing more \( K_{v4.3} \) \( K^+ \) channels showed more necrosis (a more serious cell injury) when \( K_{v4.3} \) \( K^+ \) channels were inhibited. Since CaMKII is coupled to \( K_{v4.3} \) \( K^+ \) channels, we speculate that more \( K_{v4.3} \) protein expression will couple more reserved CaMKII; when \( K_{v4.3} \) \( K^+ \) channels are inhibited, \( K_{v4.3} \) uncoupling will release more CaMKII, leading to more CaMKII activation and an increase in the apoptosis–necrosis switch.

The polyomaviruses BK virus, JC virus and SV40 encode the large T-antigen, which induces tumours in various animal models and humans [31]. The present study suggests that inhibition of \( K_{v4.3} \) \( K^+ \) channels could be a potential method to treat polyomavirus-associated tumours.

**AUTHOR CONTRIBUTION**

Qi Li, Ying Zhang, Yue Sheng, Rong Huo, Bo Sun, Xue Teng and Na Li performed the cell culture, Western blot and cell viability assay experiments. Ying Zhang and Rong Huo performed the real-time PCR experiments. Ying Zhang, Qi Li and De-Li Dong performed the patch-clamp experiments. Bao-Feng Yang gave constructive suggestions. De-Li Dong performed the real-time PCR experiments. Ying Zhang, Qi Li, Yue Sheng, Rong Huo, Bo Sun, Xue Teng and Na Li performed the cell culture, Western blot and cell viability assay experiments. Ying Zhang and Rong Huo performed the real-time PCR experiments. Ying Zhang, Qi Li, Yue Sheng, Rong Huo, Bo Sun, Xue Teng and Na Li performed the cell culture, Western blot and cell viability assay experiments. Ying Zhang and Rong Huo performed the real-time PCR experiments. Ying Zhang, Qi Li, Yue Sheng, Rong Huo, Bo Sun, Xue Teng and Na Li performed the cell culture, Western blot and cell viability assay experiments.
Large T-antigen up-regulates Kv4.3 and Kv4.3 and contributes to cell death through CaMKII


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SUPPLEMENTARY ONLINE DATA

Large T-antigen up-regulates Kv4.3 K\(^+\) channels through Sp1, and Kv4.3 K\(^+\) channels contribute to cell apoptosis and necrosis through activation of calcium/calmodulin-dependent protein kinase II

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Figure S1 Comparison of basal caspase-3 activity of HEK-293 and HEK-293T cells

The basal caspase-3 activity of HEK-293T cells was higher than that of HEK-293 cells. **P < 0.01 compared with HEK-293 cells.

Figure S2 Comparison of basal caspase-3 activity of HEK-293 and HEK-293T cells when both cells were treated with scrambled siRNA

Under these conditions, the basal caspase-3 activity of HEK-293T cells was still higher than that of HEK-293 cells. **P < 0.01 compared with HEK-293.

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Figure S3  4-AP (4 mM) treatment for 24 h had no effect on Bax and Bcl-2 protein expression in HEK-293 and HEK-293T cells.