SPANDIDOS PUBLICATIONS

Pharmacological separation of hEAG and hERG K^+ channel function in the human mammary carcinoma cell line MCF-7

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Abstract. Pharmacological inhibitors of the human ether-a-go-go (hEAG) potassium channel, astemizole and imipramine, have been used to demonstrate that hEAG plays a role in cancer cell proliferation. Astemizole and imipramine are, however, relatively non-specific ion channel blockers, as astemizole can also block the related potassium channel, human ether-a-go-go-related (hERG). Therefore, we aimed to determine the molecular target of astemizole, in the human mammary carcinoma cell line MCF-7. We initially confirmed the expression of KCNH1 and KCNH2 mRNA and hEAG and hERG channel protein in MCF-7 cells. Using a [^{3}H]-thymidine incorporation assay we determined that astemizole inhibited MCF-7 cell proliferation, whereas the hERG-specific channel blocker E-4031 had no effect. We then determined that E-4031 inhibited the regulatory volume decrease (RVD) observed in these cells following exposure to hypotonic solutions, confirming that functional hERG channels are present and may be important for cell volume regulation in MCF-7 cells. Our results suggest, for the first time, that hERG is involved in cell volume regulation. In addition, the function of hEAG and hERG in MCF-7 cell proliferation can be separated pharmacologically by utilizing the channel inhibitors astemizole and E-4031. The hEAG channel function in MCF-7 cells appears to be involved in the regulation of cell proliferation, whereas hERG is involved in cell volume regulation.

Introduction

The aberrant expression of specific potassium (K^+) channels has been found in many tumours of different histological origins (1). For example, expression of the human ether a go-go (hEAG) K^+ channel has been identified in cancers from the breast, brain, skin, soft tissue, cervix and colon, but not in normal tissues from the same regions (2-6). A related K^+ channel, the human ether a go-go-related (hERG) K^+ channel, has also been identified in many primary carcinomas, but not in adjacent normal tissue (7-12). The expression of hEAG and hERG, which are protein products of KCNH1 and KCNH2 genes respectively, has also been demonstrated in human cancer cell lines including the breast cancer cell line MCF-7 (3,13). Although there have been some insights into how the aberrant expression of certain K^+ channels occurs and affects cancer cells (14), it is still poorly understood.

In proliferating cells the function of certain K^+ channels is important for regulating cell cycle progression (1,15-17). Inhibiting the activity of specific K^+ channels often reduces cell proliferation and arrests cells at a certain phase of the cell cycle. For example, pharmacologically-inhibiting hEAG-channel current with channel blockers reduces the proliferation of many different cancer cell lines and arrests cells in the G_1 phase (3,18-20). In many cases, blocking hERG-channel current with pharmacological agents also inhibits cell proliferation (9,12,21). In uterine cancer cells, the blocking hERG channels arrests cells in the G_2/M phase (12), but in leukemic cells arrest occurs during the G_1/S transition (22). In addition, RNA interference (RNAi) has been used to demonstrate the importance of hEAG and hERG for cancer cell proliferation. Short interfering RNA (siRNA) molecules targeted against KCNH1 (23) and KCNH2 (14) mRNA reduce the proliferation of numerous cancerous and non-cancerous cell lines expressing KCNH1 or KCNH2, respectively. The aberrant overexpression of hEAG and hERG K^+ channels in many cancer cells, combined with their functional role in regulating cancer cell proliferation, implicates them in the pathophysiology of cancer (16,17).

One mechanism proposed to link K^+ channel activity with cellular proliferation is via alterations in the cell volume. Normally, cells maintain an optimum volume despite changes in their extracellular environment by activating membrane transport proteins that provide the required gain or loss of electrolytes and water. Activation of these membrane transport proteins results in a net regulatory volume increase (RVI) or decrease (RVD) as necessary (24). Changes in the cell volume occur as cells progress through the cell cycle, accompanied

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by changes in K^+ channel activity (25,26), while the ability of cells to undergo RVD reportedly also changes as cells progress through the cell cycle (27). Therefore, K^+ channel activity, volume regulation and cellular proliferation may be interconnected and their relationship altered in cancerous cells.

To study the role of hEAG and hERG in cell cancer cell proliferation the pharmacological agents E-4031, astemizole and imipramine have frequently been employed. E-4031 is a selective hERG channel blocker when used at nanomolar concentrations (28). At a concentration of 1 μM, E-4031 has been demonstrated to block the proliferation of neoplastic hematopoietic cells and numerous uterine cancer cell lines expressing hERG channels (9,12). Astemizole, a non-sedating anti-histaminergic drug and imipramine, a tricyclic antidepressant, block hEAG channel currents with IC_{50} values of ~200 nM and 2 μM, respectively (29). Astemizole (3-10 μM) and imipramine (10-30 μM) have been shown to block the proliferation of MCF-7 and IGR1 melanoma cells (3,18-20,23). However, astemizole and imipramine are relatively non-selective channel blockers. Astemizole blocks not only hEAG, but also hERG channels (28,30). The sensitivity of hERG channels to astemizole [IC_{50} of ~1.5 nM in native heart cells (31)] is much greater than that of hEAG channels. In addition to blocking hEAG channels, imipramine also blocks hERG (32) and chloride channels (18), and some members of the calcium-activated K^+ channel family (K_{Ca}) (33,34). Therefore, in cells that express the two channels, it cannot be firmly concluded that the anti-proliferative effect of astemizole or imipramine occurs via the blockade of hEAG and not hERG. However, since more selective hEAG channel blockers are not available, the former drugs continue to be widely used in the study of K^+ channel regulation of cell proliferation (18,20).

In the present study, we sought to determine the contribution of hEAG and hERG K^+ channels in the regulation of cell proliferation in MCF-7 cells, the mammary adenocarcinoma cell line commonly used to study the pathophysiology of breast cancer. Potassium channel activity is known to be involved in the proliferation and in the RVD response of these cells, since the two processes are inhibited by non-specific K^+ channel blockers (35,36) Our observations suggest that hEAG is involved in the proliferation of MCF-7 cells, whereas hERG is involved in cell volume regulation.

Materials and methods

Cell culture. MCF-7 cells were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, USA), routinely cultured in minimum essential medium (MEM) containing 5% fetal bovine serum (FBS), 10 μM non-essential amino acids, penicillin (100 U/ml), streptomycin (100 U/ml) (Invitrogen, Burlington, ON, Canada) and 100 μg/ml human insulin (Sigma-Aldrich, Oakville, ON, Canada), and maintained in a humidified atmosphere at 37˚C with 5% CO_2. The culture medium was renewed every 2 days. For RNA extraction and Western blotting, $1x10^6$ cells were seeded in 10-cm dishes. For proliferation assays, $2.5x10^4$ cells were seeded in each well of a 24-well plate. For cell volume measurements, the dissociated cells were plated on glass coverslips 4-7 h before the experiments were conducted.

RNA extraction. Total RNA was extracted from MCF-7 cells using a TRIzol™ reagent (Invitrogen). RNA was DNase-treated with a Turbo DNA-free™ kit (Ambion, Austin, TX) according to the manufacturer's protocol to remove any contaminating DNA.

Reverse transcription and polymerase chain reaction (RT-PCR). RNA (2 μg) was reverse transcribed using an M-MLV reverse transcriptase enzyme (200 units, Invitrogen), 5 mM dNTP (Invitrogen) and 1 μM oligo(dT) (GE Healthcare Life Sciences, Baie d'Urfé, PQ, Canada) in a total volume of 100 μl. PCR was used to detect the expression of KCNH1 and KCNH2 mRNA transcripts. Custom primers (Invitrogen) were designed to amplify specific regions in the two transcripts and the sequences used were: KCNH1 forward 5'-GTGGTGGCC ATTCTAGGAAA-3' and reverse primer 5'-GGAGAAGGA ATGGGAGAAGG-3', and KCNH2 forward 5'-ATGTGAC GGCGCTCTACTTC-3' and reverse primer 5'-GAGTACA GCCGCTGGATGAT-3'. PCR-amplification was performed in a total volume of 25 μl containing 100 pmol of corresponding forward and reverse primers, 200 μM dNTP mix (Invitrogen), 1X PCR buffer (Fermentas Canada, Burlington, ON, Canada), 1.5 mM $MgCl_2$ (Fermentas), 2.5 units Taq DNA polymerase (Fermentas) and 2 μl cDNA. Following an initial denaturation at 95˚C for 1 min, samples were denatured at 94˚C for 45 sec, allowed to anneal at 60˚C for 45 sec and extended at 72˚C for 1 min for 40 cycles. The reactions were completed by a final extension at 72˚C for 10 min. PCR products (12.5 μl) were visualized by gel electrophoresis using 1.5% agarose gels stained with ethidium bromide. Visualized PCR amplicons were gel-extracted using a QIAquick gel extraction kit (Qiagen, Mississauga, ON, Canada) and sequenced by a commercial sequencing facility to confirm identity (DalGEN Microbial Genomics Centre, Dalhousie University, Halifax, NS, Canada).

Western blotting. Total protein lysate was extracted from MCF-7 cells. Briefly, 85-90% confluent monolayers of MCF-7 cells were rinsed twice with PBS, detached using cell scrapers and spun down to form a pellet. Cell pellets were resuspended in RIPA lysis buffer containing protease inhibitors (Roche Applied Science, Indianapolis, IN) and incubated on ice for 30 min. Finally, the protein sample was centrifuged at 10,000 x g for 15 min, and the supernatant containing total cellular protein stored at -80˚C. Protein concentrations were measured using the Bradford method (Bio-Rad, Hercules, CA). Immunoblotting for the hEAG1 and hERG proteins was performed using rabbit anti-human EAG1 and ERG primary antibodies (Alomone Labs, Jerusalem, Israel) at 1:200 dilution, and horseradish peroxide-conjugated goat anti-rabbit IgG secondary antibody (Jackson Immunoresearch, West Grove, PA) at a 1:5,000 dilution. An ECL Plus kit (GE Healthcare Life Sciences) was used for detection.

Drugs. Astemizole, imipramine and E-4031 were all obtained from Sigma-Aldrich. ERGtoxin was obtained from Alomone labs. Astemizole stock solution (100 mM) was made in DMSO, whereas imipramine (100 mM) and E-4031 (3 mM) stocks were made in filter-sterilized H_2O. Drug stocks were diluted in culture medium to reach final concentration.

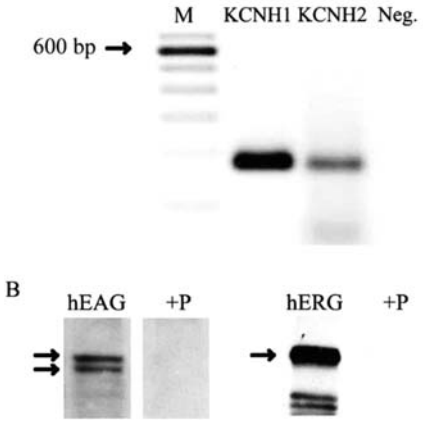

Figure 1. Molecular identification of KCNH1 and KCNH2 mRNA and protein in MCF-7 cells. (A) MCF-7 cells express mRNA transcripts for KCNH1 (lane 1, 177 bp) and KCNH2 (lane 2, 172 bp). Lane M is a DNA ladder and lane 3 is the negative control for the PCR reaction. (B) Western blotting confirms the presence of the two glycosylated forms of the hEAG (lane 1, arrows indicate 130 and 110 kDa) and hERG protein (lane 3, arrow indicates 155 kDa) in total cell lysate from MCF-7 cells. When primary antibodies were incubated with hEAG and hERG control antigen prior to immunoblotting, no protein was detected (lanes 2 and 4, respectively), confirming the specificity of the antibodies and the identity of protein bands detected.

ERGtoxin (1 μM) stock solution was made in 0.1% bovine serum albumin, 100 mM sodium chloride and 10 nM Tris. The final concentration of DMSO vehicle did not exceed 0.003% (v/v).

[^{3}H]-thymidine incorporation assay. The incorporation of radioactive thymidine into DNA was used to assess the ability of hEAG and hERG channel blockers to inhibit MCF-7 cell cycle progression through the S phase. For the [^{3}H]-thymidine incorporation assays, cells were first seeded in regular 5%-FBS MEM-containing supplements. After 24 h the media were changed to 5%-charcoal/dextran-FBS (DCC-FBS, Hyclone, Logan, UT) in phenol red-free MEM-(Invitrogen) containing supplements for 72 h. Cells were then partially synchronized in 1%-DCC-FBS phenol red-free MEM-containing supplements for 48 h. Following synchronization, the cells were treated with astemizole, imipramine, E-4031 or ERGtoxin for at least 36 h in 5%-DCC-FBS phenol red-free MEM-containing supplements in the presence of 1 μCi/ml [methyl-^{3}H]-thymidine (TRK-300, GE Healthcare Life Sciences) and 1 μM non-radioactive thymidine. DNA was extracted and radioactivity was measured as previously described (37). Briefly, cells were placed on ice and visually inspected under a microscope for signs of cytotoxicity. The cells were washed twice with 1 ml of cold PBS and then incubated on ice in 0.5 ml of cold 10% (w/v) trichloroacetic acid (TCA) for at least 1 h. The TCA was removed and the wells were rinsed with 1 ml 100% ethanol and air dried. Finally, 0.1 M NaOH with 1% sodium dodecyl sulfate was used to solubilize the macromolecules. The solution was transferred to scintillation vials containing 2 ml scintillation fluid that had been acidified with TCA to neutralize the NaOH. Radioactivity was measured on a Beckman LS 5000TA scintillation counter (Beckman Coulter Canada, Mississauga, ON, Canada) as counts per minute (cpm). For each experiment, each treatment was performed on 6 separate wells. The CPM values were converted to a percentage of control values on the same plate. Each experiment was performed independently 5-11 times.

Cell volume measurements. Cell volume measurements were performed as previously described (36). Briefly, MCF-7 cells were plated on glass coverslips and equilibrated in an isosmotic solution (in mM: 75 NaCl, 2.5 KCl, 1.2 $CaCl_2$, 0.5 $MgCl_2$, 5 glucose, 10 HEPES, 150 mannitol and pH 7.4) for 5 min. A hyposmotic solution (in mM: 75 NaCl, 2.5 KCl, 1.2 $CaCl_2$, 0.5 $MgCl_2$, 5 glucose, 10 HEPES and pH 7.4) was then applied containing vehicle (DMSO), imipramine, astemizole or E-4031. Images of single cells were taken every 60 sec for 20 min. The area of individual spherical cells was determined from captured images and the radius was determined using the equation, radius = $\sqrt{}$ (area/π). Cell volume was calculated from the radius using the equation, volume = 4/3 x π x radius^3. Cell volumes were normalized to time zero and the percentage RVD was calculated using the equation, percent RVD = 100[1-(V_{min}-1)/(V_{max}-1)], where V_{max} is the maximum volume in the hyposmotic solution and V_{min} is the minimum volume reached before returning to the isosmotic solution.

Statistical analysis. Data were analyzed using Student's t-test for unpaired values. For the [^{3}H]-thymidine incorporation experiments, the Student's t-test for unpaired values was performed to determine the significance of changes in cell proliferation for each trial (6 control vs. 6 treated wells) prior to data normalization. A value of $P<0.05$ was considered significant.

Results

Molecular identification of KCNH1 and KCNH2 mRNA and protein in MCF-7 cells. RT-PCR was used to detect KCNH1 and KCNH2 mRNA expression from the total MCF-7 RNA (Fig. 1A). For KCNH1, the primers amplified a 177 bp amplicon spanning exons 9-10 of the two transcript variants (Ref Seq accession: NM 172362 and NM 002238). For KCNH2, the primers amplified a 172 bp amplicon spanning exons 7-8 of transcript variant 1 (NM 000238), exons 3-4 of transcript variant 2 (NM 172056) and exons 8-9 of transcript variant 3 (NM 172057). KCNH1 and KCNH2 amplicons were excised from the gel, isolated and sequenced, confirming the predicted sequence was amplified (data not shown) and that MCF-7 cells express mRNA transcripts for KCNH1 and KCNH2 K^+ channels. Western blotting confirmed that MCF-7 cells translate KCNH1 and KCNH2 mRNA into the hEAG and hERG protein (Fig. 1B). It has previously been shown that hEAG can be detected as two glycosylated forms (130 and 110 kDa) (38). Western blotting results confirm the presence of the two glycosylated forms of hEAG in the MCF-7 total cellular protein. hERG was detected as a predominant protein band of ~155 kDa in the MCF-7 total protein. Two other protein bands of lower molecular weight (~110-120 kDa) were also detected. Specificity of the hEAG and hERG antibodies was confirmed by pre-incubation of the primary antibodies with their respective control antigens prior to Western blotting (Fig. 1B).

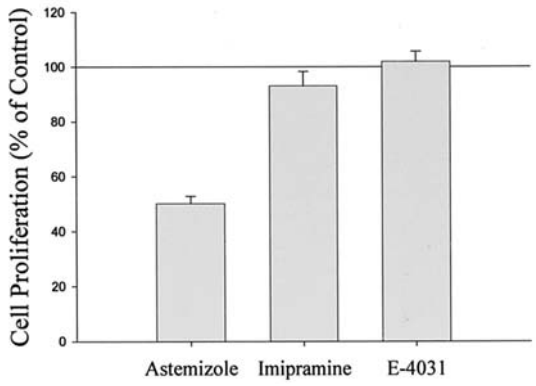

Figure 2. Proliferation of MCF-7 cells in the presence of hEAG and hERG channel blockers. The rate of DNA synthesis was measured using [^{3}H]-thymidine incorporation. Concentrations of astemizole, imipramine and E-4031 used were 30 μM, 100 μM and 300 nM, respectively. The mean data from 5-11 experiments is shown. The error bars are the standard error of the mean (SEM).

Role of hEAG and hERG in MCF-7 proliferation. The [^{3}H]-thymidine incorporation method was used to investigate the effects of astemizole, imipramine and E-4031 on the proliferation of MCF-7 cells. Fig. 2 shows the data averaged across 5-11 independent experiments. Consistent with previous reports (3,19,20), astemizole (3 μM) decreased the proliferation of MCF-7 cells (Fig. 2). Compared to the control conditions, astemizole decreased [^{3}H]-thymidine incorporation, over the 36-h treatment period by ~50% (Fig. 2). This effect of astemizole was consistent across all trials ($P<0.001$, n=11). Imipramine (20 μM) caused only a very small decrease in [^{3}H]-thymidine incorporation when averaged across all trials (Fig. 2). However, in some imipramine trials significant decreases in [^{3}H]-thymidine incorporation were observed. In four out of nine trials, imipramine significantly decreased [^{3}H]-thymidine incorporation ($P<0.05$, range 0.007-0.031), whereas in five out of nine trials a level of significance was not attained ($P>0.05$). The specific hERG channel blocker, E-4031, had no effect on [^{3}H]-thymidine incorporation even at high concentrations (3 μM, Fig. 2). The results with E-4031 were consistent, in five out of five trials with $P>0.05$. We also investigated the effect of ERGtoxin, a hERG-specific peptide channel blocker (39). ERGtoxin at concentrations as high as 300 nM did not significantly reduce [^{3}H]-thymidine incorporation (data not shown). These [^{3}H]-thymidine incorporation results suggest that hERG does not contribute to the proliferation of MCF-7 cells.

Role of hEAG and hERG in MCF-7 cell volume regulation. The lack of effect of E-4031 on cell proliferation raises the question of whether hERG channels are functional in MCF-7 cells. It has previously been shown that KCNQ1 K^+ channels are involved in the RVD of MCF-7 cells (36). However, the involvement of hEAG and hERG has yet to be reported. Upon exposure to hyposmotic solution, MCF-7 cells initially increased in size and then underwent an RVD response (Fig. 3A, solid circles). In contrast, when MCF-7 cells were exposed to the hyposmotic solution containing E-4031 (300 nM) they increased in size but failed to undergo RVD (Fig. 3A, open circles). Similar results were obtained with

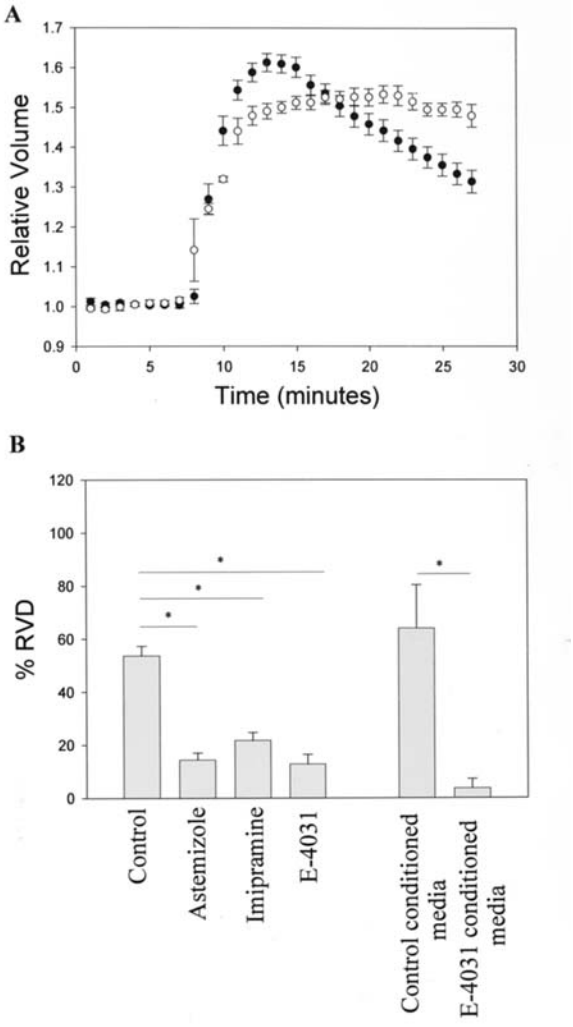

Figure 3. Regulatory volume decrease of MCF-7 cells in the presence of hEAG and hERG channel blockers. Concentrations of astemizole, imipramine and E-4031 used were 30, 100 and 300 μM, respectively. (A) The solid circles are the mean relative cell volume of control cells and the open circles are cells treated with E-4031. (B) The mean data from 4-21 experiments converted to % RVD. The error bars are the SEM, $^*P<0.05$.

astemizole (30 μM) and imipramine (100 μM). Fig. 3B is a summary of the data showing the significant inhibition of the RVD response by astemizole, imipramine and E-4031. These results suggest that hERG is involved in cell volume regulation, and therefore demonstrates that hERG channels are functional in MCF-7 cells.

One possible explanation for the inability of E-4031 to inhibit cell proliferation may be drug instability during the long-term culture. Therefore, we treated MCF-7 cells in culture with 3 μM E-4031 or control media for 36 h, following which the E-4031 containing and control media were removed from the cells, diluted 10-fold with hyposmotic solution (final E-4031 concentration 300 nM) and applied to isolated MCF-7 cells. E-4031 pre-incubated with MCF-7 cells in this way inhibited the RVD response as before ($P<0.05$, Fig. 3B), confirming that E-4031 is still active and stable in the culture after at least 36 h. The RVD response of MCF-7 cells treated with a 10-fold dilution of the control media was not significantly different ($P>0.1$) to the control cells treated with hyposmotic solution alone.

The aberrant expression of hEAG and hERG K^+ channels has been identified in numerous cancer types. In cancer cells, it has been suggested that the functional activity of hEAG and hERG contributes to cell proliferation (1,40,41). This observation is based primarily on the fact that hEAG and hERG channel blockers (astemizole, imipramine and E-4031), as well as siRNA which is targeted against the respective channel genes, inhibit cancer cell proliferation (see Introduction). However, astemizole and imipramine inhibit hEAG and hERG. Therefore, in cells that express the two channels, such as MCF-7 cells (Fig. 1), the anti-proliferative effect of these drugs cannot easily be attributed to the inhibition of a single channel type.

In MCF-7 cells, the cause of the anti-proliferative effect of astemizole appears to be the inhibition of hEAG. Astemizole (3 μM) inhibited thymidine incorporation by ~50%, whereas E-4031 had no effect, even at micromolar concentrations (Fig. 2). This suggests that hEAG, but not hERG is involved in MCF-7 cell proliferation. This is in contrast to other reports that suggest that hERG channels, aberrantly expressed in cancer cells, contribute to the increased cell proliferation phenotype of these cells (1,40,41). It is unclear why hERG does not contribute to the proliferation of MCF-7 cells. However, it is possible that the functional effects of hEAG and hERG in cancer cell proliferation are cell-type specific. The minor inconsistent effect of imipramine on MCF-7 proliferation when averaged across the nine independent experiments most likely reflects that this agent is a less potent hEAG channel blocker. Nevertheless, in four out of nine trials imipramine significantly decreased [^{3}H]-thymidine incorporation compared to the controls. We therefore suggest that imipramine, at a concentration of 20 μM, is on the threshold of effectively inhibiting cell proliferation via the blockade of hEAG. Higher doses of imipramine were tested but were cytotoxic, which is in agreement with previous reports that suggest that imipramine at concentrations >20 μM causes apoptosis (18).

Despite their apparent lack of involvement in cell proliferation, functional hERG channels appear to be present in MCF-7 cells, as indicated by their involvement in cell volume regulation. The presence of E-4031 (300 nM) clearly inhibits the ability of MCF-7 cells to undergo RVD following their initial swelling response (Fig. 3A and B). RVD is a homeostatic mechanism which permits cells to maintain a constant cell volume, essential for normal cell function. When a cell encounters a hyposmotic environment, it initially swells prior to undergoing an RVD response resulting from the activation of K^+ and Cl^- channels. While numerous K^+ channels have been indicated in RVD (24), previous studies have shown that the ability of MCF-7 cells to undergo RVD was dependent on the K^+ efflux via KCNQ1 channels (36). In addition to demonstrating the importance of an additional K^+ channel type in mediating RVD in MCF-7 cells, we believe these data are the first indicating a functional role for hERG in RVD in any cell type.

Changes in cell volume have been suggested to be important in regulating passage through the cell cycle (24). Our results demonstrate that in MCF-7 cells, regulatory cell volume changes are inhibited by the hERG channel blocker E-4031, although blocking hERG with E-4031 has no effect on cell proliferation. These results suggest that cell volume changes sensitive to K^+ channel inhibitors may not be essential for cell proliferation in MCF-7 cells. Consistent with this hypothesis, the KCNQ1 channel blocker chromanol 293B, which inhibits the RVD response in MCF-7 cells (36), has no effect on the proliferation of these cells (data not shown).

In our assay astemizole and imipramine inhibited the RVD response (Fig. 3). This result may be the consequence of either hEAG or hERG current inhibition and since there are no hEAG channel-specific blockers currently available, we cannot, in this case, pharmacologically separate the function of hEAG and hERG channels.

In conclusion, by using astemizole and E-4031, we have demonstrated that the function of hEAG and hERG channels can be separated pharmacologically. Our results are in agreement with other studies that suggest that hEAG channel function is involved in the proliferation of cancer cells (3,20,23,42). We also demonstrate that, in MCF-7 cells, functional hERG channels are not involved in cell proliferation, although they appear to be involved in cell volume regulation. Therefore, in MCF-7 cells hEAG and hERG are functional channels that play important roles in cell proliferation and cell volume regulation, respectively.

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