

Functional expression of potassium channels in cardiomyocytes derived from embryonic stem cells

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Abstract

Royan B₁ stem cell can be differentiated to specialized cell types including cardiomyocytes. This developmental change is accompanied with expression of various K⁺ channel types. The aim of this study was to detect functional expression of K⁺ currents from stem cell stage and one week and two weeks after differentiation into cardiomyocyte. Mouse stem cell derived cardiomyocytes (ES-cardiomyocytes) were isolated to single cell suspension for K⁺ current recording using whole cell patch-clamp technique. The predominant depolarizing current in ES-cardiomyocytes was a tetraethylammonium (TEA) (10 mM) sensitive current which was partially blocked by nifedipine (1 μM) and attenuated by increasing concentration of EGTA (10 mM) in the pipette solution. Pharmacology and electrophysiological properties of this oscillatory sustained current very well matched with characteristics of Ca²⁺ activated K⁺ current. In addition there was another kind of sustained outward K⁺ current which was resistance to TEA but was inhibited by 3,4-diaminopyridine. The characteristic features of this current indicate that this current was due to activation of delayed rectifier K⁺ channels. RT-PCR study also confirmed expression of these two types of K⁺ channels in ES-cardiomyocytes. Therefore, present study shows functional expression of two types of K⁺ ionic current in ES-cardiomyocytes.

Keywords: Embryonic stem cells; Royan B₁ line; Cardiomyocytes; K⁺ current; Patch-clamp; RT-PCR

INTRODUCTION

Embryonic stem cells are pluripotent cell lines initially derived from the inner cell mass of mouse blastocyst stage embryos and can be differentiated to various cell types including cardiomyocytes (1-4). These cells express specific markers and display similarities to their adult counterparts (5,6). Embryonic stem cell derived cardiomyocytes (ES-cardiomyocytes) have been suggested as an important pharmacological research tool to study early steps in cardiomyocytes development, as well as for drug development and toxicity testing. During cardiomyocytes development *in vitro*, early embryonic cardiomyocytes show spontaneous beating that disappears at a later stage (5,6). Significant changes in voltage gated ion

channels have been recognised in developing heart (6). Calcium, potassium and sodium ion channels in the cardiomyocytes are also one of the targets for drug discovery (5). In addition one of the major adverse effects of drugs that induce sudden cardiac death is associated with prolongation of QT interval in the electrocardiogram (7,8). Although prolongation of QT interval can occur through modulation of several types of ion channels, inhibition of the delayed rectifier K⁺ current which is conducted by human *ether-à-go-go* related gene (h-erg) potassium channel, is the most common mechanism responsible for drug induced QT prolongation in human. The h-erg forms the major portion of ion channel proteins of the 'rapid' delayed rectifier current that conducts K⁺ ions out of the muscle cells of the heart and

this current is critical in correctly timing to return to the resting state (repolarization) of the cell membrane during the cardiac action potential (9). This channel is also sensitive to drug binding, as well as decreased extracellular potassium levels, both of which can result in decreased channel function and drug induced long QT syndrome (7,8). Therefore, preclinical testing of new drugs on ion channels, and specially the h-erg channel is now an important part of safety screening and that is why a major area of the cell-based *in vitro* toxicity testing is cardiotoxicity. The current method of choice for obtaining high quality data of the functional effect of drugs at ion channels is patch-clamp technique (10). For application of patch-clamp, either isolated cells from myocardium or a specific cell line is needed. Studies of ionic channel expression during the early period of development of mammals embryos are limited because of small size of the embryonic heart and by lack of existence of specific permanent cell line to model of the earliest stages of cardiomyogenesis (5). Disadvantages of cardiomyocyte cell lines which are driven from myocardial tumors or by virus transfection are that they can be passaged only for a limited time and furthermore they don't express all types of ion channels and the characteristics of a typical cardiomyocyte (5). An alternative approach to study the early stages of cardiac myogenesis and drug toxicity testing is implementation of ES-cardiomyocytes (2-4, 6,7).

ES-cardiomyocytes are capable of exhibiting action potential resembling the same shapes and pharmacological properties with those described for adult cardiomyocytes of ventricular, atrial and sinus nodal types (11). Differentiation of stem cell to cardiomyocytes can be confirmed by various means including morphology at ultra-structural level, gene and specific protein expression and cell function (beating activity). In this regard previous studies showed that ES-cardiomyocytes present spindle, round and tri- or multiannular morphology with characteristic striations of sarcomeric structures of cardiac muscle cells, in addition to presenting Z-disk specific protein (α -actinin, desmin, and troponin) (2-4, 12). These authors further elucidated that 95% of the

protein detected on the stem cell derived cardiomyocytes and neonatal derived cardiomyocytes precisely paired with one another, whereas only 20% of these proteins matched up with undifferentiated stem cells. In addition, RT-PCR of differentiated cardiomyocytes shows the expression of cardiac specific proteins such as cardiac α - and β -myosin heavy chain, myosin light chain-2, ventricle and atrial natriuretic factor (2-4, 12). In contrast to existence of many reports on expression of ionic channel protein in cardiomyocyte derived stem cells, there are only few reports which functionally has characterised these channels. It had been reported that the early differentiated cardio-myocytes exhibit an outward rectifying transient K^+ current sensitive to 4-aminopyridine, and an inward Ca^{2+} current but no Na^+ current. The Ca^{2+} current shows all features of L-type and T-type currents. In addition, an inward rectifying currents, acetylcholine-induced and ATP-modulated K^+ current also has been reported (1,11, 13-17). In the previous reports existence of one type of K^+ currents in Royan B1 ES-cardiomyocytes was demonstrated (18). The aim of the present study was to further investigate functional expression of two types of outward K^+ currents, including the delayed rectifier K^+ channels, during early stage of ES-cardiomyocytes derivation from genetically modified Royan B₁ stem cell line (α -MHC-GFP-puromycin resistant), in hope to further approve the usage of ES-cardiomyocytes for cardiotoxicity screening.

MATERIALS AND METHODS

Mouse embryonic stem cell culture

Royan B₁-MHC-GFP stem cells were kept in an undifferentiated state by culturing on a feeder layer of mitomycin C treated mouse embryonic fibroblast in ES medium containing Dulbecco's modified eagle medium (DMEM, Gibco 10829-018), supplemented with 15% fetal calf serum, 0.1 mM beta-mercaptoethanol, 2 mM glutamine, 0.1 mM non essential amino acids and 1000 iu/ml leukemia inhibitory factor.

Stem cell differentiation into cardiomyocyte

The ES cells were differentiated into beating cardiomyocytes by hanging drop

method as previously described (2-4, 18,19). On day 7 for obtaining pure cardiomyocytes, embryonic bodies were cultured on 24-well plates (TPP, Switzerland) in presence of puromycin (1 µg/ml) for 7 ± 1 days or 14 ± 1 days.

Cell preparation for patch-clamping

On the day of experiment, the embryonic stem cells and ES-cardiomyocytes were trypsinized for 3 min at 37°C. Cells were suspended in HEPES solution (see solutions) and kept at 4°C to be used for patch-clamp recording.

Recording

Aliquot of cell suspension (0.2 ml) was placed into the recording chamber and left to settle on to the bottom of the chamber. Calcium bath solution was perfused continuously through the chamber by gravity flow at rate of 2 ml/min. All drugs were applied via the bath solution by switching the inflow to another reservoir.

Membrane currents were recorded by the whole-cell configuration of the patch-clamp technique (20). Patch pipettes were pulled from borosilicate glass capillaries (1.5 mm O.D. x 0.85 mm I.D.; Hirschmann Laborgerate) using a two stage pipette puller (L/M-3P-A patch-clamp puller) and then fire-polished (L/M-CPZ-101 Coating and Polishing System). Pipettes were filled with pipette solution and secured into an electrode holder with a silver-silver chloride wire electrode making contact with the pipette solution. A silver-silver chloride ground electrode was placed directly into the bath solution to complete the circuit.

Cells were viewed using an inverted microscope (Leica Microscope DM-IIRB) placed on an air table (T-250 Vibration Isolation Laboratory table). A hydraulic micromanipulator (MHW-3-1) and a coarse manipulator (Narishige) fixed onto the side of the microscope were used to lower the electrode into the bathing solution and onto the cell membrane. Once the pipette was in contact with the bathing solution the pipette current automatically was zeroed. A 10 mV step potential was then applied to the pipette and current flow through the pipette was measured on the oscilloscope and the pipette resistance calculated using Ohm's law ($V=IR$). Pipettes

with resistance of 1-4 MΩ were used for recording (20-22). The pipette tip was then placed onto the surface of a cell and a high resistance seal (giga-seal) was formed between the pipette glass and the cell membrane by applying gentle suction. Further suction was then applied to rupture the membrane within the pipette to allow access to the whole-cell. The holding potential was usually maintained at -60 mV and test voltage-steps were applied for 300 ms every 10 s.

In addition whenever possible resting membrane potential were recorded by switching the mode switch on current monitoring panel of amplifier to current clamp mode.

Current sampling and analysis

Membrane currents were recorded using a patch-clamp amplifier (L/M-EPC-7) which had a feedback resistor of 500 MΩ. Voltage-pulses were generated by a stimulator in the amplifier via a computer program. Signals filtered by a 10 KHz, 3 pole Bessel filter inserted into the current monitor pathway, viewed on an oscilloscope (Hameg HM 303-4) and capture on-line to a computer (IBM compatible Pentium 166 Mhz MMX). The software for data capture and analysis was Win Tida data acquisition and analysis software for windows (List-Electronic). A record consisted of a series of digital samples digitised by an analog-to-digital (A-D) convertor (ICT-16, Data Acquisition Interface) at a defined rate.

RT-PCR for gene expression analysis

Total RNA of cultured cells was extracted using the RNeasy Mini Kit (QIAGEN, Tehran, Iran) according to the manufacturer's protocol. The concentration of extracted RNA was determined by measuring the absorbance at 260 nm in a spectrophotometer (CeCil Instrument, England). RNA samples were digested with DNaseI (Fermentas, Iran) to remove contaminating genomic DNA. First strand cDNA synthesis was performed by RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Iran). cDNA synthesis reaction contained 2 µg of total RNA, 1 µL random hexamer primer (100 µM), 4 µL 5X reaction buffer, 2 µL dNTP Mix (10 mM), 1µL RNase inhibitor (20 U/µl) and 1 µl M-MuLV reverse

transcriptase; adjusted to a total volume of 20 μ l using DEPC treated water. The cDNA synthesis performed at 42°C for 1 h. After cDNA synthesis PCR performed for β -tubulin as housekeeping gene and *Kcnma1*, *Kcna1* as target genes. Primer sequences, annealing temperature and the lengths of amplified products are shown in Table 1. Amplification condition was as follows: initial denaturation at 95°C for 4 min followed by 35 cycles of denaturation at 95°C for 45 s, annealing for 45 s, extension for 1 min at 72°C and a final extension at 72°C for 10 min. PCR products were analyzed by gel electrophoresis on 2% agarose gel.

Patch-clamp solutions and drugs

HEPES storage solution (mM); NaCl 126, KCl 6.0, N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid] (HEPES) 10, CaCl₂ 0.05, and glucose 10, titrated to pH 7.4.

Bath solutions (mM): Ca²⁺ bath solution; NaCl 135, KCl 5.0, CaCl₂ 1.5, MgCl₂ 1.2, HEPES 10, glucose 10. Both HEPES and bath solutions were titrated to pH 7.4 with NaOH (1 M). Osmolarity was 203 \pm 5 mosm/kg.

Pipette solutions: K⁺ current recording (mM); KCl 130, MgCl₂ 2, HEPES 10, Na₂ATP 3, ethylene glycol-bis (β -aminoethyl ether) N,N,N',N'-tetra-acetic acid (EGTA) 0.2. The pipette solution was titrated to pH 7.4 with KOH (1 M). Osmolarity was 203 \pm 5 mosm/kg. The pipette solution was filtered with a 0.2 μ m pore syringe filter (Acrodisc, Gelman Sciences) and stored and frozen in 1 ml vials.

Tetraethylammonium chloride (TEA) (Sigma, China) was initially dissolved in distilled water as 100 mM stock solutions, and then diluted in bath solution to give 10 mM TEA. 3,4-diaminopyridine (Sigma) was initially dissolved in DMSO as 100 mM stock solutions, and then diluted in bath solution to give 1 mM 3,4-diaminopyridine solution. This solution contains 0.1% DMSO. Nifedipine (Sigma) was initially dissolved in DMSO as 10 mM stock solutions,

and then diluted in distilled water to give 1 mM stock solution. Then 50 μ l of this solution was dissolved in 50 ml bath solution to 1 μ M solution. This solution contains 0.01% DMSO.

EGTA prepared in distilled water as 1.5% stock solutions. To solubilized the EGTA this solution was titrated with NaOH (1 M) solution. This stock solution was used for preparation of pipette solution. Unless stated, all the chemicals were from Merck.

Data analysis

To analyze the patch-clamp records for each signal, a fixed zero level was defined. The voltage signal was measured relative to zero volts, while the current zero level was calculated from a portion of each record by averaging points on a defined cursor position at the holding potential level prior to the test voltage step. The current generated by stepping from the holding potential was analyzed by measuring the averaged current in a defined portion of the record.

The mean and standard error of mean (SEM) current for each test potential was calculated and plotted against the voltage (I/V curve). If appropriate, the data were statistically compared for differences using analysis of variance (ANOVA) and/or Student's *t*-test. Difference considered statistically significant for *P*<0.05.

RESULTS

In the current study we have investigated expression of potassium channels and ionic current activation in mouse embryonic stem cells and ES-cardiomyocytes. Undifferentiated stem cells had a resting membrane potential ranging from -21 mV to -40 mV (31 \pm 1.6, n=15). ES-cardiomyocytes relatively had a more negative resting potential ranging from -30 mV to -66 mV (42 \pm 5.4, n=228) (18). There are statistically significant differences between resting membrane potential of undifferentiated

Table 1. Primers that used for gene expression analysis by RT-PCR.

Gene symbol	Primer sequences (5'-3')	Size (bp)	Annealing temperature (°C)	Accession number
<i>Kcnma1</i>	TCGTCAAACCCAATAGAATCCT	108	65	NM-010610.2
	AATAAACCGCAAGCCAAAGTAG			
<i>Kcna1</i>	GCGTTACTGAGCCTGAGAC	164	65	NM-010595.3
	AATTCATAACAGCAGCACATC			
β -tubulin	TCACTGTGCCTGAACTTACC	318	63	NM-011655.5
	GGAACATAGCCGTAACCTGC			

stem cells and ES-cardiomyocytes (Student's *t*-test, $P < 0.05$). In this study we have looked at ion channel activity induced by depolarization. When the holding potential (HP) was -60 mV a voltage dependent outward current were elicited by square depolarizing pulses. This outward current had a turbulence appearance and was sustained for the duration of a 300 ms test-pulse. The current rectification was observed in the ES-cardiomyocytes beginning from -20 mV depolarization. Such current rectification was not observed in the undifferentiated stem cells and only slight linear current voltage relation was observed (Fig. 1). The maximum current that recorded from the stem cell was 0.2 nA while the maximum current amplitude between the ES-cardiomyocytes varied from 0.2 nA to above 1 nA, therefore we excluded cells with current amplitude below 0.3 nA for pharmacological studies because we assumed that they are possibly not mature cardiomyocytes.

To investigate the development of voltage activated K⁺ currents in ES-cardiomyocytes we compared the current amplitude in one week (7 ± 1 days) and two weeks (14 ± 1 days) old ES-cardiomyocytes and we found no difference in current amplitude between these two groups. For identification of the outward K⁺ current described above, cell chamber were perfused with 10 mM TEA for 10 min and then the same

stimulation protocol was repeated. After perfusion of TEA the sustained outward current was substantially inhibited in both one week (Fig. 2) and two weeks old ES-cardiomyocytes. For example in one old week ES-cardiomyocytes the sustained current was reversibly reduced from 2 nA to 0.24 nA by application of 10 mM TEA when cells were depolarized to +30 mV (Fig. 2A and 2B). After washing the cells with fresh bath solution this inhibitory effect of TEA was reversed (Fig. 2C).

Fig. 3 shows mean current-voltage relationship before and after perfusion of TEA in one week old ES-cardiomyocytes. For example, at +50 mV depolarizing step, TEA reduced the mean current by 78% ($n=6$). Despite this inhibitory effect of TEA, some times slight outward current remained which was resistance to TEA block. The fluctuating outward sustained current described above was similar to the currents reported for large conductance Ca²⁺ activated K⁺ currents. Therefore, Ca²⁺ dependency of this current was investigated by increasing concentration of intracellular EGTA to 10 mM. This investigation was made in two old weeks ES-cardiomyocytes, in presence of 0.2 and 10 mM EGTA. Before start recording, an extra 15 min time was allowed for EGTA to equilibrate with intracellular cell. In cells loaded with high EGTA concentration, not only the fluctuating

(A)



(B)

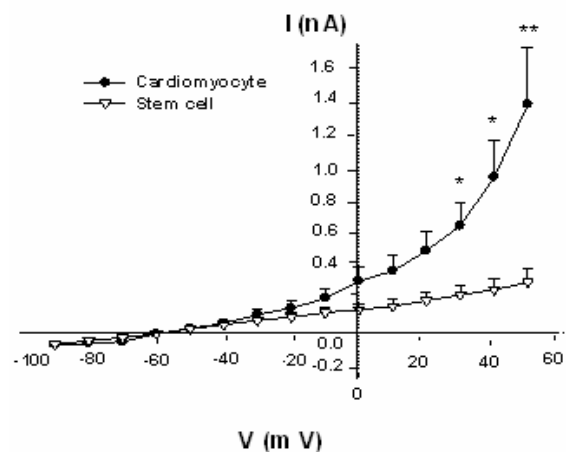


Fig. 1. (A) Current recorded from single undifferentiated stem cell when depolarized to +50 mV from holding potential of -60 mV (the recorded current is mainly leak current). (B) Current-voltage (I/V) curve for outward sustained current recorded from one old week ES-cardiomyocytes ($n=6$) compared with undifferentiated stem cells ($n=7$). The sustained current was elicited by depolarizing from holding potential of -60 mV to various test potentials for 300 ms. All the values are mean \pm SEM. Mean current amplitudes were measured relative to holding current at -60 mV. There are statistically significant differences between current amplitudes in these two groups of cells (Student's *t*-test, $*P < 0.05$, $**P < 0.01$).

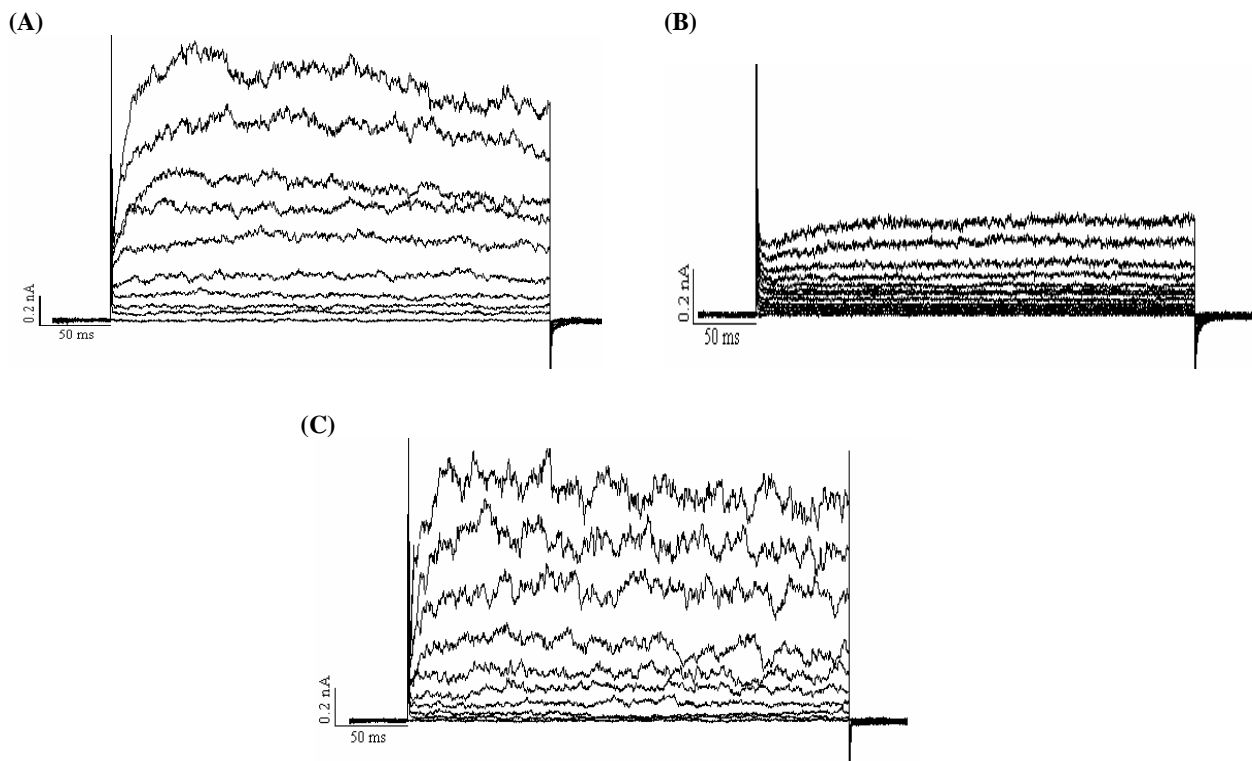


Fig. 2. Block of sustained outward current in 7 ± 1 days old week ES-cardiomyocytes by TEA with 0.2 mM EGTA in KCl recording pipette. The sustained current was elicited by depolarising from the holding potential (-60 mV) to test potential up to +50 mV for 300 ms using 10 mV step increments. (A) Original control current records. (B) TEA (10 mM) blocked this current. (C) Current recorded after wash.

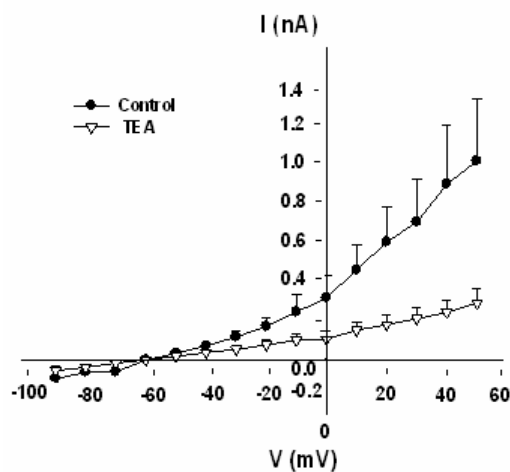


Fig. 3. Block of sustained outward current by TEA with 0.2 mM EGTA in recording pipette in 7 ± 1 days in ES-cardiomyocytes. Current-voltage (I/V) curve of the sustained current in control (circle) and presence of 10 mM TEA (triangle). The sustained current was elicited by depolarising from the holding potential (-60 mV) up to test potential of +50 mV for 300 ms using 10 mV step increment. All the values are mean \pm SEM (n=6). Mean current amplitudes were measured relative to the holding current.

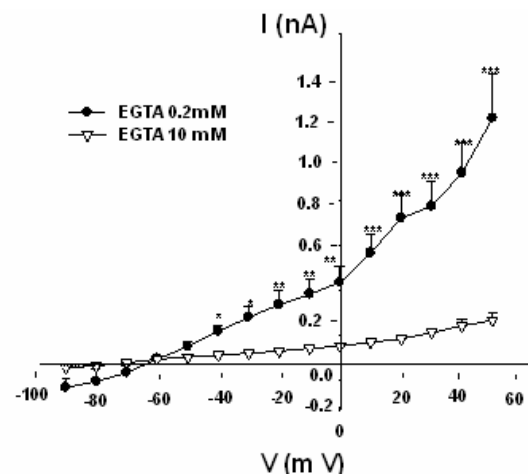


Fig. 4. Sustained outward current was reduced when there was 10 mM EGTA in recording pipette. Current/voltage (I/V) curve of the sustained current in control (with 0.2 mM EGTA), and test (10 mM EGTA) group cells. This comparison was made in one old week stem cell derived cardiomyocytes. All the values are mean \pm SEM (n=6). Mean current amplitudes were measured relative to the holding current at -60 mV. Star shows statistically significant differences at some test potential between two groups of cells. Key: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Student's *t*-test).

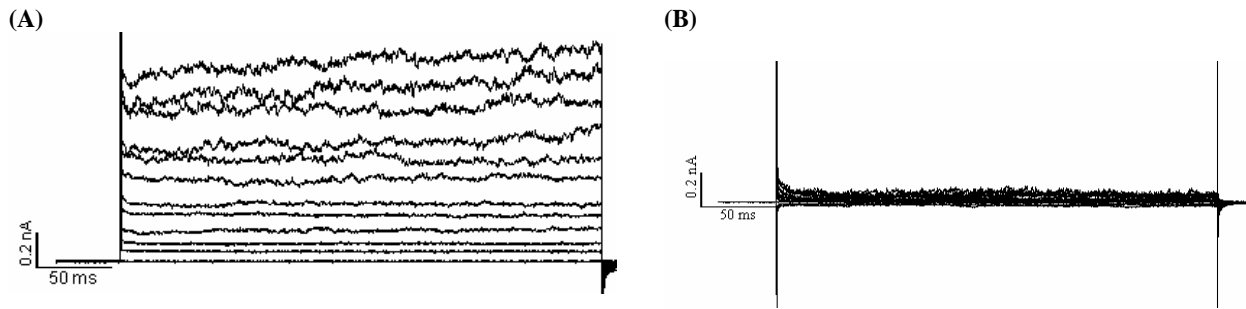


Fig. 5. Inhibition of sustained outward current by nifedipine (0.2 mM EGTA in recording pipette) in one old week ES-cardiomyocytes. (A) Original control current records. The sustained current was elicited by depolarising from the holding potential (-60 mV) to test potential up to +50 mV for 300 ms. (B) Nifedipine (1 μ M) inhibited the current, but no recovery occurred on wash-out.

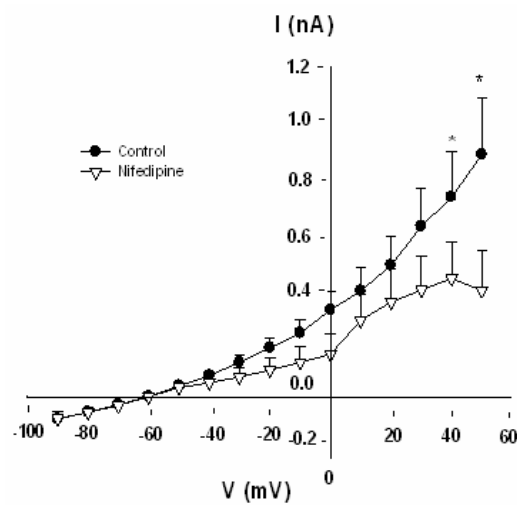


Fig. 6. Inhibition of sustained outward current by nifedipine (0.2 mM EGTA in recording pipette) in one old week ES-cardiomyocytes. Current-voltage (I/V) curve for the sustained current in; control (circle), and in the presence of nifedipine 1 μ M (triangle). The sustained current was elicited by depolarising from the holding potential (-60 mV) to various test potentials up to +50 mV for 300 ms. Nifedipine (1 μ M) partially inhibited the current. All values are mean \pm SEM (n=5). Mean current amplitudes were measured relative to the holding current at -50 mV. Star shows where the inhibition of current is statistically significant. (* $P < 0.05$, Student's *t*-test).

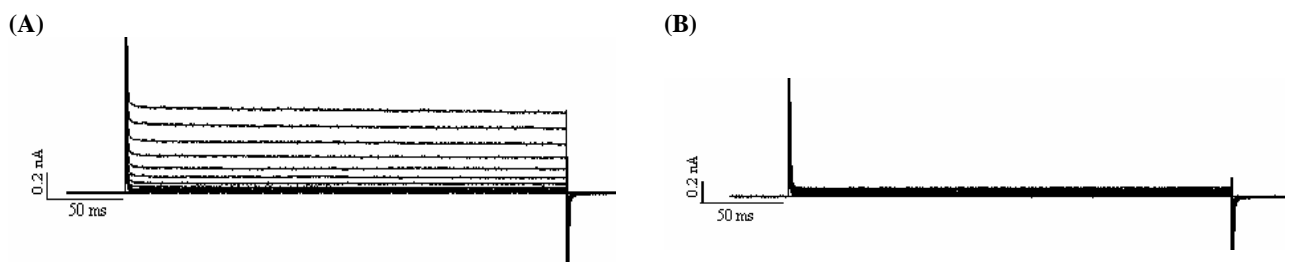


Fig. 7. Effect of 3,4-diaminopyridine on the TEA resistance current. 3,4-diaminopyridine (1 mM) reversibly blocked the current. Original current records before (A) and after addition of 3,4-diaminopyridine (B) when depolarised up to +40 mV for 300 ms from the holding potential (-70 mV) with 10 mM EGTA in the pipette solution. This study was made in two old weeks ES-cardiomyocytes.

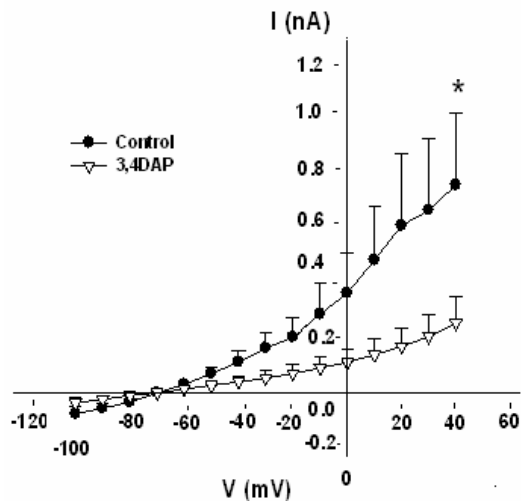


Fig. 8. Current voltage (I/V) curve for TEA resistance current in; control (circle), and after perfusion of 3,4-diaminopyridine, (1 mM). This study was made in two old weeks ES-cardiomyocytes. Current was recorded by depolarised up to +40 mV for 300 ms with 10 mM EGTA in the pipette solution. Mean current amplitudes were measured relative to the holding current at -70 mV. All the values are mean \pm SEM (n=7 for each group). Star shows where statistically significant differences exist between control current and current recorded after perfusion of 3,4-diaminopyridine (* P <0.05, Student's *t*-test).

current disappeared but the current amplitude was also significantly reduced in comparison with low EGTA cells (Fig. 4). Nifedipine is L-type Ca^{2+} channel blocker and it also reduces Ca^{2+} activated K^+ current (23). Therefore, to examine if the fluctuating sustained current is Ca^{2+} sensitive, the one old week ES-cardiomyocytes chamber perfused with 1 μM nifedipine for 10 min and the current amplitude was compared with pre-treatment current. Nifedipine (1 μM) reduced the outward sustained current from 1.05 nA to 0.06 nA in one week old ES-cardiomyocytes (Fig. 5). The mean effect of nifedipine on current voltage relationship in five separate cells reduced the mean current by 56% at test potential of +50 mV (Fig. 6).

To identify the type of channel responsible for TEA resistant current, 3,4-diaminopyridine was added to the bath solution to investigate whether these current are due to activation of delayed rectifier K^+ channels. Therefore, high EGTA pipette solution (10 mM) was used to attenuate the Ca^{2+} activated K^+ current and it was shown that 3,4-diaminopyridine substantially reduced this sustained current (Fig. 7 and 8).

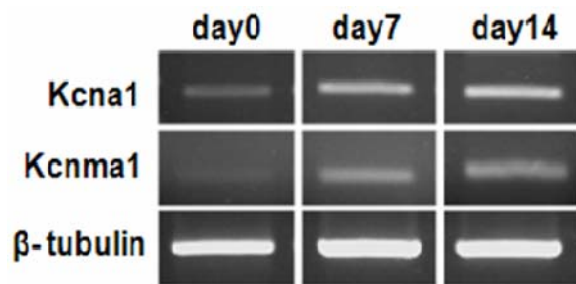


Fig. 9. Expression pattern of genes by reverse transcription – polymerase chain reaction (RT-PCR). Evaluation of genes expression large conductance Ca^{2+} activated K^+ channel (kcnma1) and delayed rectifier K^+ channel (kcna1) performed in three stages including mouse embryonic stem cells (day 0), one old week ES-cardiomyocytes (day 7) and two old weeks ES-cardiomyocytes (day 14).

Equivalent volume of nifedipine and 3,4-diaminopyridine vehicle (DMSO) had no effect on the current amplitude.

We also assessed the expression of mentioned channel at mRNA level and the results of Fig. 9 revealed that both Kcnma1 and Kcna1 channels are being expressed in one and two old weeks ES-cardiomyocytes in comparison with the stem cells. However, there is also a little expression of Kcna1 channel in the stem cells (Fig. 9).

DISCUSSION

Embryonic stem cells are capable of differentiating to variety of cells including cardiomyocytes (1-5). Therefore these cells can be a new approach for cardiotoxicity testing and drug screening. One of the objectives of this research was identification and comparison of ionic K^+ currents in undifferentiated stem cells and in one and two old-week pure ES-cardiomyocytes by means of patch-clamp technique to reveal the potential of these cells for drug screening and developmental studies. Patch-clamp technique is an advanced method which can be used to accurately evaluate ion

channel function, as well as pharmacological action of drugs especially in excitable cells such as cardiomyocytes.

Unlike stem cells, a voltage activated outward current was identified in ES-cardiomyocytes. This current was activated by depolarization to -20 mV or more positive test potentials. Blockade of this current by TEA confirms that this outward current is a type of K⁺ current. Electrophysiological evidence such as sustained and turbulence current, indicated presence of large conductance Ca²⁺ activated K⁺ channel in the ES-cardiomyocytes. In order to further investigate if this current is Ca²⁺ activated channel, the experiment was repeated in the presence of 10 mM EGTA which substantially and significantly lowered current amplitude. Furthermore, extracellular perfusion with L-type Ca²⁺ channel blocker 'nifedipine' also reduced this outward sustained current. During depolarization L-type Ca²⁺ channel are activated in the cardiomyocytes and Ca²⁺ influx has major role in cardiomyocytes contraction (24). Inhibitory effect of intracellular EGTA and extracellular nifedipine confirms that this type of current is Ca²⁺ dependent. In addition this voltage dependent outward rectifying K⁺ current was inhibited by TEA which is another pharmacological evidence which indicate that the K⁺ current recorded here is mainly from large conductance Ca²⁺ activated K⁺ channel. Nevertheless, in some cells TEA (10 mM) did not wipe out the outward rectifying current, indicating presence of different sustained outward current probably a type of delayed rectifier K⁺ current which are also abundant in cardiomyocytes (19,25). Therefore, to identify the nature of this TEA resistance current, high EGTA (10 mM in the pipette) concentration was used to subside Ca²⁺ activated currents, and recording was carried out in the presence of 3,4-diaminopyridine, a pharmacological tool used for identification of delayed rectifier current. Inhibition of TEA resistance current by 3,4-diaminopyridine indicated the presence of delayed rectifier current in the ES-cardiomyocytes.

Undifferentiated stem cells are non excitable cells (6) and they don't fire action potential and as it was expected they didn't produce any significant current following depolarization and only revealed slight linear current voltage

relations. This finding is consistent with the report indicating that undifferentiated stem cells exhibit no electrical activity (6).

Cardiomyocytes on the other hand belongs to excitable cell groups and not only can fire action potential upon depolarization but also can spread it to adjacent cells and that is how they produced a unified contraction in the cell culture tissue (1-5). It has been reported that intracellular recording from one week old cells show a membrane potential pattern similar to those observed in pacemaker cardiomyocytes (6,11). Two weeks old ES-cardiomyocytes have less spontaneous activity but comparison of outward ion channel activity show no difference between one and two weeks old ES-cardiomyocytes.

In addition intracellular recording technique reported that in two weeks old ES-cardiomyocytes action potential pattern is more similar to action potential that was seen in ventricular cell in adult cardiomyocytes (11,20,26).

One of conspicuous changes in the trans-membrane potential of cardiomyocytes is a dramatic hyperpolarization of resting potential paralleling heart development (5). Changes in resting membrane potential and development of ionic current in the ES-cardiomyocytes indicate that these cells are converted to excitable cells as their ion channels being activated by cell depolarization (20). However, comparison of current amplitude in various cells indicates that some of the cells have full functional ionic channels. In some other cells although the channel activation is not complete but still show a great channel activity upon depolarization and they are still on the way of development. This finding is consistent with the results reported from other ES-cardiomyocytes that not all the cells express functional ion channel activity (27). One of the advantages of patch-clamp technique is that it not only can distinguish differentiated cells from undifferentiated cells but also it makes sure that the differentiated cardiomyocytes have been selected for pharmacological studies.

The RT-PCR results shows a prominent increase in *Kcna1* (large conductance Ca²⁺ activated K⁺ channel) and *Kcnj1* (delayed rectifier K⁺ channel) channels expression in ES-cardiomyocytes. The patch-clamp studies not only confirms the expression of these channels

but more importantly shows that these channels are functional and are activated upon depolarization. On the other hand, the RT-PCR results show small expression of Kcna1 (delayed rectifier K⁺ channel) in the stem cells, the patch-clamp results however, indicate that although there is some expression of mRNA for Kcna1 channel, but unlike ES-cardiomyocytes, it hasn't been expressed into functional ion channel in the stem cells.

CONCLUSION

Previous reports demonstrated that ionic currents are specific markers for different tissues, particularly in the heart (2-5, 12). In the present study we described development of K⁺ ionic currents in Royan B₁ ES-cardiomyocytes including large conductance Ca²⁺ activated K⁺ channels and delayed rectifier which have significant role in repolarization of action potential. Therefore, ES-cardiomyocytes not only can be used for cardiomyocyte developments but also it is a suitable cell line for screening of drugs acting on ion channels.

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