INTRODUCTION

Skeletal muscle formation depends on the fusion of mononucleated myoblasts into multinucleated myotubes. Myoblast fusion is also the basis of muscle growth and repair during postnatal life. The ability of myoblasts to fuse and thereby inject their nucleus into existing muscle fibers led to several preclinical and clinical trials aimed at treating both muscle and non-muscle-related disorders. Identifying the pattern of events that induce myoblast differentiation and their commitment to fuse would benefit the search for improving myoblast-based therapies.

Using primary myoblast cultures derived from single human satellite cells (Baroffio et al., 1993), we have previously shown that membrane potential and the biophysical properties of specific ionic channels are important actors in the fusion process. We found that human myoblasts hyperpolarize before fusion through the sequential expression of two different K+ channels, ether-à-go-go (EAG) K+ channels (Bijlenga et al., 1998; Occhiodoro et al., 1998) and Kir2.1 inward-rectifier K+ channels (Liu et al., 1998; Fischer-Lougheed et al., 2001). The hyperpolarization induced by Kir2.1 is a prerequisite for myoblast fusion to occur, as pharmacological blockade of Kir2.1 channels (Liu et al., 1998) or inhibition of their expression using an antisense RNA vector (Fischer-Lougheed et al., 2001) suppress fusion.

Myoblast fusion is a strictly Ca2+-dependent process (Shainberg et al., 1969) and our recent results indicate that the purpose of the fusion-linked hyperpolarization is to set the resting potential of myoblasts in a range that allows Ca2+ to enter through a1H T-type Ca2+ channels (Bijlenga et al., 2000). These channels are expressed just before fusion, and have intrinsic properties that produce a substantial permanent Ca2+ current in a defined domain of hyperpolarized membrane potentials, hence the term window current. This window current is large enough to cause a detectable increase in intracellular Ca2+ and its inhibition prevents fusion (Bijlenga et al., 2000).

The presence of a window current depends on an overlap of the voltage range for channel activation and inactivation. In human myoblasts, T-type Ca2+ channels activate near –80 mV, and a maximum window current is observed at –58 mV (Bijlenga et al., 2000). At more depolarized voltages (near –40 mV), T channels inactivate fully and no Ca2+ current persists with time. We proposed that, by setting the resting membrane potential within the window range, the hyperpolarization generates the intracellular Ca2+ increase that is necessary for fusion to occur (Bernheim and Bader, 2002). At the time, however, we could not tell whether the coupling between the membrane potential of myoblasts and their differentiation was

SUMMARY

We have previously shown that human myoblasts do not fuse when their voltage fails to reach the domain of a window T-type Ca2+ current. We demonstrate, by changing the voltage in the window domain, that the Ca2+ signal initiating fusion is not of the all-or-none type, but can be graded and is interpreted as such by the differentiation program. This was carried out by exploiting the properties of human ether-à-go-go related gene K+ channels that we found to be expressed in human myoblasts. Methanesulfonanilide class III antiarrhythmic agents or antisense-RNA vectors were used to suppress completely ether-à-go-go related gene current. Both procedures induced a reproducible depolarization from –74 to –64 mV, precisely in the window domain where the T-type Ca2+ current increases with voltage. This 10 mV depolarization raised the cytoplasmic free Ca2+ concentration, and triggered a tenfold acceleration of myoblast fusion. Our results suggest that any mechanism able to modulate intracellular Ca2+ concentration could affect the rate of myoblast fusion.

Key words: Ca2+ current, Herg, Muscle differentiation, Myoblast fusion, Window current

Acceleration of human myoblast fusion by depolarization: graded Ca2+ signals involved

Jian-Hui Liu1,2, Stéphane König1,*, Marlène Michel1,*, Serge Arnaudeau1, Jacqueline Fischer-Lougheed1, Charles R. Bader2 and Laurent Bernheim1,†

1Département de Physiologie, Centre Médical Universitaire, Hôpital Cantonal Universitaire, CH-1211 Geneva 4, Switzerland
2Division de Recherche Clinique Neuro-Musculaire, Département des Neurosciences, Cliniques et Dermatologie, Hôpital Cantonal Universitaire, CH-1211 Geneva 4, Switzerland
*These authors contributed equally to the work
†Author for correspondence (e-mail: laurent.bernheim@medecine.unige.ch)

Accepted 24 April 2003
tightly or not. In other words, we could not tell whether the Ca\(^{2+}\) signal generated was interpreted by the differentiation program as being of the all-or-none type, or whether different Ca\(^{2+}\) influxes could result in different fusion rates. This is of importance, because if graded Ca\(^{2+}\) signals can be interpreted by the differentiation program, then other mechanisms able to change intracellular Ca\(^{2+}\) concentration could in principle modulate the rate of myoblast fusion.

The only way to evaluate convincingly the coupling between myoblast membrane potential and Ca\(^{2+}\)-induced differentiation is to generate a controlled, accurate and long-term small change of membrane potential. Given the profile of the Ca\(^{2+}\) current window domain (from ~80 mV to ~40 mV with a peak near ~60 mV, see Fig. 5A) and the resting potential of fusion-competent myoblasts (~74 mV), our objective was to generate a depolarization of about 10 mV, that would bring the membrane potential nearest to the ideal voltage value to produce the largest window current and consequently the largest Ca\(^{2+}\) signal.

We found that this is not as easily achieved as one would hope. Attempts to depolarize myoblasts by increasing extracellular K\(^{+}\) concentration ([K\(^{+}\)]\(_o\)) or by partial blockade of Kir2.1 K\(^{+}\) channels with Cs\(^{+}\) produced erratic results probably for the following reasons. Increasing [K\(^{+}\)]\(_o\) changes the Kir2.1 conductance in addition to shifting the equilibrium potential for K\(^{+}\) (Hille, 1992). Regarding partial Cs\(^{+}\) blockade, we believe that the difficulty stems from the fact that the Cs\(^{+}\) block of Kir2.1 channels is voltage dependent (Liu et al., 1998) and that in the voltage domain of interest (~74 mV to ~64 mV) the block is very sensitive to small changes in Cs\(^{+}\) (Liu et al., 1998).

Fortunately, we observed that myoblasts express yet another member of the K\(^{+}\) channel superfamily: the human ether-à-go-go related gene (HERG; KCNH2 – Human Genome Nomenclature Database) K\(^{+}\) channel. We found that these channels contribute to the resting potential to a lesser extent than Kir2.1 channels, that they can be specifically blocked, and that their inhibition induces a depolarization precisely in the desired window domain.

The results presented here show that an increase in Ca\(^{2+}\) influx parallels an acceleration of myoblast fusion, and demonstrate, within the resolution of the system, that there is a tight coupling between membrane potential, Ca\(^{2+}\) signals and myoblast differentiation.

**MATERIALS AND METHODS**

**Dissociation and culture procedures**

Human skeletal muscle biopsies (about 500 mg) were obtained during corrective orthopedic surgery of pediatric patients without any known neuromuscular disease. Myoblast clonal cultures are prepared from satellite cells, amplified in a proliferation medium and induced to fuse by culture in a differentiation medium (Baroffio et al., 1993).

**Electrophysiological recordings**

Whole-cell configuration of the patch-clamp technique was used to measure membrane potential and ionic currents (Hamill et al., 1981). Signals were recorded with an Axopatch 200B amplifier. The pipette resistances were 2-5 MΩ (compensations between 30 and 70% were used). For experiments in which accurate testing of the membrane potential was required, an extra fire polishing of the electrode was performed to improve the seal resistance. This may explain why resting potentials measured in this study are slightly more hyperpolarized than in a previous study (Liu et al., 1998). Cell capacitances were obtained from direct reading of the whole-cell capacitance potentiometer of the Axopatch 200B amplifier. Currents were recorded at 20-22°C, low pass-filtered at 1 kHz, and sampled at 5 kHz. To improve patching procedure, myoblasts were treated with 0.05% trypsin, and replated 1-2 hours before recording. The 30 mM K\(^{+}\) extracellular solution was made up of (mM): N-methyl-D-glucamine (NMG)-Cl (75), KCl (30), MgCl\(_2\) (2), HEPES (5), NaOH (50), acetic acid (50) and glucose (8). The pH was adjusted to 7.4 with NMG. The 5 mM K\(^{+}\) extracellular solution was made up of (mM): N-methyl-D-glucamine (NMG)-Cl (100), KCl (5), MgCl\(_2\) (2), HEPES (5), NaOH (50), acetic acid (50) and glucose (8). The pH was adjusted to 7.4 with NMG. The intracellular (pipette) solution was made up of (mM): KCl (120), NaCl (5), MgCl\(_2\) (2), HEPES (5), BAPTA (20), glucose (5) and Mg-ATP (5). The pH was adjusted to 7.3 with KOH.

Dofetilide was a gift from Pfizer, UK. E4031 was purchased from Alomone Laboratories, Israel.

**Northern blot analysis**

Data was extracted with Trizol (Invitrogen) according to manufacturer’s instructions. Total RNA (2 µg/lane) was resolved in a 1.5% agarose gel, transferred to a Zeta-Probe Blotting membrane (BioRad), crosslinked and hybridized as described by Matter et al. (Matter et al., 1990). The membrane was hybridized with a 32P-labeled 1.8 kb probe derived from the cDNA encoding HERG (Wang et al., 1998) and corresponding to nucleotides 2272-4070 (GenBank Accession Number, U04270).

**HERG antisense expression vector**

A bicistronic vector (pEF-IE) was constructed by inserting an IRES-EGFP (internal ribosomal entry site – enhanced green fluorescent protein) cassette into the eukaryotic expression vector pEF-BOS (Utsugi et al., 1989; Fischer-Lougheed et al., 2001). The antisense vector, pEF-HERGAS-IE, was obtained by inserting a 237 bp fragment of HERG open reading frame in antisense orientation into BamHI/SalI sites upstream of the IRES-EGFP. This sequence corresponds to an N-terminal region of low homology with human EAG and other related genes, and was generated from an HERG-pcDNA3 clone (kind gift of G. A. Robertson, University of Wisconsin, Madison) by PCR using forward and reverse primers 5'-GGGTTCTACAGACAAAC-3' and 5'-'TTGTCCATGGCCTGC TTCG-3'.

**Effect of HERG antisense on fusion**

Myoblasts were electroporated with 9 pmoles vector/2x10\(^5\) myoblasts using a GenePulserII (BioRad) as previously described (Espinos et al., 2001). About 15x10\(^6\) cells were electroporated, plated in proliferation medium for 48 hours, then selected for EGFP-expression by cell sorting (FACStar+, Becton Dickinson), and re-plated in proliferation medium. After reattachment, fusion was induced with differentiation medium. Cells were fixed promptly upon appearance of the first myotubes in the control cultures, at day 5, and fusion index determined (the lag time for beginning of fusion increases in electroporated cells) (Fischer-Lougheed et al., 2001).

**Pericam vectors**

The EGFP sequence from vectors pEF-IE and pEF-HERGAS-IE was replaced by the sequence coding for ‘inverse-pericam’ (Nagai et al., 2001). The inverse-pericam was amplified by PCR using primers containing cloning sites BamHI and NotI respectively, 5'-GGGGATCCAGCTGGACCACTG-3' and 5'-GGGGGCGGCGGC CGAATTCTACTTGT-3'.

**Cyttoplasmic Ca\(^{2+}\) measurements**

Myoblast were transfected by electroporation and plated on 25 mm glass coverslips in differentiation medium. The inverse-pericam
fluorescence from myoblasts was imaged with a Zeiss Axiocam S100TV microscope using a 63× plan-Neofluar 1.25 NA oil-immersion objective (Carl Zeiss AG, Feldbach, Switzerland). Cells were excited at 480±10 nm by an Optoscan Monochromator (Cairn Research, Faversham, UK) through a 505DCXR dichroic mirror (Chroma Technology Corp, Brattleboro, VT). Fluorescence emission at 535 nm (535AF45 Omega Optical, Brattleboro, VT) from the inverse-pericam was imaged using a cooled, 12 bits TE/CCD interlined CoolSNAP-HQ camera (Photometrics, Ropper Scientific, Trenton, NJ). Image acquisition and analysis was performed with Metamorph/Metafluor 4.6 software (Universal Imaging, West Chester, PA). For convenience, as inverse-pericam fluoresces less when free Ca2+ increases, pseudo fluorescence ratio images were calculated by dividing the fluorescence image at time 0 (F₀) by the fluorescence image at a given time (F). In this case, an increase in ratio-image fluorescence (F₀/F) reflects an increase in cytoplasmic Ca2+ concentration.

Statistics

Results are expressed as the means±s.e.m. Statistical analysis was performed using the Student’s t-test.

RESULTS

The testing of the coupling of differentiation of myoblasts to their membrane potential relies on our ability to block selectively HERG channels. As this is the first report on HERG in myoblasts, we shall first provide evidence for its presence and describe some of its properties.

HERG current in human myogenic cells

A Northern blot analysis revealed the presence of HERG transcripts of ≈4 kb in undifferentiated myoblasts, in cells triggered to fuse, and in myotubes (Fig. 1A). An approximate twofold decrease in HERG mRNA content was observed after induction of myoblast differentiation but no obvious change in expression was observed thereafter.

Whole-cell voltage-clamp recording techniques were then used to reveal HERG current in fusion-competent myoblasts. Fusion-competent myoblasts are obtained by plating the cells at low density for 2-3 days in serum-free differentiation medium (Krause et al., 1995). In this medium, the cells become fusion competent, but fusion itself is impeded because cells cannot contact each other. Under these conditions, the cells hyperpolarize as a consequence of the successive expression of ether-a-go-go and Kir2.1 K+ channels (Fischer-Lougheed et al., 2001). We show here that these cells also express functional HERG K+ channels. Fig. 1B illustrates an example of dofetilide-sensitive K+ currents recorded in a fusion-competent myoblast. Dofetilide-sensitive currents were obtained by subtraction the K+ current remaining in the presence of 5 μM dofetilide from the total K+ current (Fig. 1C will demonstrate that 5 μM dofetilide blocks completely HERG current).
Dofetilide, like E4031, is a methanesulfonanilide class III antiarrhythmic agent known to selectively inhibit HERG channels (Sanguinetti and Jurkiewicz, 1990; Kiehn et al., 1996). Dofetilide (5 μM) was used, as published dose-response curves indicate IC₅₀ values between 0.1 and 0.6 μM (Kiehn et al., 1996; Weerapura et al., 2002) and, furthermore, this concentration fully blocks overexpressed HERG current (Fig. 1C: the current-to-voltage relationships is linear, i.e. corresponding to a passive resistance, in the presence of 5 μM dofetilide). Currents were recorded during the typical voltage protocol required for activating HERG currents (Trudeau et al., 1995; Smith et al., 1996). It can be seen from the current-to-voltage relationships that this current has strong inward rectifying properties. The extracellular K⁺ concentration was raised to 30 mM to increase the magnitude of the current (inward rectifier K⁺ channels are sensitive to extracellular K⁺ concentration) and thereby facilitate its visualization.

Out of 16 fusion-competent myoblasts tested, seven expressed a measurable dofetilide-sensitive K⁺ current. In these cells, the mean current density recorded during a step to –65 mV from a holding potential of –5 mV was 1.3±0.3 pA/pF (mean capacitance was 37±6 pF). Although dofetilide-sensitive K⁺ currents were detected in only 44% of the cells tested, we believe that functional channels are synthesized in all fusion-competent myoblasts, but that the current they generate is too small to be detected using the whole-cell voltage-clamp recording techniques (see below).

To confirm that the dofetilide-sensitive current recorded in fusion-competent myoblasts flows through HERG channels, we compared the endogenous current with the current produced by an HERG expression vector (Wang et al., 1998). The vector was transfected into proliferating myoblasts by electroporation and current recordings were performed 48 hours later (Espinos et al., 2001; Fischer-Lougheed et al., 2001). Fig. 1C illustrates whole-cell current traces recorded in myoblasts transfected with the HERG construct. The same voltage protocol as in Fig. 1B was used. It can be seen that the current recorded in myoblasts overexpressing HERG is very similar to the endogenous dofetilide-sensitive current. The only difference is a 10-fold larger current amplitude, which we attribute to the presence of an increased number of functional channels in transfected cells.

These results show that HERG transcripts are present in human myogenic cells, and that a K⁺ current with pharmacological and biophysical properties similar to an identified HERG current can be recorded in fusion-competent myoblasts.

**Fusion-competent myoblasts depolarize when HERG current is inhibited**

The possible contribution of HERG channels to the membrane hyperpolarization that precedes myoblast fusion was examined...
by measuring the resting potential of fusion-competent myoblasts before and after application of dofetilide (Fig. 2A). Only hyperpolarized fusion-competent myoblasts expressing Kir2.1 inward rectifier K+ channels were selected for these experiments, as myoblasts that do not express Kir2.1 channels do not fuse (Fischer-Lougheed et al., 2001).

Before dofetilide application, fusion-competent myoblasts had a mean resting potential of –74 ± 2 mV (n = 14). Addition of 5 μM dofetilide to the superfusion medium depolarized all myoblasts tested (n = 14). Depolarizations ranged between 5 mV and 18 mV, with a mean amplitude of 10 ± 1 mV. Similarly, 10 μM E4031 depolarized fusion-competent myoblasts by 10 ± 2 mV (minimum 6 mV, maximum 17 mV; n = 6). To exclude the possibility that the depolarization was due to an inhibition of Kir2.1 channels, we tested the effects of dofetilide and E4031 on Kir2.1 current. Fusion-competent myoblasts were stepped to –125 mV from a holding potential of –65 mV to activate Kir2.1 current. Addition of 5 μM dofetilide or 10 μM E4031 to the superfusion medium did not significantly reduce the current. The mean Kir2.1 current density was –2 ± 0.4 pA/pF in control conditions and –1.9 ± 0.3 pA/pF after dofetilide application (n = 12; P = 0.54). When E4031 was tested, mean current densities were –1.4 ± 0.6 pA/pF and –1.4 ± 0.5 pA/pF in control conditions and after E4031 applications respectively (n = 7; P = 0.82). The other major K+ channel type that contributes to the membrane potential is EAG, and although these channels are not activated at –74 mV (Bernheim et al., 1996), we tested their sensitivity to E4031. We found that this drug did not significantly affect EAG channels (P = 0.18).

These results indicate that HERG channels contribute to the resting membrane potential of myoblasts. They also suggest that all fusion-competent myoblasts express functional HERG channels, as the 20 cells tested were depolarized by either dofetilide or E4031 (note that the probability of measuring a depolarization consecutively in 20 myoblasts with a detectable HERG current is very low, 0.44^20, i.e. less than one in 13 million).

The observation that an inhibition of HERG channels depolarizes fusion-competent myoblasts by 10 mV implies that functional HERG channels are physiologically continuously activated at a hyperpolarized resting potential of –64 mV. The experiments described in Fig. 2B, C were performed to demonstrate that a steady activation of HERG channels is indeed present at this hyperpolarized potential. The superfusion medium contained 5 mM K+ concentration to mimic physiological conditions, and the electrophysiological recordings were performed in myoblasts overexpressing HERG channels to increase the magnitude of the current (under physiological conditions the current is small in native cells; in Fig. 1B, we had to set the extracellular K+ concentration to 30 mM in order to visualize the current). Fig. 2B shows examples of dofetilide-sensitive current traces, and Fig. 2C shows current-to-voltage and conductance-to-voltage relationships. The relationship at steady-state demonstrates that a HERG conductance able to carry a steady outward K+ current under physiological conditions is present at voltages between –15 and –80 mV. This observation confirms that the biophysical properties of the channel are compatible with the role we propose for HERG, namely to assist Kir2.1 channels in driving the resting membrane potential of fusion-competent myoblasts near –74 mV.

### Inhibition of HERG channels increases the rate of myoblast fusion

Given the results described in the previous section, we were now in a position to test the effect of a 10 mV depolarization of myoblasts on their ability to fuse. When transferred to serum-free differentiation medium, human myoblasts fuse into multinucleated myotubes within 48 hours. If there is a tight coupling between membrane potential, Ca2+ signal and the differentiation program, the fusion rate should increase.

It can be seen in Fig. 3 that, under control condition, less than 4% of the myoblasts have fused after 24-27 hours of exposure to differentiation medium (Fig. 3A, B, circles). A lag time of ~1 day was always observed in human myoblasts triggered to fuse in culture. By contrast, in sister cultures treated with methanesulfonanilide agents, large multinucleated myotubes were already observed after 1 day in differentiation medium (see pictures in Fig. 3). In the presence of dofetilide (10 μM), 38% of the myoblasts have fused after 24 hours (Fig. 3A, triangles). A similar result was observed with E4031 (10 μM). When this drug was added to the culture medium, the fusion index was 42% after 27 hours (Fig. 3B, triangles). These effects of dofetilide and E4031 were observed in three independent experiments. Additional support for the hypothesis that there is a tight coupling between membrane potential and myoblast fusion was provided by testing the effects of increasing concentrations of dofetilide on the fusion rate. We found that 0.1 μM increased the fusion rate by about 50% of the maximum, and that the maximum increase is reached at 1 μM (Fig. 3A, inset). Thus, methanesulfonanilide agents clearly accelerate myoblast fusion in a dose-dependent manner and, as these agents are relatively specific, this effect may be linked to an inhibition of HERG channels. The final (steady-state) fusion index, however, was not affected by the blockade of HERG.

### The rate of myoblast fusion can also be increased by inhibition of HERG channel expression

To verify that dofetilide and E4031 increase the rate of myoblast fusion by acting specifically on HERG channels, we examined how myoblasts fused when HERG channel expression was inhibited by antisense RNAs. For these experiments, a bicistronic vector expressing EGFP (enhanced green fluorescent protein) was engineered to contain a fragment of the HERG protein (protein) was engineered to contain a fragment of the HERG promoter of pEF-BOS (Utsuki et al., 1989). The EF1α promoter was selected because it functions stably throughout myoblast differentiation (Fischer-Lougheed et al., 2001). Myoblasts were transfected by electroporation and the green fluorescence of EGFP was used to identify the transfected cells (Espinosa et al., 2001). To obtain enough transfected cells to perform a fusion test, green fluorescent myoblasts were collected using a cell-sorter (FACStar+) and seeded at an adequate density to allow rapid transfer into the fusion-inducing medium. Control cells were transfected with the empty bicistronic vector only expressing EGFP. The results of fusion tests after transfection are presented in Fig. 4A. It can be seen that when HERG channel expression was inhibited there was a 15-fold increase of the rate of myoblast fusion. A similar result was observed in another independent experiment. Thus, the effect of a reduction in the expression of HERG is equivalent to a pharmacological blockade of HERG channel activity.
To confirm the specificity of the effect of the HERG antisense, we tested the effect of dofetilide on the resting potential of fusion-competent myoblasts transfected with either the HERG antisense vector or the control empty bicistronic vector. We expected that myoblasts transfected with HERG antisense would not respond to dofetilide and, in addition, that they should be more depolarized than control myoblasts. We choose the strategy to measure resting potential, rather than the HERG current, because under physiological conditions the endogenous HERG current in fusion-competent myoblasts is too small to allow a reliable evaluation of the antisense effect on the current magnitude (as mentioned earlier, even in 30 mM extracellular K⁺, HERG was detectable in only 44% of the myoblasts). The low number of expressed channels also excludes an evaluation by western blotting: the HERG protein expression being at the limit of detection under control conditions (not shown). Fig. 4B shows that, in myoblasts transfected with the control vector, dofetilide depolarized the resting membrane potential by 11±1 mV (from −74±2 mV to

Fig. 3. Dofetilide and E4031 increase the rate of myoblast fusion. Fusion was induced with differentiation medium. The fusion index is defined as the number of nuclei in myotubes divided by the total number of nuclei counted in a given microscope field. Cultures were fixed for 5 minutes at −20°C with 100% methanol, and stained with Haematoxylin. Nuclei were counted in 20 randomly chosen microscope fields in separate cultures. (A,B) Fusion in control conditions is represented by circles. In sister cultures, dofetilide (A, 10 μM) or E4031 (B, 10 μM) were added to the differentiation medium during the entire duration of experiments (triangles). Error bars are omitted because they are smaller than symbols. Photographs represent Haematoxylin-stained cultures in differentiation medium. Myotubes (arrows) were observed only in cultures of cells transfected with the HERG antisense vector. Scale bars: 40 μm. The smaller plot in A represents the percent of fusion increase with respect to control (0 μM dofetilide) after 18 hours in differentiation medium (100% is the maximum fusion increase) in presence of 0.1, 1 and 5 μM dofetilide.

Fig. 4. HERG antisense depolarizes fusion-competent myoblasts and increases the rate of fusion. (A) Control: myoblasts transfected with a bicistronic vector containing only EGFP. AS-HERG: myoblasts transfected with a bicistronic vector expressing HERG antisense and EGFP. Myoblast transfection and fusion index determination are detailed in the Materials and Methods section. Photographs represent Haematoxylin-stained cultures in differentiation medium. Myotubes (arrows) were observed only in cultures of cells transfected with the HERG antisense vector. Scale bars: 40 μm. (B) Resting membrane potential of fusion-competent myoblasts before (−) and after (+) application of 5 μM dofetilide. Myoblasts were transfected as in A.
Membrane potential and myoblast fusion

–62±2 mV, n=8, P<0.001), whereas, in myoblasts transfected with HERG antisense, dofetilide did not significantly affect the resting potential (–65±2 mV before dofetilide application and –64±2 mV after application, n=9, P=0.28). As expected, the resting potential of myoblasts transfected with antisense was more depolarized than the resting potential of cells transfected with the control vector (–65±2 mV versus –74±2 mV). Incidentally, these results on the resting potential in myoblasts treated with HERG antisense demonstrate that HERG antisense does not interfere with Kir2.1 K+ currents.

In conclusion, these experiments with the antisense vector confirm that the rate of myoblast fusion is increased by a 10 mV depolarization. They also further confirm that dofetilide and E4031 are acting specifically on HERG channels.

**Inhibition of HERG channel increases the cytoplasmic Ca²⁺ concentration**

We mentioned that, in human myoblasts, T-type Ca²⁺ channels activate near –80 mV and the peak window Ca²⁺ current occurs at –58 mV (Bijlenga et al., 2000). When HERG channels are activated, the resting potential of fusion-competent myoblasts is approximately –74 mV (‘Rp’ in Fig. 5A). This means that the window Ca²⁺ current is relatively small (~2.1 fA/pF). Full inhibition of HERG channel activity depolarizes myoblasts by ~10 mV (horizontal arrow in Fig. 5A). According to the biophysical properties of T-type Ca²⁺ channels (Fig. 5A), this 10 mV depolarization will increase the magnitude of the window T-type Ca²⁺ current from 2.1 to 7.7 fA/pF (the shift from –74 to –64 mV is in a rising phase of the window Ca²⁺ current), and this should lead to a substantial increase in cytoplasmic Ca²⁺ concentration.

The experiments described in Fig. 5B,C show that, as expected, inhibiting HERG channel activity raises the cytoplasmic Ca²⁺ concentration in fusion-competent myoblasts. Free Ca²⁺ fluctuations were assessed using the ‘inverse-pericam’ fluorescent indicator (Nagai et al., 2001). ‘Pericams’ are a family of GFP-based Ca²⁺ indicators, which allow long-term Ca²⁺ measurements without compartmentalization of the probe. Although it does not allow measuring absolute Ca²⁺ concentration, the ‘inverse pericam’ was chosen because its high Ca²⁺ affinity permits to monitor accurately small cytoplasmic Ca²⁺ changes around basal level. It can be seen in Fig. 5B (circles) that, in response to 5 μM dofetilide, cytoplasmic Ca²⁺ rises within a few minutes and remains elevated. The mean increase of fluorescence ratio was 7.3±1.4% in the 22 myoblasts tested (Fig. 5C). To verify that the dofetilide-induced Ca²⁺ increase was indeed due to HERG channel inhibition, the same protocol was applied to myoblasts transfected with an HERG antisense RNA vector (AS-HERG). For this purpose, a bicistronic vector expressing both HERG antisense and inverse-pericam was constructed. The expected result was that, although basal Ca²⁺ might be higher, dofetilide should not increase cytoplasmic Ca²⁺ in myoblasts unable to synthesize HERG channels. Fig. 5B (square) shows that, in a myoblast expressing both antisense and inverse-pericam, dofetilide did not affect cytoplasmic Ca²⁺ concentration as the fluorescence ratio remained stable in the nine myoblasts tested (~0.4±1.3%, Fig. 5C). Note that this result is significantly different from the fluorescence ratio measured in control cells (7.3±1.4%, P=0.003).

Taken together, these results indicate that the inhibition of

![Fig. 5. Membrane potential and Ca²⁺ influx through window current during myoblast fusion.](image)
HERG channel activity, via a 10 mV depolarization of myoblast membrane potential, increases cytoplasmic Ca\(^{2+}\) concentration and consequently accelerates the rate of human myoblast fusion.

**DISCUSSION**

A hyperpolarization of the resting membrane potential is observed when human myoblasts differentiate and initiate the fusion process. Without this hyperpolarization, fusion does not occur (Liu et al., 1998; Fischer-Lougheed et al., 2001). We recently proposed that this hyperpolarization generates the Ca\(^{2+}\) influx required for fusion by setting the membrane potential in a range where a window current through T-type Ca\(^{2+}\) channels is activated (Bijlenga et al., 2000). In this model, because of the voltage-dependence of the window current, small variations of the resting potential (between –75 and –60 mV) should have a marked effect on Ca\(^{2+}\) influx. We could not tell, however, whether the differentiation program leading to myoblast fusion was able to respond in a graded manner to graded Ca\(^{2+}\) window current signals, i.e. whether there exist a coupling between Ca\(^{2+}\) entry and myoblast differentiation, or whether Ca\(^{2+}\) entry is an all-or-none differentiation signal.

The present work demonstrates that (1) there is a coupling between membrane potential and differentiation, and 2) the result of this coupling can be an acceleration of a differentiation process, myoblast fusion. This demonstration was possible as reproducible small depolarizations could be induced in myoblasts by full blockade of a K\(^{+}\) current, HERG. Incidentally, this is the first report of the presence of this current in myoblasts and we shall briefly put our observations in perspective in the next section.

**Functional HERG channels are expressed in human myogenic cells**

The HERG channel is a member of the voltage-gated ether-à-go-go K\(^{+}\) channel family. HERG channels are characterized by a slow current activation and deactivation, paired with a fast C-type inactivation mechanism (Trudeau et al., 1995). These unusual characteristics confer to HERG its peculiar electrophysiological properties. The HERG gene was shown to be expressed in the heart (Wymore et al., 1997), and mRNA for HERG is present in a number of different species and tissues (Wymore et al., 1997; Arcangeli et al., 1995; Arcangeli et al., 1997; Shi et al., 1997; Bianchi et al., 1998; Zhou et al., 1998; Overholt et al., 2000).

We found that the HERG gene is expressed in proliferating myoblasts, fusion-competent myoblasts and in myotubes. From our northern blot analysis, it appears that the amount of mRNA declines after the induction of differentiation. This may explain the reported absence of detectable signal in human skeletal muscle by Curran et al. (Curran et al., 1995). However, using the more sensitive assay of RNase protection, ERG presence could be demonstrated in rat muscle (Wymore et al., 1997).

The presence of functional HERG channels in myogenic cells was assessed using electrophysiological tools and pharmacological agents. Methanesulfonanilide antiarrhythmic agents were used, such as dofetilide and E4031, which block HERG channels with a well documented specificity (Sanguinetti and Jurkiewicz, 1990). In myoblasts, both drugs inhibit a K\(^{+}\) current that has the typical activation and inwardly rectifying properties of the HERG current. The expression of HERG channels in human myogenic cells is confirmed by the comparison of the endogenous dofetilide-sensitive K\(^{+}\) current to the corresponding current flowing through cloned HERG channels overexpressed after electroporation of proliferating human myoblasts. The biophysical properties of both currents are undistinguishable, strongly suggesting that they reflect the activity of the same channel. We also tested the effect of antisense RNA directed against the endogenous dofetilide-sensitive K\(^{+}\) current. Unfortunately, the very small current amplitude (as shown earlier, 56% of the cells have no detectable current under conditions that favor the visualization of HERG, i.e. 30 mM extracellular K\(^{+}\)) precluded interpretation of the results of these experiments.

**HERG channels are present in all fusion-competent myoblasts and contribute to their resting potential**

We showed that HERG channels contribute to the resting potential of fusion-competent myoblasts. Their contribution is lesser than that of Kir2.1 channels, as a full blockade depolarizes myoblasts only by 10 mV instead of 30-40 mV for Kir2.1 channels (Liu et al., 1998; Fischer-Lougheed et al., 2001). The existence of rather specific blocking agents for HERG channels (dofetilide and E4031) and the use of HERG antisense allowed us to estimate that HERG channels contribute ~14% (10 out of 74 mV) of the K\(^{+}\) current required to drive the membrane potential of myoblasts to ~74 mV.

We detected HERG current in only 44% of the cells tested. Nevertheless, we suggest that all fusion-competent myoblasts have functional HERG channels contributing to their resting potential. This is based on the observation that every fusion-competent cell specifically tested for its resting potential (n=37) was depolarized when HERG channel activity or expression was reduced (22 myoblasts treated with dofetilide, six with E4031 and nine myoblasts transfected with HERG antisense). The probability is extremely low (0.44\(^{28}\) or one in 9.6 billion) that the depolarizing effect of dofetilide and E4031 observed in a total of 28 cells would have resulted from recording exclusively from myoblasts expressing a detectable HERG current.

We can explain the fact that we did not always detect an HERG current during voltage-clamp of fusion-competent myoblasts by the low number of overall K\(^{+}\) channels expressed in these cells (Fischer-Lougheed et al., 2001). Indeed, if the input resistance of a myoblast at rest is in the order of 2 G\(\Omega\), a 10 mV depolarization would be produced by blocking an HERG current of 5 pA. Such currents may not always be detected due to the noise associated with the whole-cell voltage-clamp technique and the superfusion of solutions, or small instabilities during the long protocols required for recording the series of dofetilide-sensitive currents necessary for establishing the current-to-voltage relationship of HERG.

It is important to mention that, although HERG blockade allowed us to demonstrate the coupling between membrane potential and myoblast differentiation, we neither demonstrate nor disprove here that HERG is physiologically involved in modulating myoblast differentiation. However, when one considers the effect of HERG inhibition, it appears that HERG channels contribute to set the myoblast membrane potential in
a region of the T-type Ca\(^{2+}\) current window domain distant from the maximum window current, i.e. in a region where increments and decrements in window Ca\(^{2+}\) current signals by membrane potential modulation are possible. This could be used by myoblasts to adapt their fusion rate in response to environmental conditions during muscle growth or repair.

**K\(^{+}\) channels, hyperpolarization, window current and differentiation**

Many studies in several preparations have linked modulation of the resting membrane potential by K\(^{+}\) channels with differentiation (Ma et al., 1998; Pancrazio et al., 1999; Mauro et al., 1997; Puro et al., 1989; Lewis and Cahalan, 1990; Pappone and Ortiz-Miranda, 1993), but a precise mechanism linking membrane potential and differentiation was not characterized. We demonstrate here that a small voltage change in the domain of the T-type Ca\(^{2+}\) window current affects the differentiation of myoblasts.

The T-type Ca\(^{2+}\) channel α1 subunit genes [α1G, α1H, α1I (Perez-Reyes, 1999)] encode channels that are all endowed with a steady-state inward window Ca\(^{2+}\) current activated at negative potentials, i.e. close to the resting membrane potential (McRory et al., 2001). It has been suggested that a window current may play a role in many cellular events, such as neurogenesis at the onset of neuronal differentiation (Chemin et al., 2002), aldosterone or atrial natriuretic factor secretion (Lotshaw, 2001; Leuranguer et al., 2000), pancreatic β-cell apoptosis in diabetes (Wang et al., 1999), or motoneuron death in spinobulbar muscular atrophy (Scotoporeanu et al., 2000). These examples of possible physiological and pathophysiological implications of window T-type Ca\(^{2+}\) currents suggest that fine tuning of the Ca\(^{2+}\) influx via a window current is not solely an attribute of differentiating myoblasts and that it may participate to a greater diversity of cell functions than thus far examined.

We thank M. Berti, P. Brawand and C. Viglino for their excellent technical assistance on cell cultures, Dr A. Kaelin for providing the HERG original vector. This work was supported by grants from the Fonds National Suisse pour la Recherche Scientifique (No. 31-65409.01 and 4046-058639) and the Fondation Suisse pour la Recherche sur les Maladies Musculaires.

**REFERENCES**


