Human Myoblast Fusion Requires Expression of Functional Inward Rectifier Kir2.1 Channels

Jacqueline Fischer-Lougheed,* Jian-Hui Liu,*‡ Estelle Espinos,* David Mordasini,* Charles R. Bader,‡ Dominique Belin,§ and Laurent Bernheim*

*Département de Physiologie et §Département de Pathologie, Centre Médical Universitaire, CH-1211 Geneva 4, Switzerland; and ‡Division de Recherche Clinique Neuro-Musculaire, Département des Neurosciences Cliniques et Dermatologie, Hôpital Cantonal Universitaire, 24 rue Micheli-du-Crest, 1211 Geneva 14, Switzerland

Abstract. Myoblast fusion is essential to skeletal muscle development and repair. We have demonstrated previously that human myoblasts hyperpolarize, before fusion, through the sequential expression of two K^+ channels: an ether-à-go-go and an inward rectifier. This hyperpolarization is a prerequisite for fusion, as it sets the resting membrane potential in a range at which Ca^{2+} can enter myoblasts and thereby trigger fusion via a window current through α1H T channels.

This work was undertaken to elucidate the molecular identity of the inward rectifier (Kir) channel involved in fusion. Using RNase protection assays, we detected mRNA for Kir2.1 and Kir2.2. Transcript levels for both Kir candidates increased during myoblast differentiation. Single-channel recordings of undifferentiated myoblasts overexpressing Kir2.1 or Kir2.2 indicated that only the conductance of Kir2.1 corresponds to that of the endogenous channel. Inhibition of Kir2.1 expression with an antisense-Kir2.1-RNA expressed from transfected vector drastically reduced the endogenous inward rectifier current and blocked fusion. In contrast, an antisense-Kir2.2-RNA had no effect on fusion. Taken together, our results demonstrate that functional Kir2.1 channels are required for human myoblast fusion.

Key words: potassium current • membrane potential • myoblast fusion • muscle • antisense

Introduction

A multinucleated skeletal muscle fiber is formed by mononucleated myoblasts, in a fusion process that is essential to skeletal muscle development, growth, and repair. In the prospect of myoblast-based grafts and gene therapies, it is important to understand the pattern of events that lead myoblasts to commit to fusion; identifying the molecular nature of the actors involved in crucial steps might benefit the search for improving fusion competence of grafted cells.

Using clonal, primary human myoblast cultures derived from single satellite cells, we have shown previously that acquisition of fusion competence of human myoblasts requires hyperpolarization of their membrane resting potential. A similar observation has been made in chick embryonic myoblasts (Shin et al., 1997; Park et al., 1999). For human cells, this hyperpolarization occurs through the sequential expression of two different K^+ currents. An ether-à-go-go current is expressed first, and hyperpolarizes the cells to an intermediate resting potential of about −32 mV (Bernheim et al., 1996; Bijlenga et al., 1998). Then, slightly before fusion, the expression of an inward rectifier K^+ current, $IK_{(IR)}$, causes fusion-competent myoblasts (FCMB)[1] to hyperpolarize to approximately −65 mV (Liu et al., 1998). A pharmacological blockade of $IK_{(IR)}$ with Ba^{2+} or Cs^+ depolarizes myogenic cells and inhibits fusion (Liu et al., 1998), suggesting that hyperpolarization through functional K^+ (Kir) channels is required for fusion to proceed. Myoblast fusion is a strictly Ca^{2+}-dependent process, and our recent results indicate that the purpose of the second ($IK_{[IR]}$-associated) hyperpolarization step is to set the FCMB membrane resting potential in a range that allows the necessary Ca^{2+} to enter (Bijlenga et al., 2000). The Ca^{2+} influx occurs via a window current through α1H T channels (Bijlenga et al., 2000), which are expressed just before fusion takes place (Liu et al., 1998).

K^+ currents with inward rectification properties have been known to be expressed in mature muscle fibers since their first description in the frog muscle by Katz (1949). These currents have been analyzed in numerous preparations of mammalian skeletal muscle cells (adult rat fibers

Address correspondence to Laurent Bernheim, Département de Physiologie, Centre Médical Universitaire, 1 rue Michel-Servet, CH-1211 Geneva 4, Switzerland. Tel.: (41) 22 702 53 92. Fax: (41) 22 702 54 02. E-mail: laurent.bernheim@medecine.unige.ch

[1]*Abbreviations used in this paper:* DM, differentiation medium; EGFP, enhanced green fluorescent protein; FCMB, fusion-competent myoblast; GM, growth medium.

[Duval and Leoty, 1978], rodent myotubes and postnatal fibers [Ohmori et al., 1981; Matsuda and Stanfield, 1989; Gonoi and Hasegawa, 1991], rat and human sarcolemmal vesicles prepared from adult muscle [Burton et al., 1987], and human muscle cultures [Trautmann et al., 1986]). By conducting small outward or larger inward currents, $IK_{(IR)}$ maintains the resting membrane potential near E_K and thereby contributes to the control of the muscle fiber excitability (Hille, 1992).

A first goal of the present work was to identify the molecular nature of the Kir channel(s) underlying the hyperpolarization of human FCMB. A second goal was to demonstrate its role in the formation of the multinucleated muscle fiber by selectively blocking Kir channel expression. The whole-cell characteristics of the native $IK_{(IR)}$ (steep inward rectification, and insensitivity to intracellular ATP or nonhydrolyzable GTP analogue GTPγS [Liu et al., 1998]) suggest that the channel belongs to the Kir2 subfamily of Kir channels (for review see Nichols and Lopatin, 1997), of which four members have been isolated thus far from human heart, nervous tissue, and retina (Perier et al., 1994; Raab-Graham et al., 1994; Wible et al., 1995; Hughes et al., 2000). Although it is commonly accepted that the main, classic Kir channel of the skeletal muscle fiber is the first member isolated, namely Kir2.1 (Kubo et al., 1993; Tang et al., 1995; Shin et al., 1997), several subtypes could, in principle, be expressed. Indeed, Northern blotting experiments on mammalian skeletal muscle RNA also suggests the presence of Kir2.2 (Takahashi et al., 1994) and possibly also Kir2.3 (Perier et al., 1994). Given the unavailability of pharmacological agents to discriminate between members of the Kir2 subfamily, we performed RNase protection assays to examine the presence and pattern of expression of transcripts of Kir2 members in human myogenic cells during differentiation. Concomitantly, single-channel recordings were performed on FCMB to characterize the endogenous Kir channel. We found that FCMB express one unique type of Kir channel. We describe here the unitary properties of this channel and demonstrate, using comparative electrophysiology at the single-channel level and RNA targeting to specifically block channel expression of Kir2 candidates, that the channel underlying the hyperpolarization of human myoblasts committed to fuse is formed by Kir2.1 subunits, and that blockade of expression of functional Kir2.1 channels drastically impedes fusion.

Materials and Methods

Dissociation and Culture Procedures

Human skeletal muscle biopsies (~500 mg) were performed by Dr. A. Kaelin (Hôpital Cantonal Universitaire, Geneva, Switzerland) during corrective orthopedic surgeries of pediatric patients (7 mo to 13 yr old) without any known neuromuscular diseases. The biopsies were obtained in accordance with the guidelines of the ethical committee of the University Hospital of Geneva, Switzerland (written, informed consent was obtained from legal guardians). Myoblast clonal cultures were prepared from satellite cells, proliferated in a growth medium (GM), and induced to fuse by transfer to a differentiation medium (DM), as described previously (Baroffio et al., 1993). FCMB were obtained by plating myoblasts at a very low density (10,000 cells per 35-mm culture dish) in DM. Under these conditions the cells were induced to differentiate, but prevented from fusing (Krause et al., 1995).

Expression Vectors

The expression vectors were constructed using pIeG, a bicistronic vector expressing enhanced green fluorescent protein (EGFP). This vector was generated by modifying pIeG (Occhiodoro et al., 1998) to express EGFP (from pEGFP-N2; Clontech Laboratories, Inc.) and to include an adapter sequence upstream of the internal ribosome entry site (IRES) to add restriction sites for subcloning. All constructs were sequenced.

pKir2.1-IE. Kir2.1 (AN: U12507) was excised with EcoRI and XhoI from clone HH-IRK1 (gift from C. Vandenberg, University of California, Santa Barbara, CA), blunted, and subcloned into the EcoRV site upstream of the IRES of pIeG.

pKir2.2-IE. Kir2.2 (from clone HIRK-A$^+$-pCRII [gift from B. Wible, Metro Health Medical Center, Cleveland, OH]; AN: L36069) was first subcloned between the ApaI and EcoRI sites of pBluescript KS(+), excised with KpnI and EcoRI, blunted, and inserted into the EcoRV site of pIeG.

Antisense Expressing Vectors

The antisense RNA vectors were generated using a bicistronic vector (pEF-IE) constructed by inserting the IRES-EGFP cassette of pIeG into the eukaryotic expression vector pEF-BOS (Uetsuki et al., 1989), provided by S. Nagata (Osaka University Medical School, Osaka, Japan). Kir2.1 and Kir2.2 sequences were inserted between the EF1α promoter and the IRES of pEF-IE, in antisense orientation.

pEF-Kir2.1αS1-IE. A 1,599-bp XbaI fragment of HH-IRK1 (containing all the coding sequence but 31 bp at the 3′-end) was inserted into the XbaI site of pEF-IE.

pEF-Kir2.2αS-IE. A 1,358-bp fragment containing the full-length Kir2.2 coding sequence was excised from pKir2.2-IE with XbaI and EcoRI, blunted and inserted at the EcoRV site of pEF-IE.

Myoblast Transfection

Proliferating cells at ~50% confluency were electroporated with 2.5–3 pmoles of DNA using GenePulserII (Bio-Rad Laboratories) and conditions reported previously (Espinos et al., 2001), i.e., 2.10^5–2.10^6 cells in 0.2 ml F-10 (Nutrient Mixture; GIBCO BRL), 350 V, 650 μF, and 200 Ω. Cells were seeded in 35-mm dishes. Electrophysiology of overexpressed channels was performed 48 h later. For analysis of endogenous currents, cells were transferred to DM 24 h after electroporation and tested 2–3 d later.

DNA Templates for RNase Protection Assays

Templates were prepared by subcloning small (179–276 bp) DNA fragments into pBluescript (Stratagene) or pGEM3Zf(+) (Promega). All constructs were confirmed by sequencing. Probe sequences were selected to avoid long regions of identity with known transcripts other than the target. The inserts were restriction fragments of cDNA clones, or PCR fragments from these clones or from cDNA of myogenic cell RNA. The transcribed probes were larger than the targets to distinguish between specific protected bands and any remaining undigested probes.

Kir2.1. A 269-nt probe complementary to 219 nt of Kir2.1 mRNA (AN: U12507, nt 1385–1603). The DNA fragment was obtained by PCR on clone HH-IRK1, and inserted into pGEM3Zf(+).

Kir2.2-C. A 335-nt COOH-terminal probe complementary to 276 nt of Kir2.2 mRNA (AN: L36069, nt 861–1136). The DNA fragment was amplified by PCR from myoblast cDNA and inserted into pGEM3Zf(+).

Kir2.2-N. A 315-nt NH_2-terminal probe complementary to 224 nt of Kir2.2 mRNA (AN: L36069, nt 55–278). A fragment excised from clone HIRK-A$^+$-pCRII in pBluescript KS(+).

Kir2.3. A 290-nt probe complementary to 263 nt of Kir2.3 mRNA (AN: U07364, nt 228–490). A PCR fragment covering the pH sensor region was amplified from myogenic cDNA and inserted into pGEM3Zf(+). For probe calibration experiments, a 318-nt synthetic Kir2.3 mRNA was generated from this vector using SP6 polymerase.

Kir2.4. A 252-nt probe complementary to 179 nt of the Kir2.4 mRNA 3′-end (AN: AF081466, nt 1312–1490). The template was generated by restriction of a pBluescript KS(+) vector containing a Kir2.4 EST (AN: AA504857; I.M.A.G.E. Consortium, UK HGMP Resource Centre).

Internal Control. A pBluescript vector containing 195 bp of human acidic ribosomal phosphoprotein P0 (AN: M17885) was provided by S. Mandriota (Geneva Medical School, Geneva, Switzerland). Transcription with T7 generates a 168-nt riboprobe, of which 121 nt hybridize to the target.

RNase Protection Assays

RNase protection assays were performed as described previously (Belin, 1997). The data were obtained from several independent assays and using RNA from different clones of cells. RNA was routinely extracted using the Chomczynski and Sacchi (1987) method, or TRIzol Reagent (Life Technologies) from skeletal muscle samples and cells in culture (proliferating myoblasts, cells differentiating at low density, high-density cultures at the onset of fusion, or 3–10-d-old myotubes).

Radiolabeled antisense cRNA probes were synthesized in vitro from linearized templates by the appropriate RNA polymerase in the presence of [α^{32}P]UTP (400 Ci/mmol; Amersham Pharmacia Biotech). The internal control probe, synthesized in the presence of excess cold UTP, had a specific activity lower than the channel probes to avoid signal saturation by the greater abundance of P0 mRNA, compared with channel mRNAs. Samples of total cellular RNA (20 μg) were hybridized concomitantly with 10^5 cpm of channel probe and 2.10^4 cpm of P0 probe. Yeast tRNA (20 μg) was used as negative control. After 30–60 min hybridization at 70°C, the samples were digested for 60 min at 30°C with 40–120 μg/ml of RNase A (Sigma-Aldrich; R5000). The reaction was terminated by addition of SDS and proteinase K, followed by phenol-chloroform extraction and ethanol precipitation. After electrophoresis on 6% polyacrylamide/8 M urea gels, the protected fragments were visualized by PhosphorImager scanning (Molecular Dynamics) or autoradiography. Quantitative evaluation of RNA expression was done by computer analysis using the program OptiQuant3 (Packard Instrument Co.). To estimate the number of transcript molecules per cell, we assumed that 10^6 cells contain ~10 μg of RNA, and we compared the intensities of the protected bands to that of a known amount of probe loaded on the same gel. From the specific activity of the probe, several protected target mRNA molecules can be determined.

Electrophysiological Recordings

Whole-cell and single-channel (outside-out) configurations of the patch-clamp technique (Hamill et al., 1981) were used to measure ionic currents. Signals were recorded with an Axopatch 200B amplifier (Axon Instruments, Inc.). The pipette resistances were 2–5 M Ω for whole-cell recordings, and 5–10 M Ω for single-channel recordings. Compensations between 30% and 70% were used in the whole-cell mode. The 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 and low-pass filtered at 0.5–1 kHz. Sampling rates were 2.5 and 5 kHz during whole-cell and single-channel recordings, respectively. To improve the patching procedure, myoblasts were treated with 0.05% trypsin and replated 1–2 h before recording.

Whole-Cell Recording. The extracellular solution was composed of (mM): 100 *N*-methyl-D-glucamine (NMG)-Cl, 5 KCl, 2 $MgCl_2$, 5 Hepes, 50 NaOH, 50 acetic acid, and 8 glucose. The pH was adjusted to 7.4 with NMG. The intracellular (pipette) solution was composed of (mM): 110 KCl, 5 NaCl, 2 $MgCl_2$, 5 Hepes, 20 BAPTA, 5 glucose, and 5 Mg-ATP. The pH was adjusted to 7.4 with KOH.

Single-Channel Recordings. The extracellular solution was composed of (mM): 80 KCl, 60 NaCl, 2 $MgCl_2$, 5 Hepes, 0.5 $CaCl_2$, and 3 glucose. The pH was adjusted to 7.4 with KOH. The intracellular (pipette) solution was composed of (mM): 5 KCl, 10 KF, 65 K-acetate, 5 NaCl, 2 $MgCl_2$, 50 NMG-acetic acid, 5 Hepes, 1 BAPTA, and 3 Mg-ATP. The pH was adjusted to 7.4 with NMG.

Effect of Antisense Expressing Vectors on Fusion

Myoblasts were electroporated with 2.5–3 pmoles vector as described above. For each condition, ~15×10^6 cells were electroporated, pooled, and seeded in GM in T175 culture flasks. The cells were detached 48 h later, and selected for EGFP expression by cell sorting (FACStar®; Becton Dickinson). The green fluorescent cells were collected and seeded in 35-mm dishes with rings to reduce the surface (~300,000 cells in 177 mm^2). After reattachment, GM was switched to DM to induce fusion. Half of the medium was changed every two days, and at day 5, cells were fixed and the fusion index determined (see below).

Fusion Index

The fusion index is defined as the number of nuclei in myotubes, divided by the total number of nuclei counted. Cultures were fixed for 5 min at −20°C with 100% methanol, and stained with haematoxylin. Nuclei were counted in 20 randomly chosen microscope fields in separate cultures. One microscope field usually contains between 100 and 150 nuclei. In *t* tests, *n* refers to the number of microscope fields counted.

Statistics

Unless specified, results are expressed as the means ± SEM. Statistical analysis was performed using the Student's *t* test.

Results

Single-Channel Properties of the Kir Channel Expressed at the Onset of Myoblast Fusion

Human myoblasts are triggered to fuse when they are transferred to a serum-free DM. By plating the cells at low density in this medium, the cells become FCMB, but fusion itself is impeded because cells cannot contact. Under these conditions, the cells that are hyperpolarized through the expression of ether-à-go-go channels start to express an inward rectifier K^+ current ($IK_{[IR]}$) (Liu et al., 1998). To define the electrophysiological signature of the channel(s) underlying this whole-cell $IK_{(IR)}$, we patched FCMB in the outside-out configuration and performed single-channel recordings.

Fig. 1 illustrates single-channel properties of the Kir channel recorded in FCMB exposed to symmetrical K^+ concentrations (80 mM). Fig. 1 A shows individual single-channel traces recorded in a hyperpolarized FCMB (3 d in DM). The patch was stepped to various potentials between –135 mV and +65 mV from a holding potential of +5 mV. Unitary conductances were found to be identical ($n = 14$) in all patches recorded, suggesting that FCMB express only one type of Kir channel. The mean channel conductance was 21.5 ± 0.3 pS ($n = 14$). As illustrated in the legend to Fig. 1 B, the unitary current–voltage relationships present steep inward rectifying properties (hardly any openings above 0 mV; Hille, 1992). Addition of Ba^{2+} inhibits channel opening (Fig. 1 C).

These results suggest that a single type of Kir channel is expressed in human myoblasts before fusion, and the strong inward rectification of the channel is in agreement with the whole-cell results, suggesting that it is a member of the inward rectifier subfamily Kir2 (Liu et al., 1998).

Two Members of the Kir2 Subfamily Are Detected in Differentiating Human Myoblasts by RNase Protection Assays

A search for presence of the four members of the subfamily Kir2 isolated thus far was first performed by RT-PCR on human myogenic cell RNA. Fragments of predicted size could be amplified for all four members, and sequencing confirmed their identity (data not shown). As the sequences of at least three members of this subfamily were not known to contain introns, amplification from residual genomic DNA in the RNA preparations cannot be excluded. Northern blot hybridizations detected a 5.5-kb Kir2.1 transcript in human myogenic cells, confirming results of others (Raab-Graham et al., 1994). However, the high homology of different Kir2 subfamily members and the low abundance of their mRNAs prompted us to analyze them by RNase protection assays. This method is indeed the most appropriate for detecting and distinguishing low levels of highly homologous transcripts.

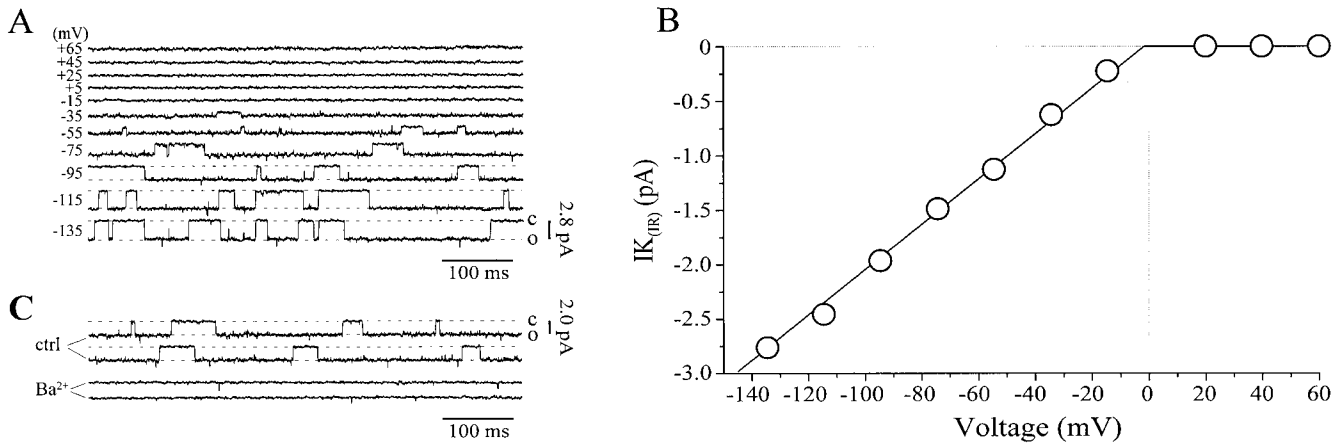

Figure 1. Single-channel properties of the inwardly rectifying K^+ channel expressed in human FCMB. (A) Single-channel recordings were performed in the outside-out configuration of the patch-clamp technique. The patch was stepped to various potentials between –135 mV and +65 mV for 700 ms from a holding potential of +5 mV. As expected, no outward current was observed for inwardly rectifying channels. (B) Same patch as in A. Unitary current amplitudes were evaluated using amplitude histograms, and plotted as a function of the potential at which the channel activity was recorded. As $[K^+]_{in} = [K^+]_{out} = 80$ mM, a linear regression was used to obtain the reversal potential of the current flowing through the channels ($E_{rev} = -2$ mV). The single-channel conductance (slope of the linear regression) is 21.0 pS. (C) Single-channel activity is blocked by external Ba^{2+}. Same patch as in A. The patch was stepped to –95 mV for 700 ms from a holding potential of +5 mV. The two upper current traces were recorded in control conditions, and the two lower traces were recorded after addition of 500 μM Ba^{2+} to bath medium.

As shown in the legend to Fig. 2 A, protected fragments of Kir2.1 and Kir2.2 transcripts were detected at all stages of myoblast differentiation, as well as in adult skeletal muscle. A quantitative analysis of transcript levels (as illustrated in the legend to Fig. 2 B), performed by comparison with that of an internal control (a house-keeping gene, P0) in several independent assays, indicates that there is a threefold increase ($P = 0.02$, $n = 6$) of Kir2.1 transcripts, and a twofold increase ($P = 0.02$, $n = 8$) of Kir2.2 between the proliferating stage and the multinucleated myotube stage. In the adult skeletal muscle, the relative transcript levels further increased between 3 to 4 and 5 to 9 times for Kir2.1 and 2.2 transcripts, respectively (data not shown).

Although no $IK_{(IR)}$ is detected at the stage of proliferation, transcripts are clearly present (Fig. 2 A). It is possible that the absence of $IK_{(IR)}$ results from translation control of Kir2.1 and Kir2.2 transcripts at this stage. On the other hand, proteins could be synthesized, but too few channels are generated to be detected by electrophysiology. We could estimate that each proliferating, mononucleated myoblast contains approximately only one Kir2.1 and two Kir2.2 transcripts. As the myotube is a multinucleated cell, the observed mild increases in respective mRNA levels during differentiation represent large increases of Kir2.1 and Kir2.2 transcripts per cell. Two variants of Kir2.2 have been reported, and we designed PCR primers and riboprobes that could discriminate between these forms. From sequencing of RT-PCR products and RNase protection assays, we did not detect the negative regulator variant (Kir2.2v; Namba et al., 1996) that was shown to inhibit Kir2.2 currents when coinjected into *Xenopus* oocytes. The other Kir2.2 variant is a 5′-end truncated sequence that was isolated by screening a human skeletal muscle cDNA library (AN: AF005214). We used two riboprobes to analyze expression of Kir2.2, one complementary to the 5′-region and the other to the 3′-region of the mRNA. The values obtained for mRNA quantification were similar with both probes, indicating that there is no detectable truncated form of Kir2.2 expressed in skeletal muscle cells.

Transcripts for Kir2.3 could not be detected at any stage of myoblast differentiation or in adult skeletal muscle with our riboprobe targeted to the pH sensing region, unique to this member of the Kir2 subfamily (Coulter et al., 1995). To evaluate the sensitivity of detection with our probe, we carried out a calibration test using a synthetic Kir2.3 RNA target. From this assay, we estimated that our probe could detect as few as two transcripts per 10 cells (data not shown), confirming that Kir2.3 is not expressed at significant levels.

Transcripts for the most recent member Kir2.4 could not be detected either. However, a faint product ~10 bases larger than expected was occasionally observed (data not shown). The level of this product was constant throughout the different stages of differentiation. If this product originates from a cellular Kir2.4 RNA, its level is predicted to be inconsequential on cell fusion.

Comparison of Single-Channel Properties of Kir2.1 and Kir2.2 Channels with the Endogenous Channel of FCMB

The results of RNase protection suggest that the endogenous Kir channel, expressed before fusion, could be either Kir2.1 or Kir2.2. Expression vectors were constructed, and single-channel recordings were performed on proliferating myoblasts transfected to express each of these two candidates. At the stage of proliferation, human myoblasts do not express any $IK_{(IR)}$, hence no native current would interfere with our recordings.

Fig. 3 illustrates the result of single-channel recordings in outside-out configuration, using the same protocol as for the recordings presented in the legend to Fig. 1. Fig. 3 A shows examples of unitary current traces elicited during steps to –95 mV, from a holding potential of +5 mV, in myo-

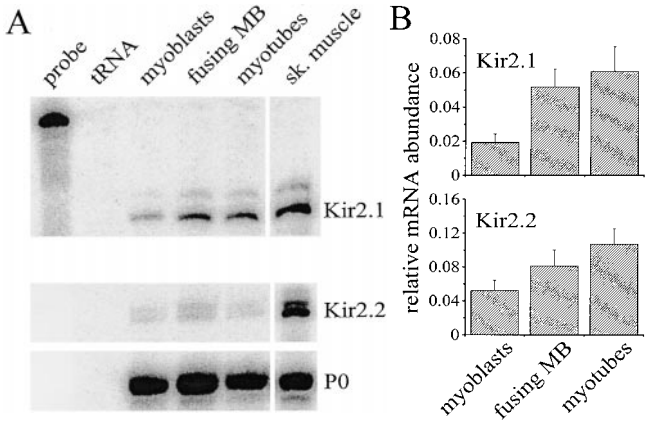

Figure 2. RNase protection analysis of Kir2.1 and Kir2.2 transcripts during human skeletal muscle differentiation. (A) Typical gels showing protected products obtained with riboprobes targeted to Kir2.1 transcripts (top) and Kir2.2 transcripts (middle) and internal control (a probe complementary to housekeeping P0 transcripts, bottom). The middle and bottom panels are from the same exposure; the top panel is from another gel. The stages of the cells from which the RNA was extracted are indicated on top. Fusing myoblasts are cultured 24 h in DM. The tRNA lane is a negative control. The probe shown in the top panel is the undigested Kir2.1 probe. In this experiment, Kir2.2 RNA abundance did not increase significantly during differentiation. (B) The relative mRNA abundance of Kir2.1 (top) and Kir2.2 (bottom), as normalized to P0 signal, increases significantly at the onset of myoblast fusion. Samples tested for Kir2.1: $n = 6$ for each stage. Samples tested for Kir2.2: $n = 8$ for myoblasts and myotubes, $n = 11$ for fusing myoblasts.

blasts transfected with Kir2.1 or Kir2.2 constructs, and in a FCMB (Kir-FC, 3 d in DM) expressing the native $IK_{(IR)}$. As shown in Fig. 3 B, the unitary current–voltage relationships obtained from traces of the channel recorded in myoblasts forced to express Kir2.1 overlap with that of the native $IK_{(IR)}$, and the conductance of the Kir2.1 channel was determined to be 20.0 ± 0.9 pS ($n = 3$), which is not significantly different from that of the endogenous channel (21.5 ± 0.3 pS, Fig. 1). The conductance of the channel formed in myoblasts overexpressing Kir2.2 subunits was determined to be 29.4 ± 0.2 pS ($n = 3$), a conductance significantly different from that observed in FCMB.

These results strongly suggest that the inward rectifier channel responsible for myoblast hyperpolarization before fusion is formed by Kir2.1 subunits.

Inhibition of Kir2.1 Expression Reduces Whole-Cell Inward Rectifier K^+ Current

To confirm the molecular identity of the channel responsible for myoblast hyperpolarization, we examined the effect on whole-cell inward rectifier K^+ current of an inhibition of Kir2.1 or Kir2.2 expression by antisense RNAs. Because of poor penetration of antisense oligonucleotides into human myoblasts and the need of their persisting presence in the medium, we chose antisense RNA vectors to block channel expression. For these experiments, bicistronic vectors expressing EGFP were engineered to contain a channel cDNA oriented antisense to the EF1α promoter of pEF-BOS (Uetsuki et al., 1989). The green fluorescence of EGFP was used to distinguish transfected cells before electrophysiological recording. The EF1α promoter was selected because it functions stably throughout myoblast differentiation, whereas expression from a cytomegalovirus promoter declined steeply when the cells were induced to fuse. Interestingly, expression from the cytomegalovirus promoter reappeared in the differentiated cells after 3 to 4 d in DM.

Myoblasts transfected with the antisense RNA vectors were induced to differentiate 24 h after transfection and whole-cell recordings performed after 2 to 3 d in DM. As shown in the legend to Fig. 4, cells treated with vectors expressing Kir2.1 antisense show significantly less average whole-cell $IK_{(IR)}$ density at –115 mV (−0.4 ± 0.07 pA/pF, $n = 57$) compared with control cells (−1.22 ± 0.21 pA/pF, $n = 59$, $P < 0.001$) or cells treated with vectors expressing Kir2.2 antisense (−1.16 ± 0.145 pA/pF, $n = 56$, $P < 0.001$). On the other hand, $IK_{(IR)}$ density expressed by cells treated with vectors expressing Kir2.2 antisense was not significantly different from control cells ($P = 0.81$). These results confirm that the native $IK_{(IR)}$ of FCMB is indeed formed by Kir2.1 subunits.

Inhibition of Kir2.1 Expression Drastically Reduces Myoblast Fusion

The data presented above demonstrate that the endogenous channel responsible for the hyperpolarization is constituted of Kir2.1 subunits, and that our antisense strategy is appropriate for testing the effect of Kir2.1 expression inhibition on the formation of multinucleated myotubes.

Myoblasts were transfected with antisense RNA vectors to block Kir2.1 or Kir2.2 channel expression. To obtain the number of efficiently transfected cells required to perform a fusion test, myoblasts were transfected with bicistronic antisense RNA vectors expressing EGFP. Green fluorescent myoblasts were then collected using a cell-sorter (FACStar®), and seeded at an adequate density to allow rapid transfer into the fusion-inducing medium (as described in Materials and Methods).

The results of cell fusion of three independent paired tests are presented in Fig. 5. In the first pair of data (left), it can be seen that the inhibition of Kir2.1 expression reduced myoblast fusion (17 ± 3%) with respect to cells transfected with the empty bicistronic pEF-IE parental vector (62 ± 1%). The second pair shows that treating cells with vectors expressing Kir2.2 antisense was without effect on the extent of fusion. The fusion index of cells transfected with the antisense-Kir2.2-RNA vector (69 ± 1%) is not statistically different from that of the control cultures (66 ± 1%). In the third test, we compared fusion in cells transfected with either the antisense-Kir2.2 or the antisense-Kir2.1-RNA vector. Fusion reached 64 ± 1% for antisense-Kir2.2 transfected cells, and only 3 ± 1% for the cultures treated with the antisense-Kir2.1-RNA vector. Equivalent results were observed in another similar set of experiments.

In conclusion, these results demonstrate the involvement of Kir2.1 channels in the formation of multinucleated myotubes.

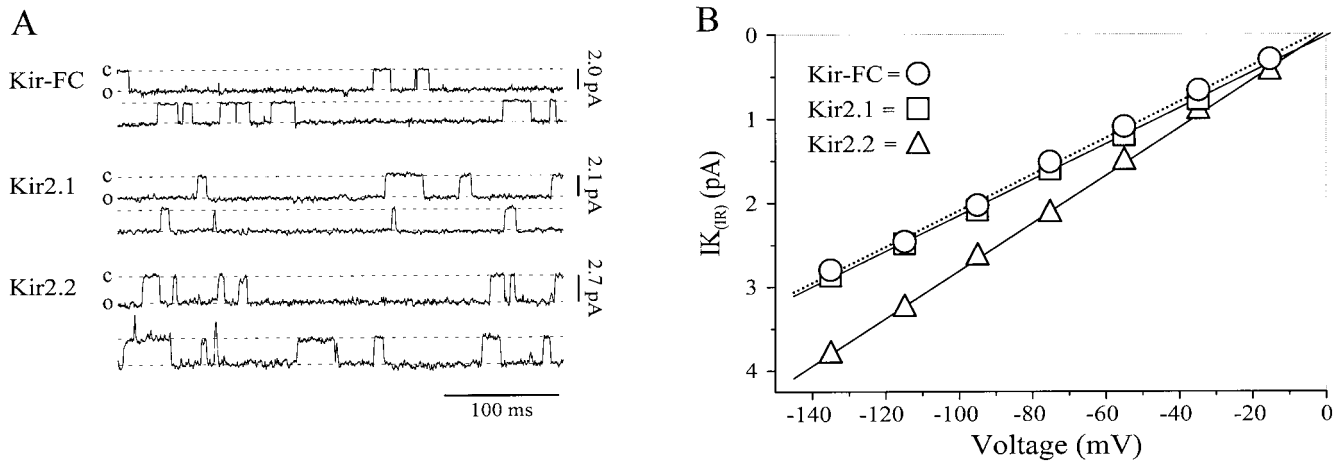

Figure 3. Single-channel properties of channels produced by overexpression of Kir2.1 or Kir2.2 in myoblasts were compared with properties of native Kir channel of FCMB. Unitary current traces of native FCBM channel (Kir-FC), and of channels Kir2.1 or Kir2.2 produced in human proliferating myoblasts forced to express these channels. Patches in outside-out configuration were stepped to various potentials between –135 mV and +65 mV for 700 ms (holding potential was +5 mV). (A) Examples of Kir channel activities (Kir-FC, Kir2.1, and Kir2.2) elicited during steps to –95 mV. (B) Unitary current amplitudes were plotted as a function of the potential at which the channel activity was recorded. Same patches as in A. The single-channel conductances (slope of the linear regression, $[K^+]_{in} = [K^+]_{out}$) were 21.2 pS for the native Kir channel, 21.5 pS for the overexpressed Kir2.1 channel, and 28.6 pS for the overexpressed Kir2.2 channel.

Discussion

We recently postulated that hyperpolarization of the membrane resting potential in the vicinity of –60 mV is required for myoblast fusion because it sets the membrane potential in a range at which the biophysical properties of expressed Ca^{2+} T channels allow Ca^{2+} to enter in the cell (via a window current) and trigger fusion (Bijlenga et al., 2000). We had indication from previous work that an inward rectifier K^+ current is involved in setting the membrane potential at these negative values (Liu et al., 1998). The goal of the present work was to identify the molecular nature of the channel underlying the inward rectifier current. Also, we wanted to obtain the molecular tools that would allow us to demonstrate the direct involvement of this channel in fusion, without having to rely on poorly selective pharmacological agents such as Cs^+ or Ba^{2+}.

One Type of Kir Channel Is Recorded in FCMB

Our results with single-channel recordings indicate that only one type of Kir channel underlies the second hyperpolarization step that occurs when human myoblasts are induced to fuse. Although the literature provides some data on single-channel recordings of inward rectifiers in skeletal muscle preparations (Ohmori et al., 1981; Matsuda and Stanfield, 1989), this is the first report on the unitary properties and role of the channel expressed in human mononucleated myoblasts before fusion. Interestingly, in one very brief report on human skeletal muscle sarcolemmal vesicles, the investigators recorded a channel with a similar conductance (18–23 pS; Burton et al., 1987). Further analysis would be required to confirm that this is the same Kir channel as recorded in FCMB, but the presence of a high level of Kir2.1 transcripts in skeletal muscle is compatible with this notion.

From the whole-cell $IK_{(IR)}$ properties (Liu et al., 1998), we hypothesized that the underlying channel was a member of the Kir2 subfamily. When expressed in *Xenopus* oocytes, channels of this subfamily differ in single channel conductances. Human Kir2.1 formed a channel with a conductance of ~30 pS (Raab-Graham et al., 1994; Wood et al., 1995), Kir2.2 elicited a channel of ~41 pS (Wible et al., 1995), and Kir2.3 has ~13 pS (Perier et al., 1994). None of these conductances in the oocyte system resembled that of the native channel of FCMB (21.5 ± 0.3 pS). However, the Kir2.2 conductance is 37% larger than the Kir2.1 conductance, which is comparable to what we found in our analysis of overexpression of Kir2 candidates in human proliferating myoblasts. In these cells, Kir2.1 was found to form a channel with a conductance nearly identical to the endogenous Kir channel of FCMB and 32% smaller than Kir2.2.

Kir2.1 and Kir2.2 Transcripts Are Present in Myoblasts before Fusion

Our RNase protection analysis confirms the presence of Kir2.1 transcripts in FCMB, but Kir2.2 transcripts are detected as well. Both mRNAs are present at all stages of myoblast differentiation (including adult skeletal muscle) and their levels increase rapidly when myoblasts are induced to differentiate. The two other members of the Kir2 subfamily, Kir2.3 and Kir.2.4, were not detected at any stage by RNase protection assays. Although Kir2.2 mRNA is clearly present in cells before fusion, overexpression of Kir2.2 in myoblasts elicited channels with a conductance that has never been observed in FCMB. This suggests that Kir2.2 subunits do not form a functional channel in FCMB.

It has been suggested that, unlike members of the Kir3 subfamily (Krapivinsky et al., 1995), Kir2.1 and Kir2.2 only form homotetrameric channels, and do not coassemble to form heteromultimeric channels (Yang et al., 1995; Tinker et al., 1996). Our results point in the same direction. Indeed, if the native Kir channel of FCMB were formed of Kir2.1 and 2.2 subunits, it is unlikely that the

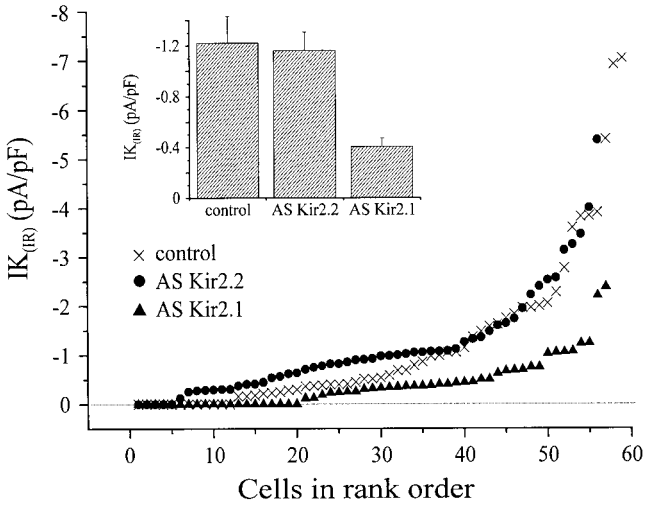

Figure 4. Inhibition of Kir2.1 expression reduces the whole-cell inward rectifier K^+ current. Human myoblasts were transfected with bicistronic vectors expressing antisense RNA to block expression of Kir2.1 (AS Kir2.l, triangles) or Kir2.2 (AS Kir2.2, circles), and $IK_{(IR)}$ density were recorded 2–3 d after the treated cells were transferred into DM to induce their differentiation. The current density is the whole-cell current amplitude divided by the membrane capacity (which is directly correlated to the surface area of the cell), thus allowing comparison between cells. Crosses represent control $IK_{(IR)}$ density recorded in FCMB kept 2–3 d in DM. $IK_{(IR)}$ amplitude was measured in whole-cell configuration during a step to –115 mV lasting 600 ms from a holding potential of –55 mV. Leak current was estimated by linear extrapolation of the current recorded at potential between −35 and –55 mV, and subtracted. The current density recorded in each cell is represented. Each symbol represents one cell. For each of the three experimental conditions (control, AS-Kir2.2, AS-Kir2.1), cells are ordered from the smallest to the largest current density recorded. About 60 cells were recorded in each condition. As previously shown, ~2/3 of the cells have no or very low $IK_{(IR)}$ (Liu et al., 1998). (Inset) Histograms of average $IK_{(IR)}$ density obtained for each population (control, $n = 59$; AS-Kir2.2, $n = 56$; AS-Kir2.1, $n = 57$).

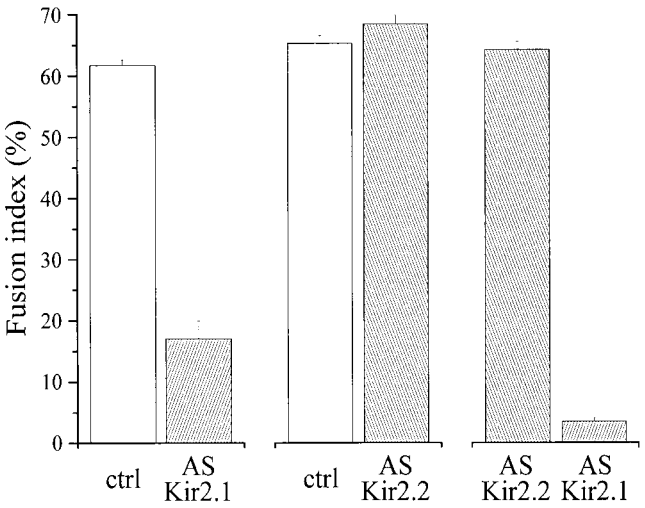

Figure 5. Myoblasts fusion is impeded when Kir2.1 expression is blocked by an antisense RNA vector. The fusion indexes are shown here for three independent experiments. Nuclei were counted in 20 randomly chosen microscope fields in each culture. (ctrl) Cells treated with pEF-IE vector. (AS Kir2.1 or AS Kir2.2) Cells treated with bicistronic vector expressing respective antisense RNA.

conductance of the channel formed by Kir2.1 subunits alone would mimic so closely that of the endogenous Kir channel of FCMB. Therefore, we presume that functional homotetrameric Kir2.2 channels are expressed at later stages of skeletal muscle development, and play a role in excitability of the mature muscle fiber.

However, it is important to mention that we cannot exclude the fact that a few Kir2.2 channels are expressed in FCMB. The fact that we never encountered a functional Kir2.2 channel in the 14 patches recorded could be explained on a statistical basis. For example, if Kir2.2 channels represent 10% of the total Kir channel population, the probability of recording a Kir2.1 channel 14 times in a row would be relatively high ($P = 0.9^{14} = 0.23$). We presently have no proof either that Kir2.2 channels are not incorporated in the plasma membrane and modulated by phosphorylation, for example, or that they require some other factor for activation or relief from inhibition.

Regulation of Kir2.1 Expression in Skeletal Muscle

Studies in various tissues have shown that expression of functional Kir2.1 channels is subject to complex regulatory mechanisms that appear to be stage and tissue dependent. The presence of Kir2.1 transcripts in proliferating myoblasts, a stage at which no $IK_{(IR)}$ is recorded, suggests that Kir2.1 expression is controlled at posttranscriptional levels. Kir2.1 channel activity has been shown to be regulated by phosphorylation, and Kir2.1 is a direct substrate for tyrosine kinase activity (Wischmeyer et al., 1998). Kir2.1 channels may already be integrated in the proliferating myoblast membrane, but the presence of growth factors could silence the channel through phosphorylation. On the other hand, mRNA translation might be tightly controlled. Recently, a K^+ channel regulatory factor was isolated and suggested to regulate synthesis of Kir2.1, among other tested potassium channels, by binding to certain RNA motifs (Keren-Raifman et al., 2000). K^+ channel regulatory factor appeared strongly expressed in skeletal muscle, but its possible role on Kir2.1 expression during myoblast terminal differentiation remains to be examined. Most interestingly, a study on mouse Kir2.1 gene structure and promoter revealed two putative, cytoplasmic polyadenylation consensus sequences in the 3′-UTR mRNA (Redell and Tempel, 1998). This observation suggests that Kir2.1 expression may be regulated through polyadenylation control, at least during early stages of maturation and development (Richter, 1999).

Kir2.1 and Kir2.2 mRNAs Are Both Present, but Only Kir2.1 Inhibition Affects Current and Fusion

To prove the molecular identity of the Kir channel of FCMB and to demonstrate its involvement in myoblast fusion, we adopted an antisense strategy. The inhibition experiments with vectors expressing antisense RNA show that the whole-cell $IK_{(IR)}$ density is strongly reduced in cells transfected with the antisense-Kir2.1-RNA vector, whereas those cells transfected with the antisense- Kir2.2-

RNA vector exhibit $IK_{(IR)}$ densities similar to control FCMB. This result confirms that the channel of FCMB is constituted of Kir2.1 subunits, and also confirms that heteromultimeric Kir2.1-Kir2.2 channels are not expressed in FCMB, because inhibition of Kir2.2 subunit expression did not noticeably affect $IK_{(IR)}$ density. From previous experiments on the effect of $IK_{(IR)}$ inhibition, we can predict that the effect of antisense-Kir2.1-RNA vector on the current would cause the voltage of FCMB to depolarize to about –45 mV (Liu et al., 1998). At this voltage, the Ca^{2+} (window) current through T channels would be markedly reduced (Bijlenga et al., 2000). We would thus anticipate an effect on fusion of antisense-Kir2.1-RNA vectors. Our results show that the formation of multinucleated myotubes is indeed impeded when myoblasts are transfected with the antisense-Kir2.1-RNA vector whereas the inhibition of Kir2.2 expression did not affect fusion. These results bring compelling evidence for the implication of functional Kir2.1 channels in human myoblast fusion, and demonstrate that myoblast membrane potential hyperpolarization is a prerequisite for fusion.

Kir2.1 and Kir2.2 Knock-Out Mice

Kir2.1 subunits have now been shown to constitute the main Kir channel in many tissues (arterial smooth muscle [Zaritsky et al., 2000], heart [Raab-Graham et al., 1994], brain [Tang et al., 1995], and lens epithelium [Rae and Shepard, 1998]). Thus far, no pathology has been associated with a dysfunctional Kir2 channel. In view of the importance of Kir2.1 channel functions in many cell types, it is easy to speculate that a deficiency in Kir2.1 would be either compensated or lethal. The Kir2.1 and Kir2.2 genes were recently disrupted in mice, and the Kir2.1$^{-/-}$mice generated presented an unexpected phenotype: the Kir2.1 knock-out mice died at birth, presumably because of a respiratory insufficiency due to a cleft palate (Zaritsky et al., 2000). Kir2.2 knock-out mice presented no particular phenotype (Zaritsky et al., 2000). This may suggest that another Kir channel can substitute for the dysfunctional protein. However, the examination of arterial myocytes isolated from the Kir2.1 $^{-/-}$ mice showed that Kir2.2 could not compensate for loss of Kir2.1 in the vascular muscle (Zaritsky et al., 2000). The skeletal muscles were apparently not affected in either deficient mice, but their muscle mass has not been reported. We would expect the isolated myoblasts of the Kir2.1-deficient animals to have a reduced fusion capacity or slower fusion rate, and it would be of interest to examine which other ionic current(s) could substitute to generate, in this context, the necessary hyperpolarization for fusion to proceed.

Further, it is interesting to note that Kir2.1 channels may play an important role in the differentiation of those few other cell types of the body that undergo a fusion process. Indeed, presence of Kir2.1 mRNA has been reported in osteoclasts, the cells responsible for bone resorption (Arkett et al., 1994), and in cytotrophoblasts, which fuse to form the placental syncytiotrophoblasts (Mylona et al., 1998). The expression of Kir2.1 channels in those mononucleated cells could suggest that a hyperpolarization of their membrane resting potential may also be required during their terminal differentiation into multinucleated cells.

We thank M. Berti and P. Brawand for their excellent technical assistance on cell cultures, Dr. A. Kaelin for providing the muscle biopsies, Dr. C.A. Vandenberg for kindly providing us with Kir2.1 and 2.3 clones, Dr. B. Wible for clone Kir2.2, and Dr. S. Nagata for pEF-BOS. We also thank Dr. W. Kelley for his useful advice, and A. Seydoux for her contribution to early parts of this project.

This work was supported by grants from the Fonds National Suisse pour la Recherche Scientifique (No. 31-054177.98 and 4046-058639), and the Fondation Suisse pour la Recherche sur les Maladies Musculaires.

Submitted: 2 February 2001
Revised: 18 March 2001
Accepted: 21 March 2001

References

Arkett, S.A., S.J. Dixon, and S.M. Sims. 1994. Lamellipod extension and K^+ current in osteoclasts are regulated by different types of G proteins. *J. Cell Sci.* 107:517–526.

Baroffio, A., J.P. Aubry, A. Kaelin, R.M. Krause, M. Hamann, and C.R. Bader. 1993. Purification of human muscle satellite cells by flow cytometry. *Muscle Nerve.* 16:498–505.

Belin, D. 1997. The use of RNA probes for the analysis of gene expression. *Mol. Biotechnol.* 7:153–163.

Bernheim, L., J.-H. Liu, M. Hamann, C.A. Haenggeli, J. Fischer-Lougheed, and C.R. Bader. 1996. Contribution of a non-inactivating potassium current to the resting potential of fusion-competent human myoblasts. *J. Physiol.* 493: 129–141.

Bijlenga, P., T. Occhiodoro, J.-H. Liu, C.R. Bader, L. Bernheim, and J. Fischer-Lougheed. 1998. An *ether-a-go-go* K^+ current, $I_{h\text{-}eag}$, contributes to the hyperpolarization of human fusion-competent myoblasts. *J. Physiol.* 512:317–323.

Bijlenga, P., J.H. Liu, E. Espinos, C.A. Haenggeli, J. Fischer-Lougheed, C.R. Bader, and L. Bernheim. 2000. T-type alpha 1H Ca^{2+} channels are involved in Ca^{2+} signaling during terminal differentiation (fusion) of human myoblasts. *Proc. Natl. Acad. Sci. USA.* 97:7627–7632.

Burton, F.L., U. Dorstelmann, and O.F. Hutter. 1987. Single channel activity in human sarcolemmal vesicles. *J. Physiol.* 392:31P.

Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.

Coulter, K.L., F. Perier, C.M. Radeke, and C.A. Vandenberg. 1995. Identification and molecular localization of a pH-sensing domain for the inward rectifier potassium channel HIR. *Neuron.* 15:1157–1168.

Duval, A., and C. Leoty. 1978. Ionic currents in mammalian fast skeletal muscle. *J. Physiol.* 278:403–423.

Espinos, E., J.-H. Liu, C.R. Bader, and L. Bernheim. 2001. Efficient non-viral DNA-mediated gene transfer to human primary myoblasts using electroporation. *Neuromuscul. Disord.* 11:341–349.

Gonoi, T., and S. Hasegawa. 1991. Postnatal induction and neural regulation of inward rectifiers in mouse skeletal muscle. *Pflügers Arch.* 418:601–607.

Hamill, O.P., A. Marty, E. Neher, B. Sakmann, and F.J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.* 391:85–100.

Hille, B. 1992. Ionic channels of excitable membranes. Sinauer Associates, Inc., Sunderland, MA. 132 pp.

Hughes, B.A., G. Kumar, Y. Yuan, A. Swaminathan, D. Yan, A. Sharma, L. Plumley, T.L. Yang-Feng, and A. Swaroop. 2000. Cloning and functional expression of human retinal kir2.4, a pH-sensitive inwardly rectifying K^+ channel. *Am. J. Physiol. Cell Physiol.* 279:C771–C784.

Katz, B. 1949. Les constantes électriques de la membrane du muscle. *Archiv. Sci. Physiol.* 2:285–299.

Keren-Raifman, T., T. Ivanina, Y. Bismuth, and N. Dascal. 2000. Expression cloning of KCRF, a potassium channel regulatory factor. *Biochem. Biophys. Res. Commun.* 274:852–858.

Krapivinsky, G., E.A. Gordon, K. Wickman, B. Velimirovic, L. Krapivinsky, and D.E. Clapham. 1995. The G-protein-gated atrial K^+ channel IKACh is a heteromultimer of two inwardly rectifying K^+-channel proteins. *Nature.* 374: 135–141.

Krause, R.M., M. Hamann, C.R. Bader, J.-H. Liu, A. Baroffio, and L. Bernheim. 1995. Activation of nicotinic acetylcholine receptors increases the rate of fusion of cultured human myoblasts. *J. Physiol.* 489:779–790.

Kubo, Y., T.J. Baldwin, Y.N. Jan, and L.Y. Jan. 1993. Primary structure and functional expression of a mouse inward rectifier potassium channel. *Nature.* 362:127–133.

Liu, J.-H., P. Bijlenga, J. Fischer-Lougheed, T. Occhiodoro, A. Kaelin, C.R. Bader, and L. Bernheim. 1998. Role of an inward rectifier K^+ current and of hyperpolarization in human myoblast fusion. *J. Physiol.* 510:467–476.

Matsuda, H., and P.R. Stanfield. 1989. Single inwardly rectifying potassium channels in cultured muscle cells from rat and mouse. *J. Physiol.* 414:111–124.

Mylona, P., L.H. Clarson, S.L. Greenwood, and C.P. Sibley. 1998. Expression of

the Kir2.1 (inwardly rectifying potassium channel) gene in the human placenta and in cultured cytotrophoblast cells at different stages of differentiation. *Mol. Hum. Reprod.* 4:195–200.

Namba, N., N. Inagaki, T. Gonoi, Y. Seino, and S. Seino. 1996. Kir2.2v: a possible negative regulator of the inwardly rectifying K^+ channel Kir2.2. *FEBS Lett.* 386:211–214.

Nichols, C.G., and A.N. Lopatin. 1997. Inward rectifier potassium channels. *Annu. Rev. Physiol.* 59:171–191.

Occhiodoro, T., L. Bernheim, J.-H. Liu, P. Bijlenga, M. Sinnreich, C.R. Bader, and J. Fischer-Lougheed. 1998. Cloning of a human *ether-a-go-go* potassium channel expressed in myoblasts at the onset of fusion. *FEBS Lett.* 434:177–182.

Ohmori, H., S. Yoshida, and S. Hagiwara. 1981. Single K^+ channel currents of anomalous rectification in cultured rat myotubes. *Proc. Natl. Acad. Sci. USA.* 78:4960–4964.

Park, J. Y., K.S. Shin, H. Kwon, J.G. Rhee, M.S. Kang, and C.H. Chung. 1999. Role of hyperpolarization attained by linoleic acid in chick myoblast fusion. *Exp. Cell Res.* 251:307–317.

Perier, F., C.M. Radeke, and C.A. Vandenberg. 1994. Primary structure and characterization of a small-conductance inwardly rectifying potassium channel from human hippocampus. *Proc. Natl. Acad. Sci. USA.* 91:6240–6244.

Raab-Graham, K.F., C.M. Radeke, and C.A. Vandenberg. 1994. Molecular cloning and expression of a human heart inward rectifier potassium channel. *Neuroreport.* 5:2501–2505.

Rae, J.L., and A.R. Shepard. 1998. Inwardly rectifying potassium channels in lens epithelium are from the IRK1 (Kir 2.1) family. *Exp. Eye Res.* 66:347–359.

Redell, J.B., and B.L. Tempel. 1998. Multiple promoter elements interact to control the transcription of the potassium channel gene, KCNJ2. *J. Biol. Chem.* 273:22807–22818.

Richter, J.D. 1999. Cytoplasmic polyadenylation in development and beyond. *Microbiol. Mol. Biol. Rev.* 63:446–456.

Shin, K.S., J.Y. Park, H. Kwon, C.H. Chung, and M.S. Kang. 1997. A possible role of inwardly rectifying K^+ channels in chick myoblast differentiation. *Am. J. Physiol.* 272:C894–C900.

Takahashi, N., K. Morishige, A. Jahangir, M. Yamada, I. Findlay, H. Koyama, and Y. Kurachi. 1994. Molecular cloning and functional expression of cDNA encoding a second class of inward rectifier potassium channels in the mouse brain. *J. Biol. Chem.* 269:23274–23279.

Tang, W., C.L. Qin, and X.C. Yang. 1995. Cloning, localization, and functional expression of a human brain inward rectifier potassium channel (hIRK1). *Receptors Channels.* 3:175–183.

Tinker, A., Y.N. Jan, and L.Y. Jan. 1996. Regions responsible for the assembly of inwardly rectifying potassium channels. *Cell.* 87:857–868.

Trautmann, A., C. Delaporte, and A. Marty. 1986. Voltage-dependent channels of human muscle cultures. *Pflugers Arch.* 406:163–172.

Uetsuki, T., A. Naito, S. Nagata, and Y. Kaziro. 1989. Isolation and characterization of the human chromosomal gene for polypeptide chain elongation factor-1 alpha. *J. Biol. Chem.* 264:5791–5798.

Wible, B.A., M. De Biasi, K. Majumder, M. Taglialatela, and A.M. Brown. 1995. Cloning and functional expression of an inwardly rectifying K^+ channel from human atrium. *Circ. Res.* 76:343–350.

Wischmeyer, E., F. Doring, and A. Karschin. 1998. Acute suppression of inwardly rectifying Kir2.1 channels by direct tyrosine kinase phosphorylation. *J. Biol. Chem.* 273:34063–34068.

Wood, L.S., T.D. Tsai, K.S. Lee, and G. Vogeli. 1995. Cloning and functional expression of a human gene, hIRK1, encoding the heart inward rectifier K^+ channel. *Gene.* 163:313–317.

Yang, J., Y.N. Jan, and L.Y. Jan. 1995. Determination of the subunit stoichiometry of an inwardly rectifying potassium channel. *Neuron.* 15:1441–1447.

Zaritsky, J.J., D.M. Eckman, G.C. Wellman, M.T. Nelson, and T.L. Schwarz. 2000. Targeted disruption of Kir2.1 and Kir2.2 genes reveals the essential role of the inwardly rectifying K^+ current in K^+-mediated vasodilation. *Circ. Res.* 87:160–166.