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The Intracellular Localization and Function of the ATP-Sensitive K^+ Channel Subunit Kir6.1

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Abstract Our aim was to determine the subcellular localization and functional roles of the K_{ATP} channel subunit Kir6.1 in intracellular membranes. Specifically, we focused on the potential role of Kir6.1 as a subunit of the mitochondrial ATP-sensitive K^+ channel. Cell imaging showed that a major proportion of heterologously expressed Kir6.1-GFP and endogenously expressed Kir6.1 was distributed in the endoplasmic reticulum with little in the mitochondria or plasma membrane. We used pharmacological and molecular tools to investigate the functional significance of this distribution. The K_{ATP} channel opener diazoxide increased reactive oxygen species production, and glibenclamide abolished this effect. However, in cells lacking Kir6.1 or expressing siRNA or dominant negative constructs of Kir6.1, the same effect was seen. Ca^{2+} handling was examined in the muscle cell line C2C12. Transfection of the dominant negative constructs of Kir6.1 significantly reduced the amplitude and rate of rise of $[Ca^{2+}]_c$ transients elicited by ATP. This study suggests that Kir6.1 is located in the endoplasmic reticulum and plays a role in modifying Ca^{2+} release from intracellular stores.

Keywords K_{ATP} channel · Kir6.1 · Endoplasmic reticulum · Mitochondria · Potassium channel opener · Ischemic preconditioning

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Introduction

ATP-sensitive potassium (K_{ATP}) channels are involved in a number of physiological and pathophysiological processes and form a link between cellular metabolism and membrane excitability. K_{ATP} channels are known to be present in many tissue types with a variety of functions (Rodrigo and Standen 2005). Characteristically, their gating is inhibited by increasing intracellular [ATP] and activated by increasing [ADP]. K_{ATP} channels are also present on endomembranes, in particular in mitochondria ("mitoK_{ATP}") (Inoue et al. 1991; Paucek et al. 1992). The K_{ATP} channel is an octameric complex comprised of four Kir6.0 subunits from the inward rectifier family of potassium channels and four sulfonylurea receptors (SURs), a member of the ATP-binding cassette family of proteins. The pore-forming subunit Kir6.0 has two different members, Kir6.1 and Kir6.2, which share 70% amino acid identity. The regulatory subunit SUR is encoded by two distinct genes, *SUR1* and *SUR2*, and is the site of action for antidiabetic drugs such as glibenclamide used in the treatment of type 2 diabetes mellitus (Babenko et al. 1998; Seino 1999; Rodrigo and Standen 2005; Ashcroft and Gribble 1998).

Ischemic preconditioning describes the phenomenon in which a short period of ischemia protects against a more prolonged one (Yellon and Downey 2003). The ability of potassium channel openers such as pinacidil and nicorandil to mimic and of inhibitors such as glibenclamide to abolish this phenomenon led to the suggestion that the effector in this process was a K_{ATP} channel. The initial focus was on the channel present in the sarcolemma; however, a number of pharmacological observations were not compatible with this hypothesis (Wang and Ashraf 1999; Fryer et al. 2000). The focus of research then turned to the potential involvement of a K_{ATP} channel present in the

mitochondrial inner membrane. There are biochemical and physiological data supporting the existence of this channel (Inoue et al. 1991; Paucek et al. 1992); however, its molecular identity has not been established. The K_{ATP} channel subunit Kir6.1 is widely distributed, and there have been a number of reports proposing that it is a subunit of the mitoK_{ATP} channel (Suzuki et al. 1997; Lacza et al. 2003; Zhou et al. 2005; Cuong et al. 2005). However, recent studies oppose this notion and, indeed, question the very existence of such a channel (Ardehali et al. 2004; Das et al. 2003; Hanley et al. 2002; Foster et al. 2008). Given this controversy, we studied the subcellular localization and potential function of Kir6.1 in intracellular membranes.

Materials and Methods

Molecular Biology

Kir6.1-GFP was generated by subcloning a *Bam*HI/*Eco*RI fragment of Kir6.1 into the *Bgl*II/*Eco*RI site of pEGFP-N1 (Clontech, Palo Alto, CA). A PCR fragment was generated using a high-fidelity polymerase (Vent DNA polymerase; New England Biolabs, Beverly, MA) corresponding to the remaining coding sequence of Kir6.1 without the stop codon and in-frame with GFP. This fragment was then subcloned into the above with an *Eco*RI/*Apa*I digest to give Kir6.1-GFP in pEGFP-N1. High performance purity (HPP) grade siRNAs (20 nmol) for Kir6.1 were custom-designed by Qiagen (Valencia City, CA) and active against both mouse and rat Kir6.1. The duplex 1 target sequence for mouse and rat was CACCACCTTGGTAGACCTGAA and the duplex 2 target sequence was CAGGAAGAGCATC ATCCCGGA. The latter was the most effective and was used in the functional studies (see below and Supplementary data). The duplex 3 target sequence was GAGGTGG TTCCTATTCACCAA (for human only).

Cell Culture

HEK293 (human embryonic kidney cell line), HepG2 (human liver cell line) and C2C12 (cell line derived from mouse skeletal muscle) cells were cultured in Eagle minimal essential medium with Earle's salts and L-glutamine supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen, Abingon, UK). The HL-1 cell line was obtained from Professor W. C. Claycomb (White et al. 2004) and grown in supplemented Claycomb medium, which contained 10% FBS (JRH Biosciences, Andover, UK), 1% penicillin/streptomycin, 0.1 mM norepinephrine (Sigma-Aldrich, Poole, UK) and 2 mM L-glutamine (Life Technologies, Paisley, UK). HL-1 cardiomyocytes were cultured on a gelatin (0.02% [wt/vol])/fibronectin (10 μg/ml) matrix. Cells were grown at 37°C in 95% air and 5% CO_2.

Biochemical Separation of Intracellular Organelles

HEK293 cells were transfected with Kir6.1-GFP (11 μg) and Sur1 (11 μg) using a calcium phosphate transfection technique (Urabe et al. 2000) and left for 48 h before analysis. Cells were fractionated using a homogenization buffer (3 mM imidazole [pH 7.4], 250 mM sucrose, 1 mM EDTA, 10 μg/ml cycloheximide, protease inhibitor [Roche, Lewes, UK]) method by modifying published protocols (Fialka et al. 1997). All steps were carried out at 4°C. Fractions were prepared from a T-75 flask of confluent HEK293 cells. Each flask was washed twice with ice-cold PBS, and cells were then collected by scraping in 1 ml ice-cold lysis buffer and homogenized by a 10 × 21-g needle. By a 10-min 1,000×*g* centrifugation step, the postnuclear supernatant (PNS) was prepared. A discontinuous OptiPrep gradient was generated by layering 1.4-ml layers of 40% OptiPrep in lysis buffer, followed by 35%, 30%, 25%, 20%, 15% and 10% OptiPrep in lysis buffer and finally topped up with lysis buffer and centrifuged at 165,000×*g* for 3 h at 4°C using the TI 41.14 rotor. Samples were fractionated in 940-ml aliquots from top to bottom to form 12 fractions (fraction 1 equates to the top fraction and fraction 12 to the bottom one). For further SDS-PAGE, the fractions were mixed with 3× reducing SDS-PAGE loading buffer (R-STB) and incubated for 10 min at 95°C.

SDS-PAGE and Western Blotting

Twenty-five microliters of each OptiPrep density fraction was separated on 10% Laemmli gel (Laemmli 1970). Gels were transferred to PVDF membranes and blocked in phosphate-buffered saline (PBS, pH 7.4) containing 5% dried milk powder. After blocking, membranes were incubated with anti-ATPB (raised to epitopes on the *β*-subunit of the mitochondrial ATPase, mouse monoclonal, 1:7,500 [Abcam, Cambridge, MA]), anti-calnexin (rabbit polyclonal, 1:2,000 [Santa Cruz Biotechnology, Santa Cruz, CA]) and anti-GFP (mouse monoclonal, 1:2,000 [Roche]) antibodies for 2 h. Membranes were then washed with PBS three times for 5 min. After washing, the primary antibody was detected by adding a horseradish peroxidase (HRP)-conjugated goat anti-rabbit specific secondary antibody (1:5,000, Santa Cruz [sc-2054]) and an HRP-conjugated goat anti-mouse specific secondary antibody (1:10,000, Jackson, Bar Harbor, ME) for 2 h. The membranes were then washed three times with PBS for 5 min and developed using the ECL™ Western blotting chemiluminescent reagent kit (Amersham, Aylesbury, UK)

per the manufacturer's instructions. Gel densitometry was performed as previously described (Leaney et al. 2001).

In the characterization of the siRNA duplexes a 35-mm dish of transfected HEK293 cells was harvested into ice-cold PBS and a fraction of this cell suspension directly lysed with SDS-PAGE lysis buffer as above.

Microscopy

Live Cells

HEK293 and C2C12 cells were transfected with Kir6.1-GFP, DsRed2-ER (targeted to the endoplasmic reticulum [ER], Clontech) and SUR1 (in pcDNA3 vector). Cells were transfected using lipofectamine (Invitrogen) and left for 24–48 h before analysis. In some cases, cells were loaded with tetramethylrhodamine, ethyl ester perchlorate (TMRE, Invitrogen) to stain mitochondria. EGFP was excited using an argon 488-nm laser, and emission was recorded using a HQ515/30 filter. DsRed2 was excited using a helium/neon laser at 543 nm, and images were collected with a LP560 filter. Colocalization analysis was performed using the approach of Manders (http://www.uhnresearch.ca/facilities/wcif/imagej/colour_analysis.htm).

Fixed Cells

Cells were fixed with 4% paraformaldehyde in PBS and washed twice with PBS before incubation with PBS + 0.2% Triton X-100. They were treated with blocking solution containing 2% BSA, 5% goat serum and 0.1% Triton X-100 in PBS. The affinity-purified rabbit antiserum for Kir6.1 was diluted 1/300 in blocking solution (Cui et al. 2001), and the secondary antibody used was a rhodamine-conjugated goat anti-rabbit antibody. The ER was stained using 100 μg/ml solution of fluorescein conjugate of concanavalin A. We also stained for mitochondria using cytochrome-*c* oxidase mouse monoclonal antibody and a FITC-linked secondary antibody (all probes were purchased from Molecular Probes, Eugene, OR). The coverslips were individually mounted onto slides using a small drop of Vectashield (Vector Laboratories, Burlingame, CA). Images were acquired and analyzed as above.

Reactive Oxygen Species Production Assay

Cells were plated into black-walled, clear-bottomed 96-well plates for 24 h before 1-h incubation with the reactive oxygen species (ROS) detection reagent 2′7′-dichlorofluorescein (DCF, 15 μg/ml in PBS from Molecular Probes). The drug plates containing diazoxide and glibenclamide were prepared as 10× stocks in DMSO in clear 96-well plates so that the final concentration of DMSO in each well was 0.02% (2.6 mM). Equivalent experiments using DMSO in PBS were used as controls. The plates were then placed in the fluorometric imaging plate reader system (Bucher Biotech, Basel, Switzerland), and the fluorescence (in arbitrary units) was measured every second. The cells were illuminated for 1 min before addition of the agonist (diazoxide or control). At 3 min, antagonist (glibenclamide or control) was added into the wells containing the agonist or control buffer.

$[Ca^{2+}]_c$ Imaging

For $[Ca^{2+}]_c$ measurements, C2C12 cells were plated into six-well plates containing 25-mm coverslips 24–48 h before they were loaded with 5 μM Fura-2 AM (Molecular Probes) containing 0.005% Pluronic (Sigma-Aldrich) in a HEPES-buffered salt (HBSS) solution composed of (in mM) 156 NaCl, 3 KCl, 2 $MgSO_4$, 1.25 KH_2PO_4, 2 $CaCl_2$, 10 glucose and 10 HEPES (pH 7.35). After 30 min, the medium was removed and replaced with the same solution without $CaCl_2$ but with 0.5 mM EGTA. The cells were left for 15 min before fluorescence measurements were obtained using a Nikon (Tokyo, Japan) epifluorescence inverted microscope inverted with a 20× fluorite objective. The excitation light from a xenon arc lamp was selected using 10-nm bandpass filters centred at 340, 360 and 380 nm housed in a computer-controlled filter wheel (Cairn Research, Faversham, UK). The emitted light passed through a long-pass filter to a cooled charged-coupled device (CCD) camera (Orca ER; Hamamatsu, Welwyn Garden City, UK). The data were collected at intervals of 5–10 s before they were digitized and analyzed with Origin 7.0 (OriginLab, Northampton, MA). The traces are presented as ratios of excitation at 340 and 380 nm, both with emission at >515 nm. The rising rate constants of ATP-induced Ca^{2+} transients were measured by calculating the largest rate between successive data points in this rapid phase.

Patch Clamping

Membrane currents were studied with the whole-cell patch-clamp technique, using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Currents were filtered at 1 kHz and sampled at 2 kHz via a Digidata 1200 interface. Data were analyzed using Clampfit software (Axon Instruments). Whole-cell patch pipettes were manufactured from borosilicate glass (OD 1.5 mm, ID 1.2 mm) using a PP-830 puller and fire-polished using a MF-830 microforge (both Narishige, Tokyo, Japan) to give pipette resistances of 2–3.5 MΩ. Pipette capacitance was reduced by coating tips with a parafilm/mineral oil suspension and compensated for electronically, while series resistance was

compensated to 70% using the amplifier. Whole-cell bath solutions (pH 7.4) contained (mM) 140 KCl, 5 HEPES, 1.2 $MgCl_2$ and 2.6 $CaCl_2$ and pipette solutions (pH 7.2) contained (mM) 140 KCl, 5 HEPES, 1.2 $MgCl_2$, 10 EGTA, 1 $CaCl_2$, 1 (Mg)ATP and 0.5 (Na)UDP.

Statistical Analyses

The colocalization data were compared against a theoretical value of zero with the rank scoring Mann-Whitney test. These data are presented as medians with 25% and 75% interquartile ranges. One-way ANOVA with a Bonferroni post-hoc test was carried out to assess for statistical differences for ROS production and Ca^{2+} handling data. Data are presented as means ± SEM. All data analyses were performed using Prism (version 4; Graphpad Software, San Diego, CA). $P < 0.05$ was defined as significant.

Results

Kir6.1 in combination with SUR2B expresses plasmalemmal currents similar to that thought to underlie the K_{ATP} channel in smooth muscle. We thus determined whether the expression of Kir6.1-GFP with SUR2B in HEK293 cells leads to membrane currents with the appropriate pharmacology indicative that the GFP fusion was not grossly altering membrane function. Using whole-cell patch clamping, the currents were qualitatively similar to those with Kir6.1 and SUR2B expression (Fig. 1).

We heterologously expressed Kir6.1-GFP in a number of cell lines and examined its subcellular distribution. The pharmacology described for the mitoK_{ATP} channel is closest to that described for the Kir6.1/SUR1 combination (Liu et al. 2001). Thus, we expressed both Kir6.1-GFP and Kir6.1-GFP/SUR1 together with ER-DsRed2 in C2C12 (Fig. 2a) and HEK293 cells (Fig. 2b). In both cell lines, a significant proportion of the Kir6.1-GFP signal colocalized with a marker of the ER (coefficients of colocalization ~30–50%). Although a significant proportion of Kir6.1 was located in the ER, there is still a fraction that is unaccounted for; and this may be located in other compartments such as mitochondria. Thus, we loaded C2C12 cells, transfected with Kir6.1-GFP and Kir6.1-GFP/SUR1, with Mitotracker red. Colocalization analysis indicated that a low proportion of Kir6.1-GFP colocalized with the mitochondrial marker (Fig. 2c). The coexpression of Kir6.1-GFP with SUR1 does not lead to prominent membrane translocation of Kir6.1-GFP; however, the combination of SUR1 with Kir6.2-GFP does show this pattern (Fig. 2d). The numerical data are summarized in Fig. 2e.

We next examined the distribution of endogenous Kir6.1 using immunofluorescent staining with an antibody raised

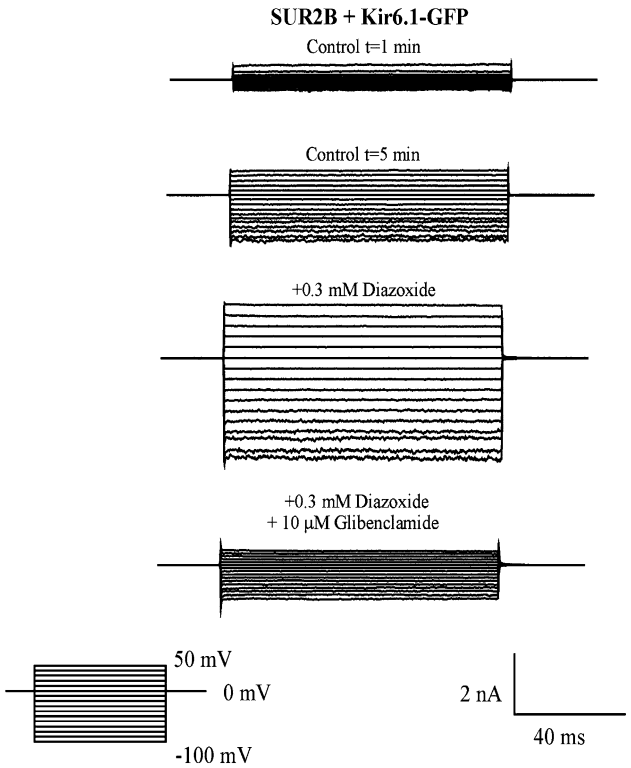

Fig. 1 An example of a whole-cell recording to show K_{ATP} channel activity in HEK293 cells transfected with Kir6.1-GFP and SUR2B. Currents were stimulated with 300 μM diazoxide, and channel activity was inhibited with 10 μM glibenclamide

to the C terminus of Kir6.1. We previously published a characterization of this antibody (Cui et al. 2001). Kir6.1 is not present in HEK293 cells (Cui et al. 2001); but C2C12, HepG2 and HL-1 cells showed significant expression of Kir6.1 using immunofluorescence (Fig. 3a–c). We labeled the ER with concanavalin A, and there was at least 50% colocalization between the channel and the marker (Fig. 3a–c). We also investigated the distribution of Kir6.1 in mitochondria using cytochrome-*c* oxidase, a component of the mitochondrial respiratory chain. The results in Fig. 3d and e show that there was only at best a modest fraction of Kir6.1 expressed in mitochondria. The data are summarized in Fig. 3f.

To further investigate the subcellular distribution of Kir6.1 using techniques other than imaging, we transiently transfected HEK293 cells with SUR1 and Kir6.1-GFP. We then isolated potential fractions corresponding to an ER and mitochondrial localization using gradient centrifugation (see "Materials and Methods"). In Fig. 4 it is apparent that Kir6.1-GFP (detected using an anti-GFP antibody) has a distribution that most closely matches the distribution of the ER marker. The mitochondrial fractions are lower on the gel; but there is some overlap, and it is impossible to exclude some possible mitochondrial localization.

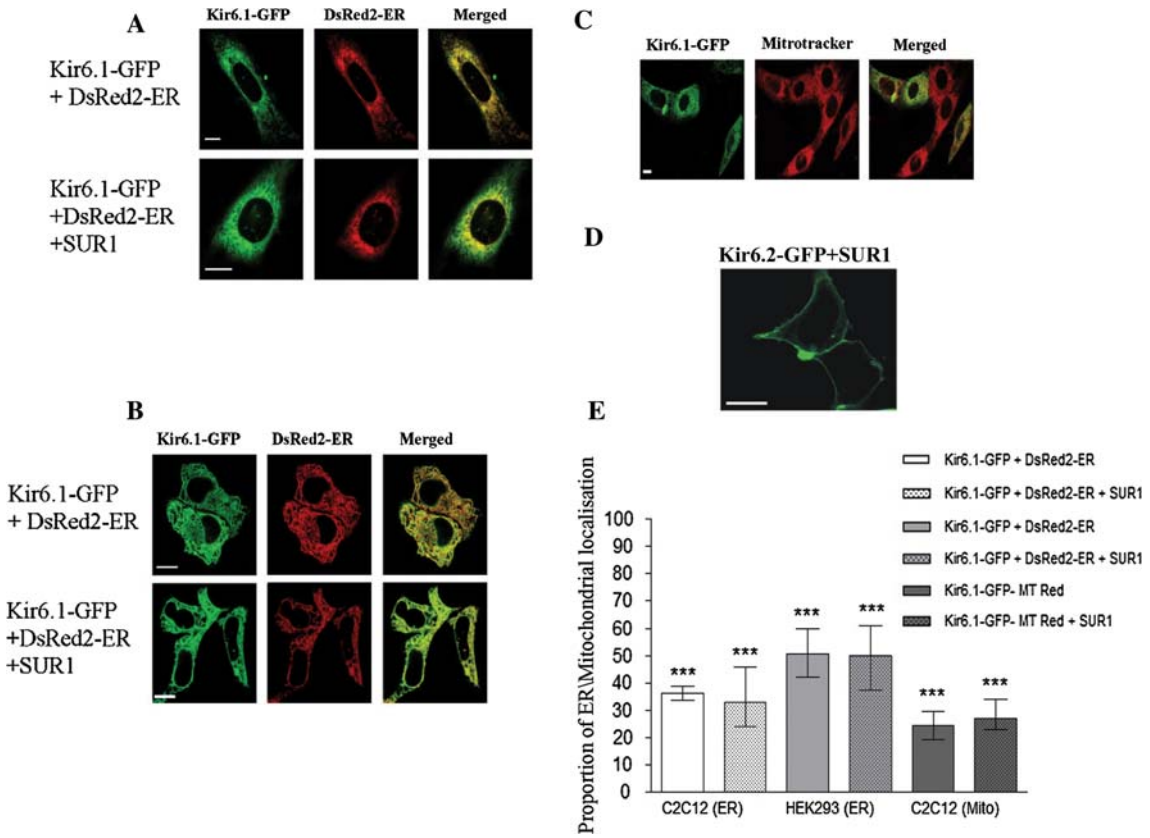

Fig. 2 Confocal images showing the proportion of colocalization between Kir6.1 (Kir6.1-GFP) and ER (DsRed2-ER) or mitochondria (Mitotracker) signals in immortalized cell lines using transient transfections (median values for colocalization coefficient are in bold). The *scale bar* in **a–d** is 10 μ. **a** C2C12 cells transfected with Kir6.1-GFP + DsRed2-ER ($n = 48$, 36%) or Kir6.1-GFP + DsRed2-ER + SUR1 ($n = 32$, 33%). **b** HEK293 cells: Kir6.1-GFP + DsRed2-ER ($n = 48$, 51%), Kir6.1-GFP + DsRed2-ER + SUR1 ($n = 32$, 50%). **c** C2C12 cells transfected with Kir6.1-GFP and treated with Mitotracker red ($n = 36$, 25%), C2C12 cells transfected with SUR1 + Kir6.1-GFP ($n = 17$, 27%). **d** HEK293 cells transfected with Kir6.2-GFP and SUR1. These K_{ATP} subunits form a protein complex and exit the ER to localize to the plasma membrane. **e** A graph to summarize the data for **a–c**. Each column represents the proportion of Kir6.1 that localizes to the ER or mitochondria. These data are presented as medians with 25% and 75% interquartile ranges

In addition to the well-known pharmacological tools for the manipulation of K_{ATP} channel function, it would be desirable to have equivalent molecular reagents. We have previously characterized Kir6.1 dominant negative constructs with two different pore mutations (GFG to AFA = DNGA or GA and GFG to SFG = DNGS or GS) (Cui et al. 2001). They are highly effective but not selective for Kir6.1 as they also have effects on Kir6.2 (Cui et al. 2001). Thus, we also developed and characterized siRNA duplexes to Kir6.1, and the controls are shown in Fig. 5. Both duplexes were able to suppress Kir6.1, but not Kir6.2, expression (Fig. 5); but duplex 2 was the most effective and was used below.

It has been proposed that activation of mitoK_{ATP} channels by diazoxide (diazoxide) leads to generation of ROS and that this is central to the protective effects of the compound. Glibenclamide antagonizes diazoxide-induced ROS production. We investigated the effects of these drugs in three cell lines (C2C12, HEPG2 and HEK293), and they all showed a significant increase in ROS production in response to 50 μM diazoxide; this effect was abolished by 10 μM glibenclamide (Fig. 6a–c). Thus, the pharmacological agents have effects on ROS production, but this is independent of the presence of the Kir6.1 subunit as the channel is not expressed in HEK293 cells. We investigated this further and knocked down the expression of Kir6.1 in C2C12 and HepG2 cells using siRNA. Knockdown of Kir6.1 did not affect diazoxide-induced ROS production in any of the three cell lines, and the data for C2C12 cells are displayed in Fig. 6d. Finally, we used the dominant negative constructs with two different pore mutations (Cui et al. 2001), and the overexpression of these cDNA constructs in C2C12 cells did not affect ROS production induced by diazoxide (Fig. 6e, f). Transfection efficiency was $\geq$ 70% as determined in parallel cotransfections with eGFP and the constructs.

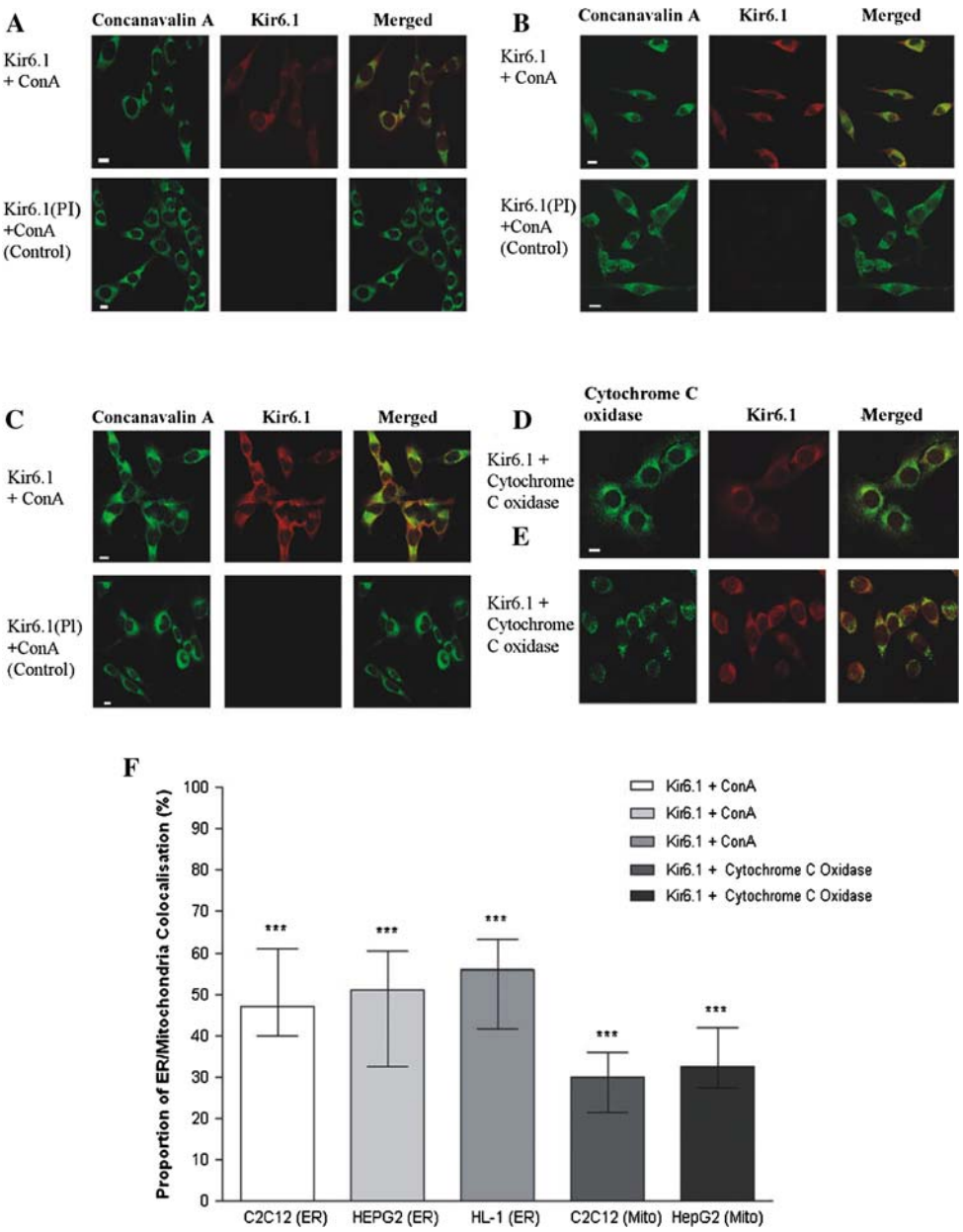

Fig. 3 Images to show the endogenous distribution of Kir6.1 in immortalized cell lines using Kir6.1 antibody, ER marker (concanavalin A FITC) and mitochondrial marker cytochrome-*c* oxidase (median values for colocalization coefficient are in bold). **a** C2C12 cells ($n = 25$, 47%) (ER). **b** HepG2 ($n = 33$, 51%). **c** HL-1 cells ($n = 34$, 56%) (ER). **d** C2C12 cells ($n = 17$, 30%) (Mitochondria). **e** HEPG2 ($n = 22$, 32%). **f** A graph to summarize the degree of colocalization between the endogenous distribution of Kir6.1 and ER and mitochondrial markers. These data are presented as medians with 25% and 75% interquartile ranges

$[Ca^{2+}]_c$ Handling

Our results show that Kir6.1 is predominantly located in the ER. We therefore asked whether it has a function in the regulation of Ca^{2+} release from intracellular stores, perhaps providing a route for charge compensation during Ca^{2+} release. Undifferentiated C2C12 cells do not generate a $[Ca^{2+}]_c$ transient to 1 mM caffeine, but 10 μM ATP routinely and reliably induced a significant increase in $[Ca^{2+}]_c$, presumably via purinergic receptors such as P_{2Y}. The absence of added calcium and the presence of EGTA in the extracellular medium ensured that the source of Ca^{2+} was derived from internal stores. Even in differentiated C2C12 cells (4 days in low serum medium) only 59% of the cells showed responses to caffeine. Given the reproducible response to ATP, we investigated the role of Kir6.1 in ATP-induced Ca^{2+} transients in nondifferentiated C2C12 cells using the dominant negative constructs as they led to a more prominent inhibition than siRNA. Apparent Fura-2 ratios were reduced in all eGFP-expressing cells compared to nontransfected cells due to some degree of GFP excitation in the UV as this blunts the dynamic range of the ratio (Bolsover et al. 2001). However, there was a significant decrease in the amplitude of the $[Ca^{2+}]_c$ transient (Fig. 7a, b) and a decrease in the rate of rise of the response (Fig. 7a, c) when comparing cells transfected with eGFP alone and those transfected with eGFP and a dominant negative construct. The

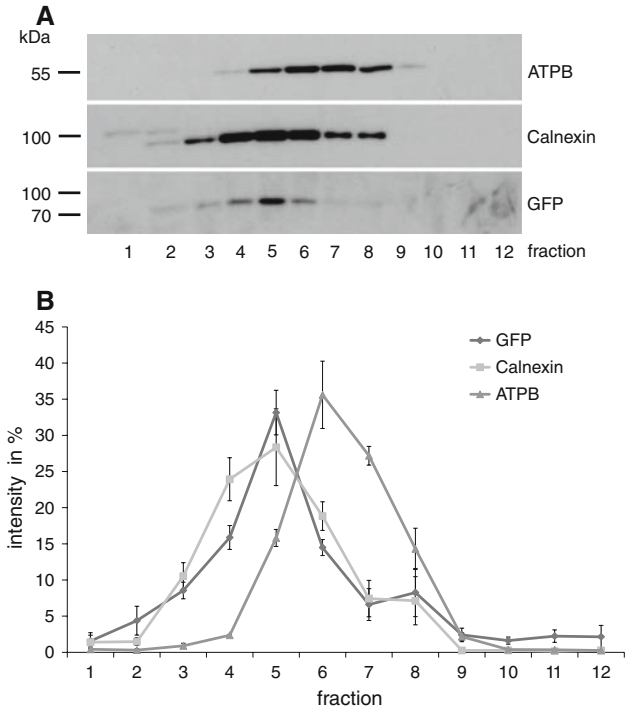

Fig. 4 Western blot analysis of OptiPrep density fractions of HEK293 cells expressing Kir6.1-GFP and SUR1. **a** The expression and localization of Kir6.1-GFP using an anti-GFP antibody (GFP) and comparison to fractions containing ER, detected with an anti-calnexin antibody (Calnexin) and mitochondria, detected by using an anti-ATPB antibody (ATPB). **b** Graph shows the signal intensity (% of total signal) in the Western blot analysis shown in **a**. The diagram contains mean data from four experiments, and the *error bars* represent the standard error of the mean

presence of GFP did not affect the rate constant for the rise of the $[Ca^{2+}]_c$ transient (for GFP-transfected cells = $1.20 \pm 0.2\ s^{-1}$ [$n = 9$], for nontransfected cells = $1.19 \pm 0.2\ s^{-1}$ [$n = 23$]).

Discussion

Since the sequencing of a number of mammalian genomes, the full palette of canonical K^+ channels is now known (Salkoff et al. 2005). One question following the cloning of the various components of the K_{ATP} channel was whether one of the known Kir6.0 and/or SUR subunits was the molecular equivalent of mitoK_{ATP}. Furthermore, the unequivocal demonstration of such would establish the true existence of these channels in mitochondria. A number of studies have suggested that Kir6.1 might be one component (Suzuki et al. 1997; Lacza et al. 2003; Zhou et al. 2005; Cuong et al. 2005). Our imaging and biochemical studies showed that Kir6.1 had a predominant intracellular location, with only at most a modest proportion in mitochondria. However, we were unable to demonstrate any correlation between functional readout and Kir6.1 expression. ROS production is often associated with mitochondrial K_{ATP} channel opening and protection against cell death at reperfusion. Indeed, the generation of such species has been suggested to form a key link between mitoK_{ATP} channel opening and the subsequent activation of downstream signaling pathways responsible for protection (Forbes et al. 2001; Costa and Garlid 2008; Costa et al. 2008). Specifically, diazoxide and glibenclamide had identical effects on ROS production in cell lines that express Kir6.1 (HL-1 and C2C12) and in those that do not (HEK293). Furthermore, siRNA treatment and the expression of 6.1 dominant negative constructs in C2C12 cells had no effect ROS production. Thus, our data do not support the idea that Kir6.1 underlies mitoK_{ATP}. It is worth noting that much of the supporting data are based on antibody recognition techniques, and one group has recently shown, using proteomic techniques, that some of the commercial antibodies detect other unrelated proteins (NADH dehydrogenase flavoprotein 1 and mitochondrial isocitrate dehydrogenase) in mitochondria (Foster et al. 2008). We raised our own antisera and undertook a number of control experiments (Cui et al. 2001). If it is not a known K^+ channel subunit, what protein components constitute mitoK_{ATP}? There is evidence that it is a multiprotein complex of a succinate dehydrogenase, mitochondrial ATP-binding cassette protein 1, phosphate carrier, adenine nucleotide translocator and ATP synthase (Ardehali et al. 2004). However, the ion selectivity and the molecular basis for the ion conduction pathway were not established, and it is not clear how it is related to the canonical elements in the H5 segment of the established K^+ channel genes and structures (Doyle et al. 1998). Furthermore, there are a number of studies that question the existence of mitoK_{ATP} and whether K^+ channel activity in the mitochondrial inner membrane has a role in cardiac protection (Das et al. 2003; Hanley et al. 2002; Foster et al. 2008).

Our imaging studies showed that Kir6.1 had a predominant intracellular location in the ER. Heterologous transient transfection of GFP-tagged proteins might overwhelm the transport capacity of the cell, and for an intrinsic membrane protein this might result in apparent retention in the ER. However, immunofluorescent staining also detected the presence of endogenous Kir6.1 in these cell lines, and this had a similar subcellular distribution to Kir6.1-GFP. In addition, Kir6.1 was ER-retained and not trafficked to the plasma membrane when coexpressed with a potential regulatory subunit, SUR1, in contrast to Kir6.2-GFP, which was clearly translocated to the plasma membrane. The apparent predominant distribution of an ion channel is determined by retention and forward export sequences present in the channel complex, in this case Kir6.0 and SUR (Ma et al. 2001). There may be

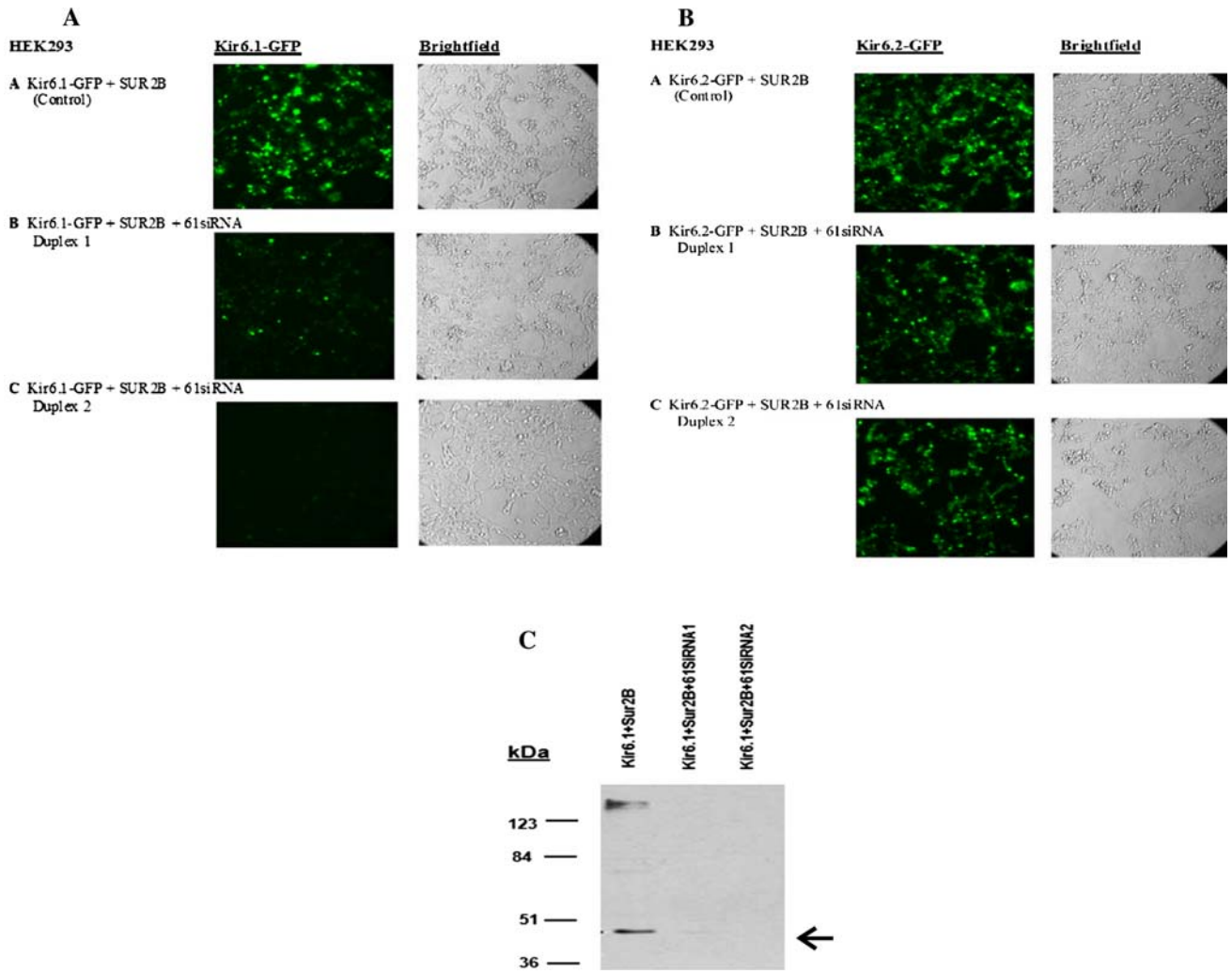

Fig. 5 HEK293 cells were transiently transfected with Kir6.1-GFP or Kir6.2-GFP with SUR2B and 61siRNA (duplex 1 or 2, see "Materials and Methods"). Images were taken with epifluorescent and brightfield microscopy. The amount of fluorescence was used to monitor the silencing effect of siRNA for Kir6.1. **a** HEK293 transfected with Kir6.1-GFP, SUR2B and 61siRNA. **b** HEK293 transfected with Kir6.2-GFP, SUR2B and 61siRNA. The 61siRNA does not affect the expression of Kir6.2-GFP and is specific to Kir6.1. **c** Western blot of HEK293 lysates, transiently transfected with K_{ATP} channel subunits and siRNA duplex 1 or 2 for Kir6.1 and probed with an antibody to the C terminus of Kir6.1

significant differences between the different Kir6.0 and SUR isoforms, especially in terms of forward export motifs which have not been well characterized. What would be the consequence of a regulated K^+ permeability opening and closing in endoplasmic and/or sarcoplasmic membranes? A major function of these intracellular organelles is to store and release Ca^{2+}. Potentially, Ca^{2+} release could generate a large diffusion potential, which if unchecked, would ultimately inhibit the release process. It is thought that there is a mechanism of "charge compensation" mediated by ion fluxes through K^+ and Cl^- channels in the sarcoplasmic reticulum (SR) membrane (Coronado and Mille, 1980; Tinker et al. 1992; Garcia and Miller 1984). Opening of additional K^+ channels may enhance Ca^{2+} release, alter the dynamics of the Ca^{2+} transient and prevent accumulation of Ca^{2+} in the SR during Ca^{2+} overload. Interestingly, we were able to obtain some preliminary data indicating an influence of modulating Kir6.1 function on Ca^{2+} transients in C2C12 cells. Specifically, the magnitude of the Ca^{2+} transient was attenuated and the rate of release slowed. Thus, it is possible that Kir6.1 contributes a pathway underlying a modest fraction of the charge compensating current in ER membranes. It is worth stating that the responses were not abolished, and it appears that there are a number of specialized ER resident channels that also accomplish this role (Coronado and Miller 1980; Tinker et al. 1992). These initial observations need to be consolidated using

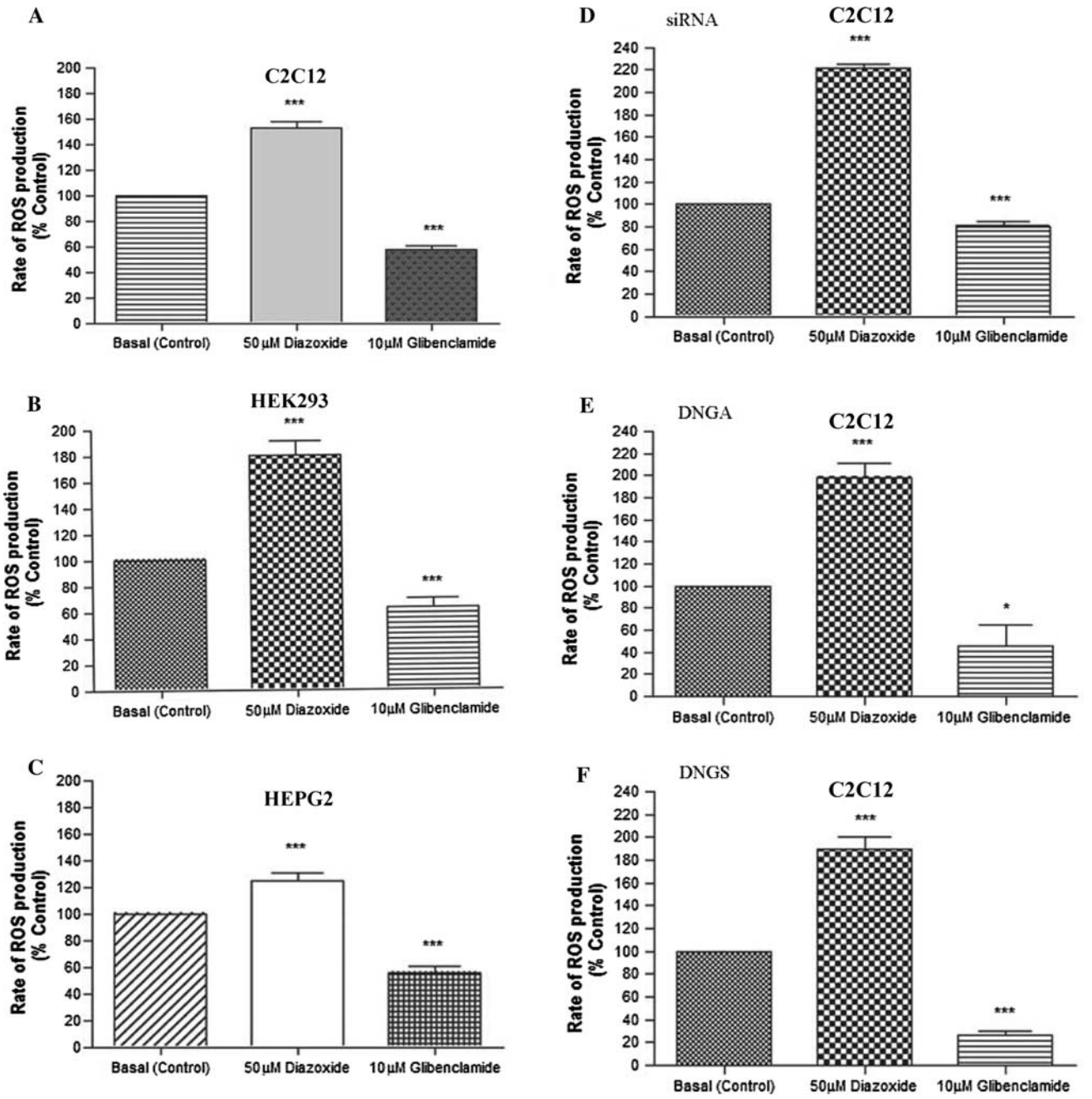

Fig. 6 Graphs to show the rate of ROS production in immortalized cell lines in response to 50 μM diazoxide and 10 μM glibenclamide. **a** C2C12 ($n = 24$). **b** HepG2 cells ($n = 48$). **c** HEK293 ($n = 53$). *Graphs* to show the rate of ROS production in C2C12 cells treated with siRNA and dominant negatives for Kir6.1. **d** C2C12 cells transfected with siRNA for Kir6.1 ($n = 24$). **e** C2C12 cells transfected with Kir6.1 with the AFA mutation (DNGA, $n = 16$). **f** C2C12 cells transfected with Kir61 with the SFG mutation (DNGS, $n = 16$)

more physiological approaches, such as genetically modified mice. It is established that a number of ion channels, including inwardly rectifying K^+ channels, depend for their activity on the provision of phosphatidylinositol bisphosphate (PtdIns[4,5]P_2) in the membrane system (Gamper and Shapiro 2007). Intracellular membranes have little PtdIns(4,5)P_2 compared to the plasma membrane (Voelker 1996), and thus, a large number of PtdIns(4,5)P_2-dependent ion channels would be expected to be inactive during transit through the secretory pathway. This has been suggested to be potentially beneficial to the cell. However, we have established that Kir6.1 has a very high PtdIns(4,5)P_2 affinity, and it may be one of the exceptions to this paradigm (Quinn et al. 2003).

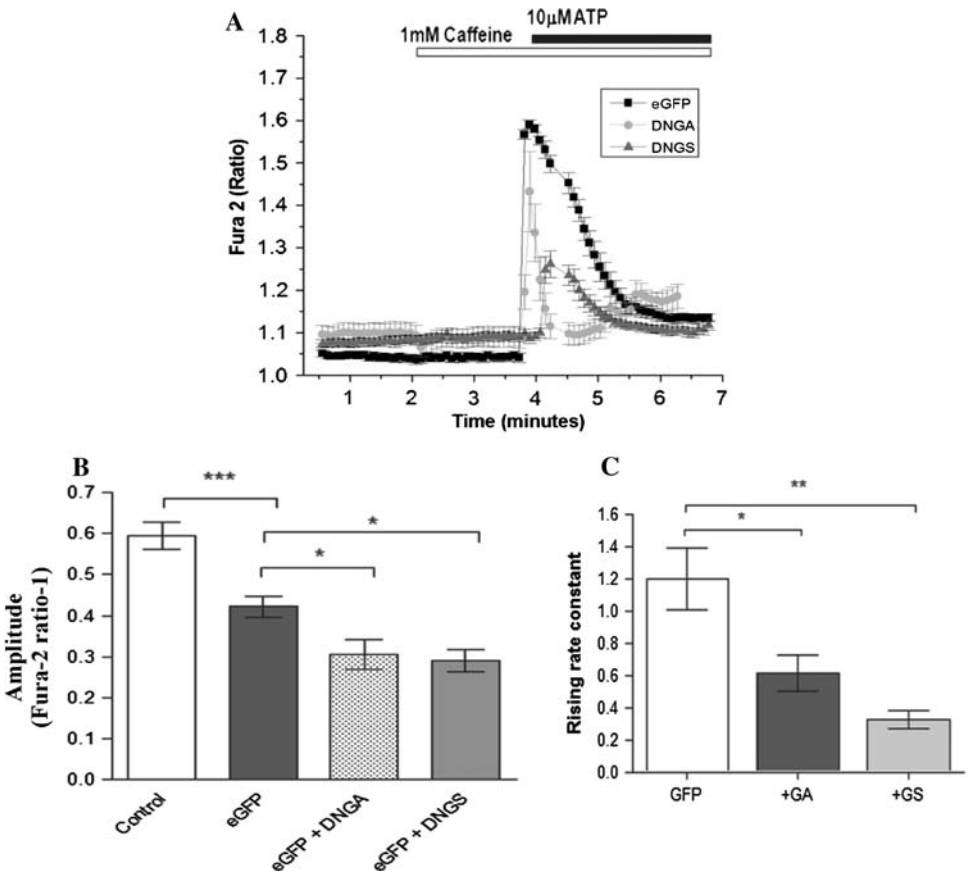

Fig. 7 **a** C2C12 cells treated with 1 mM caffeine and 10 μM ATP. Caffeine did not induce $[Ca^{2+}]_c$ transient; however, ATP did. A comparison of representative transients on the same time base. **b** A graph to summarize the relative magnitude of ATP-induced Ca^{2+} responses in control ($n = 23$) and transfected (eGFP $n = 9$) cells, eGFP + DNGA ($n = 8$) and eGFP DNGS ($n = 5$). **c** Histograms to show the rising rate constants (τ, s^{-1}) of the $[Ca^{2+}]_c$ transients in transfected cells

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References

Ardehali H, Chen Z, Ko Y, Mejia-Alvarez R, Marban E (2004) Multiprotein complex containing succinate dehydrogenase confers mitochondrial ATP-sensitive K^+ channel activity. Proc Natl Acad Sci USA 101:11880–11885

Ashcroft FM, Gribble FM (1998) Correlating structure and function in ATP-sensitive K^+ channels. Trends Neurosci 21:288–294

Babenko AP, AguilarBryan L, Bryan J (1998) A view of SUR/KIR6.X, KATP channels. Annu Rev Physiol 60:667–687

Bolsover S, Ibrahim O, O'luanaigh N, Williams H, Cockcroft S (2001) Use of fluorescent Ca^{2+} dyes with green fluorescent protein and its variants: problems and solutions. Biochem J 356:345–352

Coronado R, Miller C (1980) Decamethonium and hexamethonium block K^+ channels of sarcoplasmic reticulum. Nature 288:495–497

Costa AD, Garlid KD (2008) Intramitochondrial signaling: interactions among mitoKATP, PKCepsilon, ROS, and MPT. Am J Physiol 295:H874–H882

Costa AD, Pierre SV, Cohen MV, Downey JM, Garlid KD (2008) cGMP signalling in pre- and post-conditioning: the role of mitochondria. Cardiovasc Res 77:344–352

Cui Y, Giblin JP, Clapp LH, Tinker A (2001) A mechanism for ATP-sensitive potassium channel diversity: functional coassembly of two pore forming subunits. Proc Natl Acad Sci USA 98:729–734

Cuong DV, Kim N, Joo H, Youm JB, Chung JY, Lee Y, Park WS, Kim E, Park YS, Han J (2005) Subunit composition of ATP-sensitive potassium channels in mitochondria of rat hearts. Mitochondrion 5:121–133

Das M, Parker JE, Halestrap AP (2003) Matrix volume measurements challenge the existence of diazoxide/glibencamide-sensitive KATP channels in rat mitochondria. J Physiol 547:893–902

Doyle DA, Cabral JM, Pfuetzner RA, Kuo A, Gulbis JM, Cohen SL, Chait BT, MacKinnon R (1998) The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. Science 280:69–77

Fialka I, Pasquali C, Lottspeich F, Ahorn H, Huber LA (1997) Subcellular fractionation of polarized epithelial cells and identification of organelle-specific proteins by two-dimensional gel electrophoresis. Electrophoresis 18:2582–2590

Forbes RA, Steenbergen C, Murphy E (2001) Diazoxide-induced cardioprotection requires signaling through a redox-sensitive mechanism. Circ Res 88:802–809

Foster DB, Rucker JJ, Marban E (2008) Is Kir6.1 a subunit of mitoK(ATP)? Biochem Biophys Res Commun 366:649–656

Fryer RM, Eells JT, Hsu AK, Henry MM, Gross GJ (2000) Ischemic preconditioning in rats: role of mitochondrial K(ATP) channel in preservation of mitochondrial function. Am J Physiol 278:H305–H312

Gamper N, Shapiro MS (2007) Regulation of ion transport proteins by membrane phosphoinositides. Nat Rev Neurosci 8:921–934

Garcia AM, Miller C (1984) Channel-mediated monovalent cation fluxes in isolated sarcoplasmic reticulum vesicles. J Gen Physiol 83:819–839

Hanley PJ, Mickel M, Loffler M, Brandt U, Daut J (2002) K(ATP) channel-independent targets of diazoxide and 5-hydroxydecanoate in the heart. J Physiol 542:735–741

Inoue I, Nagase H, Kishi K, Higuti T (1991) ATP-sensitive K^+ channel in the mitochondrial inner membrane. Nature 352:244–247

Lacza Z, Snipes JA, Miller AW, Szabo C, Grover G, Busija DW (2003) Heart mitochondria contain functional ATP-dependent K^+ channels. J Mol Cell Cardiol 35:1339–1347

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Leaney JL, Dekker LV, Tinker A (2001) Regulation of a G-protein gated inwardly rectifying potassium channel by a Ca^{2+}-independent protein kinase C. J Physiol Lond 534:367–379

Liu Y, Ren G, O'Rourke B, Marban E, Seharaseyon J (2001) Pharmacological comparison of native mitochondrial K(ATP) channels with molecularly defined surface K(ATP) channels. Mol Pharmacol 59:225–230

Ma D, Zerangue N, Lin YF, Collins A, Yu M, Jan YN, Jan LY (2001) Role of ER export signals in controlling surface potassium channel numbers. Science 291:316–319

Paucek P, Mironova G, Mahdi F, Beavis AD, Woldegiorgis G, Garlid KD (1992) Reconstitution and partial purification of the glibenclamide-sensitive, ATP-dependent K^+ channel from rat liver and beef heart mitochondria. J Biol Chem 267:26062–26069

Quinn KV, Cui Y, Giblin JP, Clapp LH, Tinker A (2003) Do anionic phospholipids serve as cofactors or second messengers for the regulation of activity of cloned ATP-sensitive K^+ channels? Circ Res 93:646–655

Rodrigo GC, Standen NB (2005) ATP-sensitive potassium channels. Curr Pharm Des 11:1915–1940

Salkoff L, Wei AD, Baban B, Butler A, Fawcett G, Ferreira G, Santi CM (2005) Potassium channels in *C. elegans*. WormBook 1–15

Seino S (1999) ATP-sensitive potassium channels: a model of heteromultimeric potassium channel/receptor assemblies. Annu Rev Physiol 61:337–362

Suzuki M, Kotake K, Fujikura K, Inagaki N, Suzuki T, Gonoi T, Seino S, Takata K (1997) Kir6.1: a possible subunit of ATP-sensitive K^+ channels in mitochondria. Biochem Biophys Res Commun 241:693–697

Tinker A, Lindsay AR, Wlliams AJ (1992) A model for ionic conduction in the ryanodine receptor channel of sheep cardiac muscle sarcoplasmic reticulum. J Gen Physiol 100:495–517

Urabe M, Kume A, Tobita K, Ozawa K (2000) DNA/calcium phosphate precipitates mixed with medium are stable and maintain high transfection efficiency. Anal Biochem 278:91–92

Voelker DR (1996) Lipid assembly into cell membranes. In: Vance DE, Vance JE (eds) Biochemistry of lipids, lipoproteins and membranes. Elsevier, Amsterdam, pp 391–423

Wang Y, Ashraf M (1999) Role of protein kinase C in mitochondrial K_{ATP} channel-mediated protection against Ca^{2+} overload injury in rat myocardium. Circ Res 84:1156–1165

White SM, Constantin PE, Claycomb WC (2004) Cardiac physiology at the cellular level: use of cultured HL-1 cardiomyocytes for studies of cardiac muscle cell structure and function. Am J Physiol 286:H823–H829

Yellon DM, Downey JM (2003) Preconditioning the myocardium: from cellular physiology to clinical cardiology. Physiol Rev 83:1113–1151

Zhou M, Tanaka O, Sekiguchi M, He HJ, Yasuoka Y, Itoh H, Kawahara K, Abe H (2005) ATP-sensitive K^+-channel subunits on the mitochondria and endoplasmic reticulum of rat cardiomyocytes. J Histochem Cytochem 53:1491–1500