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Reduced expression of Kir6.2/SUR2A subunits explains K_{ATP} deficiency in K^+-depleted rats

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Abstract

We investigated on the mechanism responsible for the reduced ATP-sensitive K^+(K_{ATP}) channel activity recorded from skeletal muscle of K^+-depleted rats. Patch-clamp and gene expression measurements of K_{ATP} channel subunits were performed. A down-regulation of the K_{ATP} channel subunits Kir6.2(−70%) and SUR2A(−46%) in skeletal muscles of K^+-depleted rats but no changes in the expression of Kir6.1, SUR1 and SUR2B subunits were observed. A reduced K_{ATP} channel currents of −69.5% in K^+-depleted rats was observed. The Kir6.2/SUR2A-B agonist cromakalim showed similar potency in activating the K_{ATP} channels of normokalaemic and K^+-depleted rats but reduced efficacy in K^+-depleted rats. The Kir6.2/SUR1-2B agonist diazoxide activated K_{ATP} channels in normokalaemic and K^+-depleted rats with equal potency and efficacy. The down-regulation of the Kir6.2 explains the reduced K_{ATP} channel activity in K^+-depleted rats. The lower expression of SUR2A explains the reduced efficacy of cromakalim; preserved SUR1 expression accounts for the efficacy of diazoxide. Kir6.2/SUR2A deficiency is associated with impaired muscle function in K^+-depleted rats and in hypoPP.

Keywords: K_{ATP} channel; Hypokalaemic periodic paralysis; Skeletal muscle; Gene expression

1. Introduction

ATP-sensitive K(K_{ATP}) channels are involved in pathophysiological conditions of various tissues [1–7]. In skeletal muscle, recent evidence supports the role of K_{ATP} channels in the physiological performance of different muscle fibre types in response to their metabolic needs [8,9]. This is related to a muscle-dependent molecular composition and properties of K_{ATP} channels that for instance may determine a muscle-specific regulation of the extracellular K^+ concentration with local vasodilation, modulation of glucose uptake and drug responses [9]. A reduced activity of sarcolemmal K_{ATP} channels has been observed in the primary and secondary forms of hypokalaemic periodic paralysis (hypoPP), neuromuscular disorders associated with abnormal insulin response leading to fibre depolarization, transient weakness and hypokalaemia [10–13].

In primary hypoPP, the phenotype is characterized by episodic attacks of flaccid muscle paralysis or weakness associated with a transient decrease in the blood K^+ concentration. Attacks are precipitated by carbohydrate-rich meals, rest after exercise, sudden exposure to heat or cold, glucose or insulin infusion and acute stress [14,15]. The episodes of muscle weakness observed in hypoPP are accompanied by a silent electromyogram and a lack of muscle action potentials, even upon electrical stimulation. Isolated muscle fibres also exhibit membrane depolariza-

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tion on acute "in vitro" exposure to low extracellular K^+ concentrations. Familial hypoPP is caused by loss-of-function mutations in at least two different genes: CACNL1A3 and SCN4A encoding for the α1 subunits of the skeletal muscle L-type Ca^{2+} channel and voltage-dependent Na^+ channels, respectively [15,16]. Muscle biopsies from hypoPP patients carrying the CACNL1A3-R528H mutation showed also an abnormal sarcolemmal K_{ATP} channel [13]. This channel exhibited reduced single-channel conductance due to the appearance of subconductance states and an impaired activation by nucleotide diphosphates. The abnormal K_{ATP} channel from hypoPP type-1 patients also exhibited smaller macroscopic K_{ATP} currents; it is therefore likely that this abnormality plays a role in the symptoms of hypoPP.

The secondary, or non familial, forms of hypoPP share a phenotype similar to that of the familial form, but are caused by K^+ deficiency secondary to diuretic abuse, toxins and other factors. The K^+-depleted rats indeed show permanent muscle weakness, paralysis induced by insulin/glucose injection and plasma K^+ concentration constantly below 3.2 mEq/l in contrast with interictal periods in familial hypoPP where the plasma K^+ concentration is normal [10,11,17,18]. As observed in humans hypoPP type-1, skeletal muscle fibres isolated from K^+-depleted rats possess a K_{ATP} channel with a reduced single-channel conductance that is insensitive to insulin stimulation [10,11]. Also, muscles from K^+-depleted rats exhibited smaller macroscopic K_{ATP} currents, however the molecular mechanism responsible for this abnormality is not known.

To address the mechanisms responsible for reduced macroscopic K_{ATP} currents in K^+-depleted rats, we combined electrophysiological recordings of sarcolemmal K_{ATP} channel activity in fast-twitch muscle with quantification of mRNA expression levels of K_{ATP} channel subunits: the inwardly rectifying K-channels (Kir6.1, Kir6.2) and sulfonylurea receptors (SUR1, SUR2A-B) in muscle samples from K^+-depleted and normokalaemic rats. The responses of muscle K_{ATP} channels of K^+-depleted and normokalaemic rats to cromakalim, a Kir6.2/SUR2A-B agonist and diazoxide, a Kir6.2/SUR1-2B agonist, were also compared.

2. Materials and methods

2.1. Animal care

Animal care was performed in accordance with the *"Guide for Care and Use of Laboratory Animals"* prepared by the National Academy of Sciences of USA. Adult male Wistar rats (260–350 g) were divided into two groups and housed at a density of three rats per cage. Normokalaemic rats ($n = 7$ rats) were fed for 23 days on a normal K^+diet (0.8%) (30 g of pellets/day), while K^+-depleted rats ($n = 7$ rats) were given a K^+-free diet. Blood samples were collected from the tail vein of all animals at the beginning of the diet regimen. Intracardiac blood samples were collected at the time of death from K^+-depleted and normokalaemic rats fasted overnight, for evaluation of serum K^+ concentrations. Standard flame spectrophotometry (Corning. EEL 450 flame photometer) was used for measurement of serum K^+ levels and values were expressed as mEq/l. Rats were considered hypokalaemic if the serum K^+ concentration was <3.2 mEq/l [10].

2.2. Muscle biopsies

The flexor digitorum brevis (FDB), tibialis anterior (TA) and extensor digitorum longus (EDL) muscles were dissected under urethane anaesthesia (1.2 g/kg). After dissection, the animals were rapidly sacrificed with an overdose of urethane. Single muscle fibres were prepared from FDB muscles by enzymatic dissociation for patch-clamp experiments, while the contralateral FDB muscles removed from the same rats were rapidly frozen in situ with liquid nitrogen and used for mRNA analysis. TA and EDL muscles were also collected from the same rats and used for patch-clamp and mRNA analyses. The normal Ringer solution used during muscle biopsy and for preparation of isolated fibres contained (mM): 145 NaCl, 5 KCl, 1 $MgCl_2$, 0.5 $CaCl_2$, 5 glucose, 10 3-(*N*-morpholino)-propanesulfonic (MOPS) sodium salt and was adjusted to pH 7.2 with MOPS acid.

2.3. Real time quantitative PCR

For each muscle sample, total RNA was isolated using Trizol reagent and treated with DNase I (4 U, 37 °C, 1 h). RNA was quantified using a spectrophotometer (Beckman DU 530) and 3 μg was used for reverse transcription. Synthesis of cDNA was performed using random hexamers (annealed 10 min, 25 °C) and Superscript II reverse transcriptase (Invitrogen-Life Technologies, Carlsbad, CA, USA) incubated at 42 °C for 50 min. We used the available rat sequences for Kir6.1 (GenBank Accession No. NM_017099), Kir6.2 (NM_031358), SUR1 (NM_013039), SUR2A (D83598), SUR2B (AF019628) and β-actin (NM_031144). Fluorescently labeled TaqMan (Applied Biosystems, Foster City, CA, USA) probes for Kir6.1, SUR1, SUR2A, SUR2B and β-actin were designed using PrimerExpress (Applied Biosystems) to amplify 124–141 bp products encompassing each probe annealing site. Specific primer and probe sequences for each gene have been reported previously [9]. To achieve a high level of specificity and to avoid detection of genomic DNA, we designed probes to span exon–exon junctions for each gene except for Kir6.2, which is intronless. For Kir6.2, a TaqMan probe was designed within a region having substantial sequence divergence with other genes and control amplifications of RNA without reverse transcription were performed to exclude genomic DNA contamination. None of the SUR and Kir primer and probe sets cross-reacted with non specific SUR or Kir sequences after 40 cycles of PCR, and no amplification was observed after 45 cycles of PCR in

control reactions containing no DNA template. Triplicate reactions were carried out in parallel for each individual muscle sample. The results were compared to a gene specific standard curve and normalized to expression of the housekeeping gene, β-actin, in the same sample. Template used for determining standard curves consisted of plasmid DNA containing the expected target sequence quantified by Pico Green fluorescence (Molecular Probes, Eugene, OR, USA).

2.4. Electrophysiology

Experiments were performed with the inside-out configuration of the patch-clamp technique. Current recordings were performed during voltage steps from the holding potential (0 mV) to test potentials ranging from −70 mV to +70 mV immediately after excision, at 20–22 °C. Current was recorded at 1 kHz (filter = 0.2 kHz) using an Axopatch-1D amplifier equipped with a CV-4 headstage (Axon Ins. Foster City, CA, USA).

Pipettes having an average tip area of $8.1 \pm 0.8\ \mu m^2$ ($n = 300$ patches) were used to measure K_{ATP} currents and the pharmacological responses of the channels. The pipette area was measured by scanning electron microscopy as previously described [10]. The patch pipette solution contained (mM) 150 KCl, 2 $CaCl_2$, 1 MOPS, pH 7.2. The bath solution contained (mM) 150 KCl, 5 EGTA, 1 MOPS, pH 7.2. Stock solutions of cromakalim (50 mM) and diazoxide (50 mM) (SIGMA, Co Milano) were prepared by dissolving the drugs in dimethylsulphoxide (DMSO). Microliter amounts of these stock solutions were then added to the bath solutions in the presence of ATPMg. DMSO applied at the maximal concentration tested (0.1%) did not affect K_{ATP} channel currents in the absence or in the presence of ATPMg.

Current amplitude was measured using the Clampfit program (Axon Ins. Foster City, CA, USA). No correction for liquid junction potentials were made as these were estimated to be <2 mV under our experimental conditions.

Concentration-response relationships experiments were performed by applying increasing concentrations of diazoxide (10^{-12}–1.5×10^{-4} M) or cromakalim (10^{-10}–2×10^{-4} M) to inside-out macropatches in the presence of internal ATPMg (0.1 mM).

2.5. Statistical analysis

All data were expressed as mean ± SD. Statistical comparisons between group of data were made using Student's *t*-test and significance was assumed when $p < 0.05$.

The mean current amplitudes were calculated by subtracting the base-line level, corresponding to the current recorded in the presence of internal ATPMg (5 mM), from the open channel level, corresponding to the current recorded in the absence of internal nucleotide, for each current trace and then digitally averaging all records using Clampfit. The mean K_{ATP} currents were analyzed by including all patches (conductive and non conductive patches) in the average. The frequency of finding functional channels in the patches was also calculated. The non conductive patches (with no K_{ATP} channels or inward rectifier K^+ channels), not resulting from membrane vesicle formation at the tip of the pipette or connective tissues interference, showed a leak current of -49.9 ± 5 pA/−60 mV (V_m) (N patches = 43) in the presence or absence of internal ATP, seal rupture following continuous voltage stimulation for 10 s and seal rupture following the rapid exposure of the patches to the air. In contrast, patches showing high resistant seals $>50 \pm 10$ GΩ which are also resistant to the air exposure and to the continuos voltage stimulation were excluded from the computed average. These are indeed the results of connective tissues interference or membrane vesicles formation at the tip of the pipette in excised patches.

Concentration-response relationships were fit with a function that assumes one stimulatory component described by the term: $(I_{drug+ATP} - 1) * 100 = E_{max}/(1 + (DE_{50}/[Drug])^n)$ where $I_{drug+ATP}$ is the K_{ATP} currents measured in the presence of the molecules under study and in the presence of internal ATPMg (10^{-4} M); E_{max} is the per cent maximal activation of the K_{ATP} currents produced by the molecules under study in the presence of internal ATPMg; DE_{50} is the concentration of the drug needed to enhance the current by 50%, calculated in respect to the maximal activation produced by the drugs in the presence of internal ATP; n is the slope factor of the curves. The algorithms for the fitting procedures used are based on a Marquardt least-squares fitting routine. Data from patches in which the current did not respond to drugs were excluded from the mean and from the concentration-response curves.

3. Results

K^+-depleted rats displayed low serum K^+ levels (2.4 ± 0.12 mEq/l; $n = 7$ rats), a delay in the righting reflex ranging between 2 and 25 s, and general weakness (evaluated by observing the movement of the animal in its cage). In contrast, rats fed a normal diet showed normal serum K^+ levels (4.6 ± 0.04; $n = 7$ rats) and a righting reflex of less than 1 s.

3.1. K_{ATP} channel activity in K^+-depleted and normokalaemic rat muscles

We used fast-twitch muscles for our studies because secondary and primary hypoPP affects this muscle type. Differences in the biophysical and pharmacological properties, and in molecular composition of K_{ATP} channels in slow-twitch and fast-twitch muscles, have recently been found [9]. For example, fast-twitch muscle has a higher K_{ATP} channel activity per unit area than slow-twitch muscle.

Patch excision from FDB muscle fibres into ATP-free solution produced a dramatic increase of inward currents in 75% of macropatches from normokalaemic rats but only in 19% of patches from K^+-depleted rats. Mean inward current recorded immediately after excision, which was calculated by averaging conductive and non conductive patches, was -248.4 ± 25 pA ($n = 69$ patches; $n = 7$ rats) for normokalaemic rats and -78.09 ± 11 pA ($n = 231$ patches; $n = 7$ rats) for K^+-depleted rats. Exposure of macropatches from both K^+-depleted and normokalaemic rats to intracellular ATPMg (5 mM) reduced the current amplitude indicating that the current flowed through K_{ATP} channels (Fig. 1a). The ATP-sensitive current was -76.1 ± 7 pA ($n = 31$ patches) in K^+-depleted rats and -250.2 ± 23 pA ($n = 42$ patches) in normokalaemic rats, respectively. There was no significant difference in the ATP-insensitive current, which was -62 ± 10 pA and -74 ± 11 pA in the normokalaemic and K^+-depleted rats, respectively. Thus, the lower inward current observed on patch excision in K^+-depleted rats results from a specific reduction in the K_{ATP} current, of approximately 69.5%. A similar reduction of the K_{ATP} current was also observed in other muscles (e.g. EDL) of K^+-depleted rats (data not shown).

ATPMg applied on the intracellular side of the patches caused a dose-dependent reduction of K_{ATP} currents in FDB muscles of normokalaemic and K^+-depleted rats. However, there was a small but significant difference in potency. The IC_{50} values for ATPMg were 15 ± 6 μM ($n = 6$ patches) and 6.3 ± 1 μM (slope = 1) ($n = 5$ patches) for FDB muscles of normokalaemic and K^+-depleted rats, respectively.

3.2. Expression of K_{ATP} channel subunits

Quantitative real time RT-PCR measurements demonstrated a significant difference in the expression levels of Kir6.2 mRNA between normokalaemic and K^+-depleted rats of FDB muscles (Fig. 1b). Expression of Kir6.2 was indeed 68% lower in K^+-depleted rats. As previously reported [9], the relative expression of Kir6.2 in normokalaemic rats was significantly higher than that of the Kir6.1 (which was negligible). Expression of Kir6.1 was unaltered in K^+-depleted rats. Similar data were obtained from other muscles from K^+-depleted rats (e.g. EDL and TA; data not shown).

We also found that the expression of the SUR2A subunit was significantly reduced in FDB muscles of the K^+-depleted rats and it was 46% lower as compared with that of the normokalaemic rats. By contrast, no significant differences in the expression levels of SUR1 and SUR2B

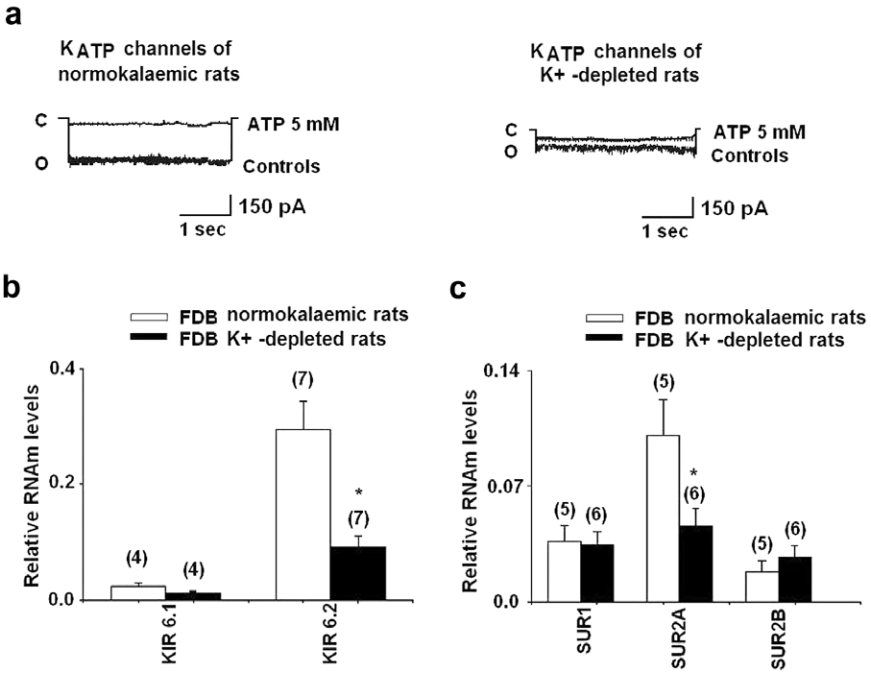

Fig. 1. ATP-sensitive K^+ currents and expression levels of subunits composing the K_{ATP} channel complexes in skeletal muscle of K^+-depleted rats and normokalaemic rats. (a) Representative K_{ATP} currents recorded in membrane patches excised from flexor digitorum brevis (FDB) muscle fibres of a normokalaemic rat and a K^+-depleted rat. O indicates the open channel level and C indicates the closed channel level. Currents were recorded in response to voltage steps from 0 mV to −60 mV in the presence of 150 mM KCl on both sides of the membrane patches. ATPMg (5 mM) applied to the intracellular side of the patches inhibited the currents. The ATP-sensitive currents recorded from the K^+-depleted rat was significantly smaller than that of the normokalaemic rat. (b and c) Expression levels of K_{ATP} channel subunits in normokalaemic and K^+-depleted rat muscles expressed as fraction of β-actin levels. Expression was evaluated by RT-PCR on contralateral muscles of the same rats used for patch-clamp experiments. The numbers above the bars indicate the number of muscles sampled. Expression of Kir6.2 and SUR2A was significantly reduced in FDB muscles of K^+-depleted rats as compared with those of the normokalaemic rats ($^*p < 0.05$). No differences were observed in expression of Kir6.2, SUR1 or SUR2B between K^+-depleted and normokalaemic rats.

subunits between the K^+-depleted and normokalaemic rats were observed (Fig. 1c).

3.3. Effects of cromakalim and diazoxide

Cromakalim and diazoxide, in the presence of 0.1 mM internal ATPMg, caused a dose-dependent activation of K_{ATP} channels in patches excised from muscles of both K^+-depleted and normokalaemic rats. The cromakalim concentration-response data were fitted with one stimulatory site function for both K^+-depleted and normokalaemic rats (Fig. 2a). In the concentration range from 10^{-8} M to 2×10^{-4} M, cromakalim produced 88% activation of K_{ATP} channels in normokalaemic rat muscle, but the efficacy of the drug in activating K_{ATP} channels in muscles of K^+-depleted rats was markedly reduced (Fig. 2a and b). No difference in the cromakalim response between normokalaemic and K^+-depleted rats was observed at drug concentrations $<10^{-8}$ M (Fig. 2a and b). The parameters of the concentration-response relationships calculated by the fitting routine for the normokalaemic rat data were: $DE50 = 1.1 \pm 0.5\times10^{-9}$ M, $E_{max} = 80 \pm 4$, $n = 1$; and for the K^+-depleted rat data were: $DE50 = 2.1 \pm 0.4\times10^{-9}$ M, $E_{max} = 63 \pm 5$, $n = 0.9$.

In the concentration range from 10^{-9} M to 10^{-4} M, the diazoxide data were fitted with a single stimulatory site function in both normokalaemic and K^+-depleted rats (Fig. 3a). At saturating concentrations, diazoxide produced similar activation of K_{ATP} channels of both normokalaemic and K^+-depleted rats (Fig. 3a and b). The parameters of the concentration-response relationships calculated by the fitting routine for the normokalaemic rat data were: $DE50 = 8 \pm 0.9\times10^{-9}$ M, $E_{max} = 57 \pm 9$, $n = 0.7$; and for the K^+-depleted rat data were: $DE50 = 9 \pm 1\times10^{-9}$ M, $E_{max} = 53 \pm 6$, $n = 0.7$. Therefore, no differences in the responses of K_{ATP} channels in muscles of K^+-depleted rats and normokalaemic rats to diazoxide were observed in the range of concentrations tested (Fig. 3a and b).

4. Discussion

In this study, we demonstrated that fast-twitch muscles of K^+-depleted rats exhibited a 70% reduction in the expression of Kir6.2, which was paralleled by a 69.5% reduction in K_{ATP} channel current. These findings indicate that reduced expression of Kir6.2 is responsible for the lower K_{ATP} channel currents in the muscles of K^+-depleted rats. It is well established that both Kir6.2 and SURs subunits are required to form a functional K_{ATP} channel

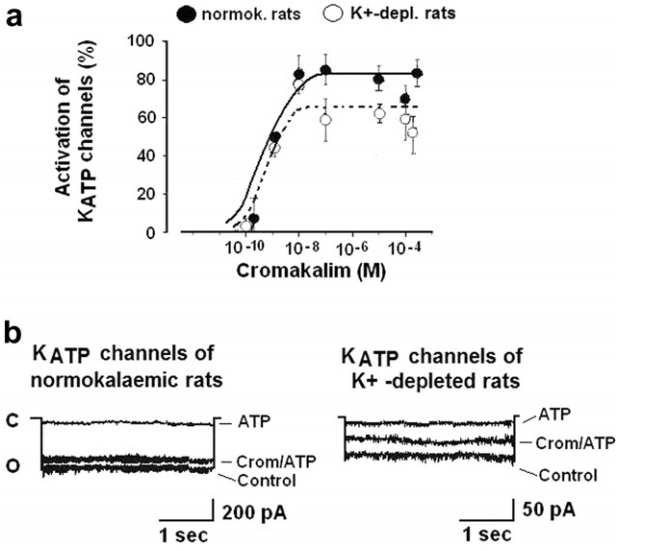

Fig. 2. Effects of cromakalim on muscle K_{ATP} channels of normokalaemic rats and K^+-depleted rats. (a) Concentration-response relationships of cromakalim measured in flexor digitorum brevis (FDB) muscles of normokalaemic (●, solid line) and K^+-depleted rats (○, dotted line). (b) Representative K_{ATP} currents recorded in membrane patches excised from FDB muscle fibres of a normokalaemic rat and a K^+-depleted rat exposed to cromakalim. O indicates the open channel level and C indicates the closed channel level. Currents were recorded in response to voltage steps from 0 mV to −60 mV in the presence of 150 mM KCl on both sides of the membrane patches. K_{ATP} currents were recorded in inside-out patches in the absence of ATPMg (Control), in the presence of internal 0.1 mM ATPMg (ATP), and in the presence of 0.1 mM ATPMg plus 0.1 mM cromakalim (Crom). A reduced response at concentrations higher than 10^{-8} M to cromakalim has been observed in the muscle K_{ATP} channels of K^+-depleted rats as compared with that of the normokalaemic rats.

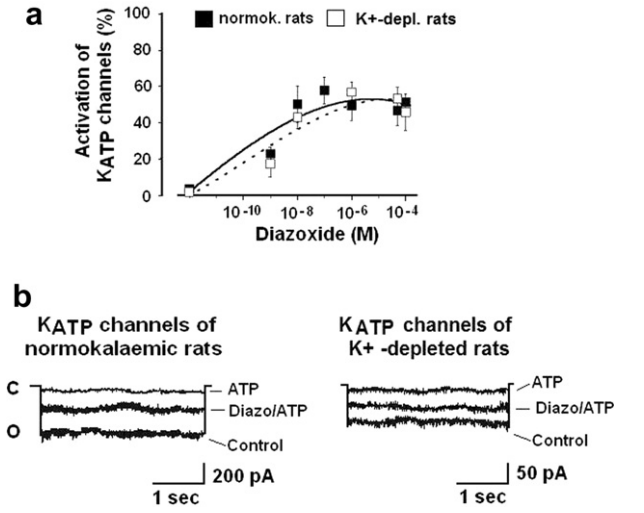

Fig. 3. Effects of diazoxide on muscle K_{ATP} channels of normokalaemic rats and K^+-depleted rats. (a) Concentration-response relationships for diazoxide measured in flexor digitorum brevis (FDB) muscles of normokalaemic (■, solid line) and K^+-depleted rats (□, dotted line). (b) Representative K_{ATP} currents recorded in membrane patches excised from FDB muscle fibres of a normokalaemic rat and a K^+-depleted rat exposed to diazoxide. O indicates the open channel level and C indicates the closed channel level. Currents were recorded in response to voltage steps from 0 mV to −60 mV in the presence of 150 mM KCl on both sides of the membrane patches. K_{ATP} channel currents were recorded from inside-out patches in the absence of ATPMg (Control), in the presence of 0.1 mM internal ATPMg (ATP), and in the presence of 0.1 mM internal ATPMg plus 0.1 mM diazoxide (Diazo). No differences were observed in the response to diazoxide between K_{ATP} channels of normokalaemic and K^+-depleted rats.

and that neither subunit is trafficked to the surface membrane in the absence of the other [19]. Thus, down-regulation of Kir6.2 is expected to result in a lower K_{ATP} channel current of skeletal muscle as we observed.

One point of interest is that members of the Kir1.x (ROMK) family of inward rectifiers, which control the renal excretion of K^+ ions, are also down-regulated by hypokalaemia. For example, K^+restriction leads to down-regulation of both Kir1.1 in the rat kidney and Kir7.1 in the basolateral membrane of the distal nephron and collecting duct [20–22]. This suggests that there may be a common K^+-dependent mechanism that regulates transcription of Kir genes in both muscle and kidney. Down-regulation of Kir genes in response to hypokalaemia may have a protective role, sparing K^+ excretion and helping to conserve plasma K^+ levels. However, in skeletal muscle this mechanism can be also a precipitating factor for hypoPP, by impeding K^+ efflux and impairing fibre repolarization.

In addition to this mechanism which is associated with chronic K^+-depletion, the hypokalaemia may directly affects the K_{ATP} channels. A brief hypokalaemic period indeed causes a direct block of the K_{ATP} and of other Kir channels; shut-off of the inward rectifier K^+ channels occurs when they are acutely exposed to low external concentrations of K^+ ions below 2 mEq/l. This is the basis of the known bistable behaviour of the membrane potential in response to the lowering of external K^+ concentrations [23]. Therefore the down-regulation of the Kir6.2 message, that also reduces the number of functional channels in the membrane, and the direct block of the channel pore due to the hypokalaemia are the mechanisms responsible for the generation of the subconductance states and low Popen characterizing the abnormal K_{ATP} channel found in K^+-depleted rats and human hypoPP patients [10,13].

A significant reduction in expression of SUR2A (46%) was also found in the muscles of K^+-depleted rats. The SUR2 gene is alternatively spliced, but the reduction in SUR2A expression was not paralleled by a similar down-regulation of SUR2B mRNA. This suggests that a transcriptional splicing mechanism is also involved in the impaired K_{ATP} channel function of K^+-depleted rat muscles.

The data on the expression of SUR mRNAs complements our electrophysiological and pharmacological findings. For example, the reduced expression of SUR2A found in the K^+-depleted rats explains why their K_{ATP} channels were less responsive to cromakalim, a well known Kir6.2/SUR2A-B agonist. The binding sites for diazoxide, possibly SUR1 and/or SUR2B subunits, were not affected by hypokalaemia as suggested by fact that the dose-response relationships of the K_{ATP} currents vs diazoxide concentrations of normokalaemic and K^+-depleted rats were not different.

The finding that K_{ATP} channels of K^+-depleted rats were more sensitive to ATP inhibition than those of normokalaemic rats may be related to the subunit composition of muscle K_{ATP} channels. We have previously shown that K_{ATP} channels in normal fast-twitch muscles are mostly composed of Kir6.2/SUR2A and Kir6.1/SUR1, with possibly a small contribution from Kir6.2/SUR2B complexes. In contrast, slow-twitch muscle lacks Kir6.2/SUR1. K_{ATP} channels containing SUR1 are more sensitive to ATP inhibition than those containing SUR2, and they are sensitive to diazoxide but not to cromakalim [24]. Down-regulation of SUR2A in K^+-depleted rats will leads to an increased fraction of Kir6.2/SUR1 channels which are more sensitive to ATP and are activated by diazoxide.

We studied the K^+-depleted rat model which is not a genetic animal model of hypoPP. However, our data may help to explain the insulin/glucose-dependent hypokalaemia, muscle weakness and paralysis which are commonly observed in both the secondary and primary forms of hypoPP. For example, in normal muscle insulin promotes a cascade of events that includes activation of $3Na^+/2K^+$ATP-ase with uptake of K^+ ions into the fibres and transient hypokalaemia which is balanced by the activation of the K_{ATP} channels that extrude K^+ ions from the fibres. In human hypoPP and in K^+-depleted rats a persistent hypokalaemia is observed following insulin and/or adrenergic stimulation because the abnormally reduced K_{ATP} channel activity is unable to counteract the low serum K^+ ions levels. The lack of K_{ATP} currents that we found and possibly of inward rectifier K^+ currents as observed by others [12], reduces the repolarizing components and may unmasks depolarizing currents activated by insulin or by other signals with fibre depolarization. In turn, depolarization is expected to cause inactivation of voltage-gated Na^+ channels and reduces muscle excitability.

K_{ATP} channel deficiency other than associated to hypokalaemia and fibre depolarization may also impairs the functionality of skeletal muscle. Kir6.2 deficiency indeed affects contractility of skeletal muscle as demonstrated by the fact that Kir6.2 knock-out (−/−) mice show reduced resting tension and impaired adaptation to exercise-induced physical stress [7]. Moreover, the SUR2A deficiency may leads to a K_{ATP} channel unresponsive to metabolic stimulus with impairment in the capability of the skeletal muscle to recover upon stress. Mutations in the SUR2A gene have been recently associated with some forms of dilated cardiomyopathy in humans and are responsible for the impaired performance of cardiac muscle in mice carrying those mutations [4,6,7].

While the reduced K_{ATP} activity found in the K^+-depleted rats is clearly related to the K^+-depletion, however the link between the gene mutations found in the familial hypoPP and the reduction of the sarcolemmal K_{ATP} channel activity found in the primary form is still an open question. One possible mechanism involves changes in the concentrations of intracellular Na^+ ions (or Ca^{2+}), as a consequence of mutations in SCN4A or CACNL1A3, that lead to down-regulation of K_{ATP} channel genes. The fact that human hypoPP patients show elevated

intracellular Na^+ ions in skeletal muscle corroborates this idea [12,14,16]. The elevated intracellular Na^+ ions may stimulates the activity of the Na^+/Ca^{2+} exchanger or other Na^+-dependent pumps and transporters with effects on the intracellular levels of Ca^{2+} ions in turn affecting the K_{ATP} activity. Additional mechanisms involving protein–protein interactions, changes of membrane voltage and/or intracellular factors that regulate the activity of ion channels cannot be excluded [25]. Indeed, there is evidence that the L-type Ca^{2+}channel is functionally coupled to the K_{ATP} channel in ventricular cells, since genetic deletion of Kir6.2 results in up-regulation of L-type Ca^{2+}channels [4,26].

In conclusion, we have demonstrated that the symptoms observed in K^+-depleted rats can be explained by down-regulation of Kir6.2 and SUR2A and a consequent reduction in the muscle K_{ATP} channel activity. Because a reduction in K_{ATP}current is also observed in muscle fibres isolated from human hypoPP patients type-1, it is possible that a similar reduction in Kir6.2 and SUR2A expression may occur in the human disease. Our experiments also suggest that diazoxide may be an effective treatment for hypoPP. Current drug therapy is based on the use of acetazolamide and dichlorphenamide which act by targeting another class of K^+ channels, the muscle BK channel [15,27]. In our experiments diazoxide was capable of directly activating muscle K_{ATP} channels by binding to SUR1 (and SUR2B subunits) which are expressed in K^+-depleted rats and contribute to functional K_{ATP} channels in fast-twitch skeletal muscle. This provides a mechanistic basis for the potential effectiveness of this drug and its structural analogs in this disorder [14,16].

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