Acetazolamide Opens the Muscular K_{Ca}^{2+} Channel: A Novel Mechanism of Action That May Explain the Therapeutic Effect of the Drug in Hypokalemic Periodic Paralysis

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Acetazolamide is a thiazide derivative clinically used in skeletal muscle disorders related to altered K^+ homeostasis such as the periodic paralyses. The mechanism of action responsible for the therapeutic effects of the drug is still unknown, however. In the present work, we investigated the mechanism of action of acetazolamide in the K–deficient diet rat, an animal model of human hypokalemic periodic paralysis (hypoPP). The in vivo administration of 2.8- and 5.6-mg/kg^{-1}/day^{-1} concentrations of acetazolamide to K–deficient diet rats prevented paralysis and depolarization of the fibers induced by insulin. In the acetazolamide-treated animals, intense sarcolemma Ca^{2+}-activated K^+ channel ($K_{Ca^{2+}}$) activity was recorded. Acetazolamide also restored the serum K^+ levels to control values. The concentrations of acetazolamide needed to enhance the $K_{Ca^{2+}}$ current by 50% in vitro were 6.17 and 4.01 $\times$ 10^{-6} M at -60 and $+30$ mV of membrane potentials, respectively. In normokalemic animals, the thiazide derivative enhanced the $K_{Ca^{2+}}$ current with similar efficacy. Our data demonstrate that the therapeutic effects of acetazolamide in the K–deficient diet rats and possibly in human hypokalemic periodic paralysis patients can be mediated by activation of the $K_{Ca^{2+}}$ channel.

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Acetazolamide is the prototype of the thiazide agents known for having limited usefulness as diuretics but showing interesting therapeutic features in different disorders such as epilepsy and mania,[1,2] episodic ataxia,[3–5] eye disorders such as glaucoma,[6,7] acute mountain sickness,[8] and neuromuscular disorders.[9–11] In particular, acetazolamide is used with some success to resolve the episodic paralysis and transient weakness that frequently accompany hyperkalemic periodic paralysis (hypoPP), paramyotonia congenita, and Becker disease, in which mutations of the sarcolemma voltage-dependent Na^+ channel and Cl^- channel, respectively, are the basis of the abnormal fiber hyperexcitability and paralysis.[11–14] The thiazide derivative is also one of the most common medications in familial hypoPP, which is an autosomal dominant disorder linked to mutations of the α_1 subunit of the skeletal muscle voltage-dependent L-type Ca^{2+} channel.[14]

HypoPP is characterized by episodes of flaccid paralysis and muscle weakness accompanied by lowering of the serum K^+ concentration. In hypoPP patients, the paralytic attacks may last for hours to days and are precipitated by maneuvers that lower the serum K^+ level such as high-carbohydrate meals, emotional stress, and insulin injection.[10] Investigations of the electrophysiologic properties of the muscle biopsies of hypoPP patients revealed that the fibers are depolarized and that further depolarization occurs after incubation of the muscle fragments with low external K^+ solution and insulin.[10,15] Similarly, in the K–deficient diet rat, an animal model of hypoPP, profound fiber depolarization and paralysis induced by insulin were observed.[16,17] We recently demonstrated that the abnormal response of the muscle fibers of hypoPP patients and K–deficient diet rats to insulin is associated with a malfunction of a K^+ channel abundantly expressed in skeletal muscle, the adenosine triphosphate–sensitive K^+ channel (K_{ATP}).[18,19]

To date, most hypoPP patients benefit by empiric treatment with acetazolamide. The thiazide derivative improves muscle strength and reduces the frequency of attacks in hypoPP patients, although a small number of patients do not respond to the drug treatment.[9–11] Further, Griggs and co-workers[20] have recently dem-

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onstrated the efficacy of dichlorophenamide, another potent inhibitor of the carbonic anhydrase enzyme, in the treatment of the episodic weakness in hypoPP patients in a randomized, double-blind, placebo-controlled trial. Although the clinical efficacy of acetazolamide and related drugs in hypoPP has been reported by several authors, the cellular mechanism of action responsible for the therapeutic effects of acetazolamide is not known.

The most popular idea to explain the therapeutic effect of acetazolamide in various disorders is that the drug action involves the inhibition of local forms of carbonic anhydrase enzyme with alkalization of the interior of the cells and subsequent acidification of the external compartment. This mechanism can explain most of the effects of the drug in several systems; for example, in the renal district, the Na^+ loss due to acetazolamide is associated with the inhibition of the carbonic anhydrase enzyme with a decrease in the availability of protons for Na^+-H^+ exchange. More recently, it has been shown that acetazolamide and other thiazide derivatives such as hydrochlorothiazide exert vasodilating effects in vascular smooth muscle through the inhibition of local carbonic anhydrase enzyme, with a consequent intracellular alkalization of the cells that finally promotes an indirect activation of a class of K^+ channel, the Ca^{2+}-activated K^+ channel ($K_{Ca^{2+}}$).[21]

Other than through inhibition of local carbonic anhydrase enzymes,[22] it seems that the therapeutic effects of acetazolamide in skeletal muscle disorders can be related to an unknown mechanism of action. In fact, it has been reported that in patients affected by hypoPP, the metabolic acidosis resulting from the acetazolamide medication is not correlated with the improvement of muscle strength, because the influence of the drug treatment on the acid base equilibrium is maximal within a few days and decreases during the treatment, although improvement of muscle strength continues through the entire treatment period (2–3 weeks or longer).[9]

In the present work, we studied the effects of in vivo chronic administration of acetazolamide on K–deficient diet rats. In particular, we examined the capability of the drug to prevent the characteristic paralysis and fiber depolarization induced by insulin in this animal model of hypoPP. The effects of in vivo administration of the drug were evaluated on macroscopic sarcolemma $K_{Ca^{2+}}$ and K_{ATP} currents of the fibers from muscles of K–deficient diet rats. During the drug treatment period, the serum K^+ levels were also monitored. The effects of the in vitro application of acetazolamide to the patches excised from the K–deficient diet rat and normokalemic rat fibers were also investigated.

Materials and Methods

Rat Housing and Diet

Adult male Wistar rats (260–300 g) were divided into 5 groups and housed 3 rats per cage. The animal care was performed in accordance with guidelines prepared by the National Academy of Sciences. The rats were fed 30 g of pellets per day based on different recipes for 23 days of treatment. Three groups of rats (K–deficient diet rats) were made hypokalemic by feeding them a special food free of K^+ as previously described.[19] The fourth and fifth groups (controls, normokalemic rats) were fed with food containing a normal concentration of K^+ (0.8%). Blood samples were collected from the tail vein of the animals at the beginning of treatment. At the time of death, intracardiac blood samples were collected from the rats after an overnight fast for the evaluation of serum K^+ concentrations. The rats were considered hypokalemic when the serum K^+ concentration was less than 3.2 mEq/L. As previously shown,[16,19] 18 or more days of treatment were needed to induce measurable hypokalemia in the rats.

Drug Treatment

The first (n = 13 rats) and second (n = 4 rats) groups of K–deficient diet rats were treated orally for 10 days, starting at day 18 from the beginning of the K^+-free diet, with the physiologic solution enriched with 2.8- and 5.6-mg/kg^{-1}/day^{-1} concentrations of acetazolamide, respectively. The third group of K–deficient diet rats was treated for 10 days only with physiologic solution (n = 5 rats). The fourth group (n = 3 rats) of normokalemic rats was treated in vivo for 10 days with a 5.6-mg/kg^{-1}/day^{-1} concentration of acetazolamide. The fifth group (n = 2 rats) of normokalemic rats was used as a control. After 10 days of treatment, the K–deficient diet rats and normokalemic rats were treated with a single intraperitoneal injection of insulin.

Spectrophotometry

Standard flame spectrophotometry (EEL 450 flame photometer; Corning, Acton, MA) was used for detection of the serum K^+ levels as previously described.[19] The values were expressed as milliequivalents per liter of ions.

Muscle Preparations and Single-Fiber Isolation

The extensor digitorum longus (EDL) and flexor digitorum brevis (FDB) muscles were dissected from the animals under urethane anesthesia (1.2 g/kg). After dissection, the animals were rapidly killed with an overdose of urethane according to guidelines prepared by the National Academy of Sciences. Single muscle fibers were prepared from FDB muscles by enzymatic dissociation.[19] Resting potential (RP) measurements were performed mainly on EDL muscles, whereas patch-clamp experiments were performed on dissociated fibers from FDB muscle.

Solutions

The normal Ringer solution contained 145 mM of NaCl, 5 mM of KCl, 1 mM of $MgCl_2$, 0.5 mM of $CaCl_2$, 5 mM of glucose, and 10 mM of 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.2. The low-K^+ solution contained 145

mM of NaCl, 0.5 mM of KCl, 1 mM of $MgCl_2$, 0.5 mM of $CaCl_2$, 5 mM of glucose, and 10 mM of MOPS, pH 7.2. The patch pipette solution contained 150 mM of KCl, 2 mM of $CaCl_2$, and 10 mM of MOPS, pH 7.2. The bath solution contained 150 or 30 mM of KCl, 5 mM of ethylene glycol bis(β-aminoethyl ether)-N,N,N,N-tetraacetic acid, and 10 mM of MOPS, pH 7.2. $CaCl_2$ was added to the bath solution to give a free Ca^{2+} ion concentration of 16 μM. The calculations of the free Ca^{2+} ion concentration in the bath were performed as previously described.[19,23] For in vitro experiments, a stock solution of acetazolamide and glibenclamide was prepared by dissolving the drugs in dimethylsulfoxide at concentrations of 20 and 4.05 mM, respectively. Charybdotoxin (ChTX) was dissolved in the low-K^+ solution at a 1-μM concentration. Microliter amounts of the stock solutions were then added to the bath solution or to the low-K^+ solution as needed. For in vivo experiments, the acetazolamide solutions were prepared daily, dissolving the drug in the normal Ringer solution at concentrations of 0.95 and 1.9 mg/ml. The suspensions obtained were continuously stirred before oral administration performed by esophageal incanulation. Insulin Zn^{2+}-free (bovine pancreas, Sigma-Aldrich, Milano, Italy) was dissolved in 1.5 ml of normal Ringer solution containing 1 g of glucose and administrated to the K–deficient diet rats and normokalemic rats by intraperitoneal injection at a concentration of 4 U/per 100 g of body weight.

Evaluation of Paralysis

The paralysis was evaluated by testing the righting reflex of the K–deficient diet-treated and -untreated rats 3 to 5 hours after the insulin and glucose injection. The righting reflex is defined as the time needed for a rat to return to its normal posture after being turned on its back.[16]

Intracellular Microelectrode Technique

The RP of EDL muscle fibers was measured by using one intracellular microelectrode in the current-clamp mode.[19] Briefly, the fibers were impaled by an intracellular voltage electrode filled with KCl (3 M) with a resistance of 10 to 15 MΩ and connected to a holder/amplifier (WPI Instruments, New Haven, CT). The muscles were incubated for 30 minutes at 30°C in low-K^+ solution or normal Ringer solution enriched with ChTX.

Patch Pipettes

The patch pipettes were prepared as previously described.[18,19] Pipettes having an average tip opening area of 5.4 ± 0.8 μm^2 (160 macropatches) were used to measure the current sustained by multiple K_{ATP} and $K_{Ca^{2+}}$ channels and their pharmacologic properties. The single-channel conductance and multiple channels (N) × open probability (p_{open}) of the channels per unit area were measured using pipettes having a tip opening area of 0.85 ± 0.1 μm^2 (32 patches).

Patch-Clamp Experiments

Experiments were performed in inside-out configurations using a standard patch-clamp technique. Recordings of channel currents were performed during voltage steps of 2 seconds going from 0 mV of holding potential to different voltages (range, −60 to +40 mV) immediately after excision in the presence of 150 mM of KCl on both sides of the membrane at 20°C. The $K_{Ca^{2+}}$ current was recorded in the presence of a 16-μM concentration of free Ca^{2+} ion in the bath solution. At this concentration, any residual activity of the K_{ATP} channel present in the macropatches was abolished.[24] Single-channel recordings were performed under constant voltage at 20°C, in the presence of 150 mM of KCl on both sides of the membrane at −60 mV of membrane potential (Vm) for the 120 to 200 seconds required for recordings and during voltage steps. The macropatch current and single-channel current were recorded at 1 (filter = 0.2 kHz) and 20 kHz (filter = 2 kHz) of sampling rates, respectively, using an Axopatch-1D amplifier equipped with a CV-4 headstage (Axon Instruments, Foster City, CA).[18,19,23]

The in vitro effects of acetazolamide on the K_{ATP} and $K_{Ca^{2+}}$ channel currents were evaluated during voltage steps and at a constant voltage of −60 mV with 150 mM of KCl on both sides of the membrane in the absence and presence of a 16-μM concentration of free Ca^{2+} ion. Before recordings, the patches were exposed to acetazolamide for about 20 seconds.

Analysis of the Macropatch Current and Single-Channel Current

The currents flowing through the macropatches excised from different fibers were digitally averaged and calculated subtracting the baseline level of the currents from the open channel level. The baseline level for the K_{ATP} current was measured in the presence of ATP (5 mM), whereas the baseline level for the $K_{Ca^{2+}}$ current was measured in the absence of free Ca^{2+} ion in the bath. The criteria for accepting the data entered in the digital average were based on the stability of the seal and on the possible noise. Macropatches containing voltage-dependent K^+ channels or inward rectifier K^+ channels were excluded from the analysis.[18,19,23] The single-channel current was measured using the cursor method provided by the Fetchan program (Axon Instruments).[18,19,23] The single-channel conductance was then calculated as the slope of the voltage-current relation of the channel in the range of potentials from −70 to +70 mV. No correction for liquid junction potential (estimated to be <1.9 mV in our experimental condition) was made.

The p_{open} was measured as the ratio between the time spent by the channel in an open state over the total time of recordings. In the presence of N in the patches, the effects of the drug on the gating properties of a single $K_{Ca^{2+}}$ channel were expressed as the $N \times p_{open}$.[23]

Statistics

The data are expressed as mean ± SE unless otherwise specified. The concentration-response relation could be fitted with the following equation:

$$(I\ Drug - I\ Control)/I\ Control = E/(1 + (DE_{50}/[Drug])^n)$$

where (I Drug − I Control) ÷ I Control) is the ratio between the current measured in the presence of acetazolamide and that measured in the absence of the drug, E is the acti-

vation of the current caused by the drug, DE_{50} is the concentration of acetazolamide needed to enhance the current by 50%, [Drug] is the concentration of the drug tested, and n is the slope of the curves. The algorithms of the fitting procedures used were based on the Marquardt least squares fitting routine. Significant differences between individual pairs of means were determined by Student *t* test.

Results

Serum K^+ Levels, Insulin Sensitivity, and K^+ Channel Activity in the K–Deficient Diet Rats

In agreement with previous findings,[10,16,19,25,26] the K–deficient diet rats used in our experiments were characterized by low serum K^+ levels of 2.4 ± 0.5 mEq/L (n = 22 rats), abnormal response to insulin causing fiber depolarization and flaccid paralysis within 3 to 5 hours after the injection (Table), delay in the righting reflex ranging between 13 and 50 seconds, and abnormally reduced muscular K_{ATP} channel current. In contrast, in agreement with previous findings[10,25] in normokalemic rats, insulin induced fiber hyperpolarization with no paralysis and a righting reflex shorter than 1 second (see Table), and the fibers showed an intense muscular K_{ATP} channel current.

Effects of In Vivo Treatment with Acetazolamide on Serum K^+ Levels, Insulin Sensitivity, and K^+ Channel Activity of K–Deficient Diet and Normokalemic Rats

The chronic treatment of the K–deficient diet rats for 10 days with 2.8-mg/kg/day (n = 13 rats) (experimental group I) and 5.6-mg/kg/day (n = 4 rats) (experimental group II) concentrations of acetazolamide pre-

Table. Effects of the In Vivo Administration of Acetazolamide on Resting Potentials and Serum K^+ Levels of K–Deficient Diet and Normokalemic Rats

Experimental Groups	Serum K^+ Level (mEq/L)	Resting Potential in Normo-K^+ Solution (mV)	Resting Potential in Low-K^+ Solution (mV)	Resting Potential in Low-K^+ Solution + ChTX (mV)	Number of Paralyzed Rats/ Total Number of Rats
Group III: Untreated K-deficient diet rats	2.0 ± 0.3	−53.2 ± 8.0 n = 12 fibers	−49.1 ± 6.0 n = 19 fibers	−51.1 ± 7.0 n = 11 fibers	5/5
Group V: Untreated normokalemic rats	4.2 ± 0.6	−86.2 ± 5.0 n = 14 fibers	−91.1 ± 3.0 n = 16 fibers	−90.2 ± 5.0 n = 13 fibers	0/3
Acetazolamide, 2.8 mg/kg/day					
Group Ia: Treated K-deficient diet rats not paralyzed by insulin	5.5 ± 0.5	−78.1 ± 6.0 n = 39 fibers	−85.1 ± 4.0[a] n = 29 fibers	−65.3 ± 3.0[b] n = 58 fibers	
Group Ib: Treated K-deficient diet rats paralyzed by insulin	2.1 ± 0.3	−57.8 ± 5.0 n = 42 fibers	−52.2 ± 4.0 n = 41 fibers	−54.3 ± 4.0 n = 53 fibers	6/13
Acetazolamide, 5.6 mg/kg/day					
Group II: Treated K-deficient diet rats	5.8 ± 0.3	−72.1 ± 5.0 n = 32 fibers	−91.1 ± 3.0[a] n = 27 fibers	−64.2 ± 4.0[b] n = 22 fibers	0/4
Group IV: Treated normokalemic rats	4.8 ± 0.5	−87.1 ± 6.0 n = 26 fibers	−92.1 ± 4.0 n = 25 fibers	−89.1 ± 6.0 n = 21 fibers	0/3

Serum K^+ concentrations and resting potentials of extensor digitorum longus muscle fibers of: group III, untreated K–deficient diet rats; group V, untreated normokalemic rats; groups Ia and Ib, K–deficient diet rats treated in vivo for 10 days with a 2.8-mg/kg/day concentration of acetazolamide; group II, K–deficient diet rats treated in vivo for 10 days with a 5.6-mg/kg/day concentration of acetazolamide, and group IV, normokalemic rats treated for 10 days with a 5.6-mg/kg/day concentration of acetazolamide. After 10 days of treatment, the K–deficient diet rats and normokalemic rats were injected intraperitoneally with a solution containing insulin (4 U per 100 g of body weight) and glucose. This provoked paralysis in all the K–deficient diet rats of group III and in a fraction of the K–deficient diet rats of group Ib treated with the low dose of acetazolamide. A 2.8-mg/kg/day concentration of acetazolamide prevented paralysis in 7 of 13 K–deficient diet rats (group Ia). A 5.6-mg/kg^{-1}/day^{-1} concentration of acetazolamide prevented paralysis in all the treated rats (group II). The resting potential was measured in normokalemic solution (5 mEq/L) and in low K^+ solution (0.5 mEq/L) in the absence and presence of a 100-nM concentration of charybdotoxin. Blood intracardiac samples were collected at the time of death for serum K^+ measurements.

[a]Significantly different from the corresponding normo-K^+ data ($p \leq 0.001$) as determined by Student *t* test.
[b]Significantly different from the corresponding low-K^+ solution data ($p \leq 0.001$) as determined by Student *t* test.

vented the characteristic insulin-induced paralysis. This effect seems to be related to dose (see Table). In fact, the treatment of the K–deficient diet rats with low dose of acetazolamide prevented the insulin-induced paralysis in 54% of the treated rats, although high-dose acetazolamide prevented paralysis in 100% of the rats. The fraction of the K–deficient diet rats belonging to the first experimental group (Ia) that did not become paralyzed by insulin as well as the K–deficient diet rats of the second experimental group showed a righting reflex shorter than 1 second and normal serum K^+ levels (see Table). In the same animals (groups Ia and II), significant fiber repolarization was detected in the normo-K^+ solution, particularly in the low-K^+ solution after treatment with acetazolamide (see Table). The in vitro application of ChTX (100 nM), a blocker of the $K_{Ca^{2+}}$ channel, to the EDL muscle fibers of the acetazolamide-treated K–deficient diet rats, in which insulin did not provoke paralysis, depolarized the fibers, suggesting that the $K_{Ca^{2+}}$ channel contributes to the RPs of the fibers (see Table). ChTX seems also to block voltage-dependent K^+ channels; however, at the RP, these channels are closed and their contribution to the RP is negligible. The application of glibenclamide (1–10 μM), a blocker of the K_{ATP} channel, to the muscle fibers of K–deficient diet rats treated with acetazolamide was without effects. Macropatch current recordings revealed that the acetazolamide treatment significantly increased the $K_{Ca^{2+}}$ channel current in the muscle fibers of the K–deficient diet rats of experimental groups Ia and II (Fig 1). In particular, at -60 mV (Vm), the $K_{Ca^{2+}}$ channel current increased from -21 ± 5 pA/μm^2 (n = 16 patches, n = 3 rats) in the untreated K–deficient diet rats (experimental group III) to -35.5 ± 5.0 and -45.7 ± 3.1 pA/μm^2 in the K–deficient diet rats of experimental groups Ia and II treated with 2.8-mg/kg^{-1}/day^{-1} (n = 41 patches, n = 4 rats) and 5.6-mg/kg^{-1}/day^{-1} (n = 18 patches, n = 2 rats) concentrations of the thiazide derivative, respectively. The lowering of the internal KCl concentration from 150 to 30 mM shifted the reversal potentials of the macroscopic current-voltage curves of the channels from K–deficient diet rats treated with acetazolamide from 0 mV to positive values, confirming that the current stimulated by the drug was carried by the K^+ ion.

In contrast, the fraction of K–deficient diet rats of the first experimental group (group Ib), in which insulin provoked paralysis, showed a delay in the righting reflex ranging from 7 to 43 seconds and marked hypokalemia (see Table). In the same animals, acetazolamide failed to restore the RPs as recorded in normo-K solution, and further depolarization occurred after exposure of the EDL muscle fibers to low-K^+ solution (see Table). The in vitro application of ChTX (100 nM) to the EDL muscle fibers of K–deficient diet rats of the Ib group did not affect the RP of the fibers

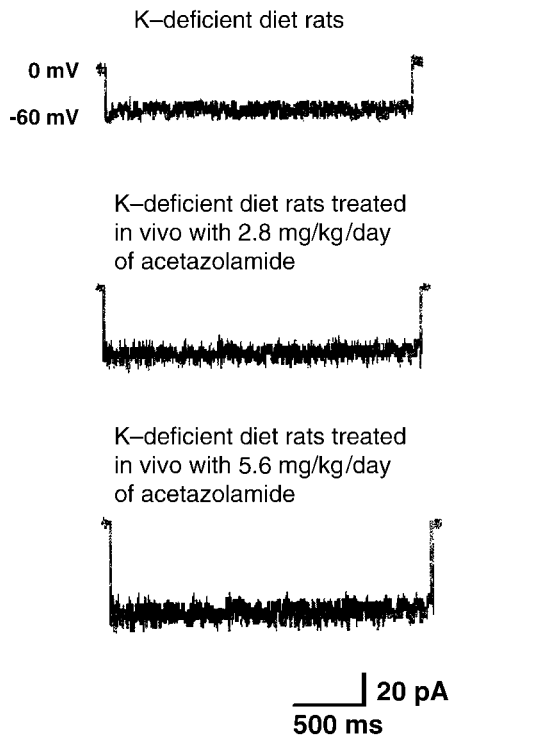

Fig 1. Effects of the in vivo chronic administration of acetazolamide on the Ca^{2+}-activated K^+ ($K_{Ca^{2+}}$) channel currents of K–deficient diet rat fibers. Digital average of $K_{Ca^{2+}}$ channel currents recorded by macropatches excised from untreated K–deficient diet rat fibers (group III) (n = 16 patches; patch area = 4.95 ± 0.7 μm^2), K–deficient diet rat fibers (group Ia) pretreated in vivo for 10 days with acetazolamide at a concentration of 2.8 mg/kg/day (n = 41 patches; patch area = 5.05 ± 0.8 μm^2), and K–deficient diet rat fibers (group II) pretreated in vivo for 10 days with a 5.6-mg/kg^{-1}/day^{-1} concentration of acetazolamide (n = 18 patches; patch area = 5.07 ± 0.6 μm^2). The currents were recorded during voltage steps going from 0 to −60 mV (Vm) of holding potential, with 150 mM of KCl on both sides of the membrane, in the presence of an internal 16-μM concentration of free Ca^{2+} ion. During the voltage steps, downward deflections of the currents were recorded (inward currents). The currents were leak subtracted. The in vivo treatment of the K–deficient diet rat with acetazolamide led to stimulation of the $K_{Ca^{2+}}$ channel currents.

(see Table). In these animals, the macropatch $K_{Ca^{2+}}$ channel current was not modified by acetazolamide treatment, being -19 ± 5 pA/μm^2 (n = 24 patches, n = 4 rats) at -60 mV (Vm). The activity of the sarcolemma K_{ATP} channels of the K–deficient diet rats of the Ia, Ib, and II experimental groups was not modified by acetazolamide treatment.

The in vivo administration of a 5.6-mg/kg/day concentration of acetazolamide to the normokalemic rats (experimental group IV) did not significantly affect the $K_{Ca^{2+}}$ channel currents and the K_{ATP} channel currents of the fibers or the RPs and serum K^+ levels compared

with those of the untreated normokalemic rats (experimental group V) (see Table).

Effects of In Vitro Treatment with Acetazolamide on the Sarcolemma $K_{Ca^{2+}}$ Channels and K_{ATP} Channels of Muscle Fibers of K–Deficient Diet and Normokalemic Rats

In the presence of a 16-μM concentration of internal free Ca^{2+} ion, the application of increasing concentrations of acetazolamide on macropatches excised from K–deficient diet rat and normokalemic rat fibers induced a dose-dependent enhancement of the sarcolemma $K_{Ca^{2+}}$ channel currents. No effects were observed on the K_{ATP} currents. In particular, in the K–deficient diet rats at −60 mV (Vm), the drug enhanced the $K_{Ca^{2+}}$ channel currents, with a DE_{50} of 6.17×10^{-6} M and a maximal E of 98% (slope factor = 1.9) (Fig 2a and c). At +30 mV (Vm), acetazolamide increased the current with a DE_{50} of 4.01×10^{-6} M and a maximal E of 54.4% (slope factor = 2.1) (see Fig 2b and d).

In the normokalemic rats, the DE_{50} of acetazolamide, at −60 mV (Vm) was 6.87×10^{-6} M, and the maximal E was 87% (slope factor = 2.1) (see Fig 3a and c). At +30 mV (Vm), acetazolamide increased the current, with a DE_{50} of 4.02×10^{-6} M and a maximal E of 44.3% (slope factor = 2.3) (see Fig 3b and d). In the absence of internal free Ca^{2+} ion, the drug did not affect the sarcolemma $K_{Ca^{2+}}$ currents of either normokalemic or K–deficient diet rats.

Single-channel analyses revealed that the enhancement of the $K_{Ca^{2+}}$ currents observed in the K–deficient diet rat and normokalemic rat fibers caused by acetazolamide was related to the increase in $N \times p_{open}$. In fact, the application of acetazolamide (10 μM) to the patches excised from K–deficient diet rat fibers increased the $N \times p_{open}$ from 0.23 ± 0.07 in the absence of the drug to 0.56 ± 0.08 (n = 8 patches) in the presence of the drug. In the normokalemic rat fibers, acetazolamide increased the $N \times p_{open}$ from 0.16 ± 0.08 in the absence of the drug to 0.41 ± 0.09 (n = 9 patches) in the presence of a 10-μM concentration of the drug. The in vitro application of acetazolamide to the patches did not modify the single-channel conductance of the $K_{Ca^{2+}}$ channel of K–deficient diet rat and normokalemic rat fibers. In the pres-

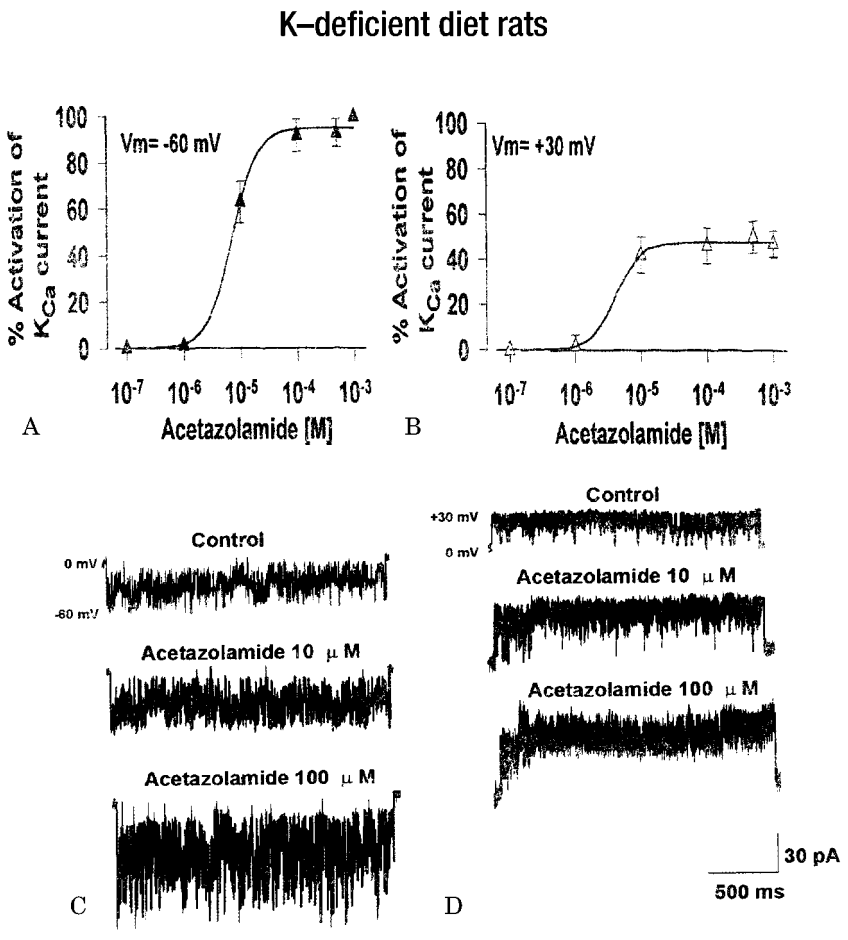

Fig 2. In vitro effects of acetazolamide on the Ca^{2+}-activated K^+ ($K_{Ca^{2+}}$) currents of K–deficient diet rat fibers. Dose-response curves of $K_{Ca^{2+}}$ current versus acetazolamide concentrations (a and b) and sample traces of macropatch $K_{Ca^{2+}}$ channel currents in the absence (control) and presence of 10- and 100-μM concentrations of acetazolamide (c and d). The channel currents were recorded by macropatches during voltage steps going from a holding potential of 0 to −60 mV (Vm) (a and c) and from 0 to +30 mV (Vm) (b and d) with 150 mM of KCl on both sides of the membrane in the presence of a 16-μM concentration of free Ca^{2+} ion in the bath. During the voltage steps going from 0 to −60 mV (Vm), downward deflections of the currents were recorded (inward currents), whereas during the voltage steps going from 0 to +30 mV (Vm), upward deflections of the currents were recorded (outward currents). Before recordings, the macropatches were incubated with drug solutions for 20 seconds. Acetazolamide dose dependently increased the $K_{Ca^{2+}}$ channel current at both voltages. At least five $K_{Ca^{2+}}$ channels were present in the patch. Each experimental point represents the mean ± SE of a minimum of four and a maximum of six macropatches.

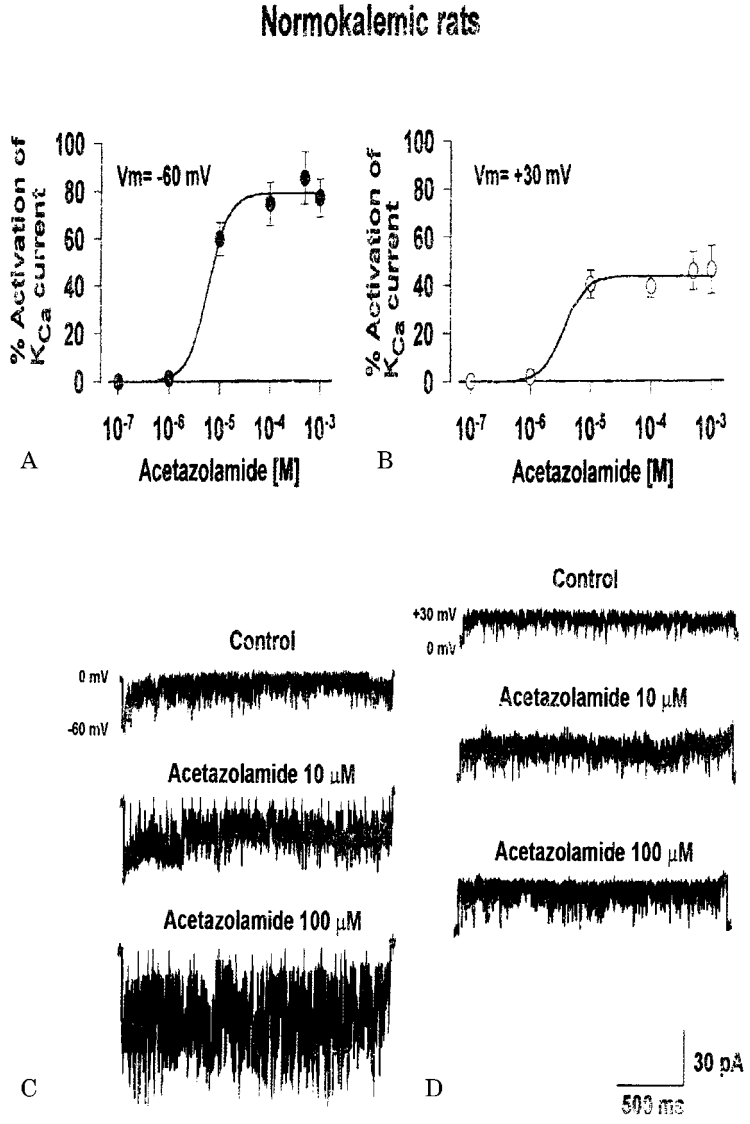

Fig 3. In vitro effects of acetazolamide on the Ca^{2+}-activated K^+ ($K_{Ca^{2+}}$) currents of normokalemic rat fibers. Dose-response curves of $K_{Ca^{2+}}$ current versus acetazolamide concentrations (a and b) and sample traces of macropatch $K_{Ca^{2+}}$ channel currents in the absence (control) and presence of 10- and 100-μM concentrations of acetazolamide (c and d). The channel currents were recorded by macropatches during voltage steps going from a holding potential of 0 to −60 mV (Vm) (a and c) and from 0 to +30 mV (Vm) (b and d) with 150 mM of KCl on both sides of the membrane in the presence of a 16-μM concentration of free Ca^{2+} ion in the bath. During the voltage steps going from 0 to −60 mV (Vm), downward deflections of the currents were recorded (inward currents), whereas during the voltage steps going from 0 to +30 mV (Vm), upward deflections of the currents were recorded (outward currents). Before recordings, the macropatches were incubated with drug solutions for 20 seconds. Acetazolamide dose dependently increased the $K_{Ca^{2+}}$ channel current at both voltages. At least four $K_{Ca^{2+}}$ channels were present in the patch. Each experimental point represents the mean ± SE of a minimum of four and a maximum of six macropatches.

ence of 150 mM of KCl on both sides of the membrane, the amplitude of the currents flowing through the $K_{Ca^{2+}}$ channels of K–deficient diet rat fibers at −60 mV (Vm) was −12.8 ± 2.0 pA (n = 8 patches) in the controls and −13 ± 2 pA in the presence of a 10-μM concentration of acetazolamide. In the normokalemic rat fibers, the current amplitude of a single $K_{Ca^{2+}}$ channel recorded in the same condition was −13.8 ± 3.0 pA (n = 9 patches) in the controls and −11.9 ± 3.0 pA in the presence of acetazolamide. The slope conductance calculated from the current-voltage relations for the $K_{Ca^{2+}}$ channel of the K–deficient diet rat and normokalemic rat fibers was 221 ± 12 pS (n = 8 patches) and 218 ± 11 pS (n = 9 patches), respectively, in the controls, and 228 ± 9 and 226 ± 6 pS (n = 9 patches), respectively, in the presence of a 10-μM concentration of acetazolamide.

Discussion

In the present work, we attempted to solve the question of how acetazolamide works in skeletal muscle disorders linked to altered K^+ homeostasis. We demonstrated that acetazolamide exerted beneficial effects on K–deficient diet rats through direct activation of the sarcolemma $K_{Ca^{2+}}$ channels. Normal RPs, hyperpolarization in low external K^+ solution, depolarization induced by ChTX, and overactivation of the muscular $K_{Ca^{2+}}$ channels are all features of the acetazolamide-treated K–deficient diet rat not paralyzed by insulin injection. In contrast, these features do not apply to acetazolamide-treated K–deficient diet rat fibers that developed weakness and paralysis after insulin administration. The proposed mechanism of action of acetazolamide involving direct activation of $K_{Ca^{2+}}$ channels is in contrast to the popular idea that the effects of

acetazolamide in various tissues, including skeletal muscle, are exclusively mediated by inhibition of carbonic anhydrase enzyme. The activation of the sarcolemma $K_{Ca^{2+}}$ channel caused by acetazolamide would contribute to the normalization of the serum K^+ levels that we measured in the K–deficient diet rats; in fact, several reports provide evidence that overactivation of $K_{Ca^{2+}}$ channels in the cultured cells can be associated with hyperkalemia.[27]

The involvement of the $K_{Ca^{2+}}$ channel as a new target of the action of acetazolamide is supported by several findings. First, the chronic administration of acetazolamide to the K–deficient diet rats prevented the characteristic paralytic attacks induced by insulin leading to a dose-dependent fiber repolarization, which is selectively reversed by the $K_{Ca^{2+}}$ channel blocker ChTX but not by glibenclamide, a well-known K_{ATP} channel blocker. Second, acetazolamide administrated in vivo to the rats selectively stimulated the $K_{Ca^{2+}}$ current in the excised macropatches, without modifying the K_{ATP} currents. Third, acetazolamide applied in vitro to the patches excised from K–deficient diet rat and normokalemic rat fibers, in which the metabolic factors are lost, directly stimulated the $K_{Ca^{2+}}$ channel activity.

The mechanism by which acetazolamide activated the $K_{Ca^{2+}}$ channel is complex, involving Ca^{2+}- and voltage-dependent actions of the drug. The Ca^{2+}-dependent effect of acetazolamide is demonstrated by the fact that in the absence of internal free Ca^{2+} ion, the drug failed to activate the $K_{Ca^{2+}}$ channel in the excised macropatches of K–deficient diet and normokalemic rats. The voltage-dependent effect of acetazolamide is demonstrated by the fact that the drug produced maximal activation of the $K_{Ca^{2+}}$ currents at a rate of approximately 94 to 98% at −60 mV, whereas at +30 mV of voltage membrane, the percentage of activation of the current was significantly lower, ranging between 44 and 54%. The differences existing in the maximal activation of the currents at different voltages can be explained by the fact that acetazolamide enhanced the p_{open} or the number of functional channels in the patches without affecting the single-channel conductance. In fact, at +30 mV, most of the channels are already in the open state and not further available for drug activation, whereas at −60 mV, the channel resides in a closed state and is therefore available for drug activation. Unfortunately, it was not possible to clearly distinguish between the effects of the drug on N or p_{open}; in fact, in view of the low p_{open} measured at −60 mV (Vm), the probability of observing all N channels simultaneously open is small.

In conclusion, our data show that acetazolamide acts as a K^+ channel opener selective for the $K_{Ca^{2+}}$ channel population. This mechanism can explain the beneficial effects observed in K–deficient diet rats and possibly in human beings affected by hypoPP. The observation that acetazolamide given in vivo to the normokalemic rats did not affect the electrophysiologic parameters of the fibers is related to the fact that the in vivo injection of insulin to the normokalemic rats sets the RP of the fibers close to the equilibrium potential for the K^+ ion (approximately 90 mV), masking the acetazolamide action. This is in line with the general idea that the effects of the K^+ channel openers are dependent on the difference existing between the RP and the equilibrium potential for K^+ ion so that the fiber depolarization favors the action of these drugs.[28] Therefore, we believe that the efficacy of the thiazide derivative in opening the $K_{Ca^{2+}}$ channel may be augmented in depolarized fibers as occurs in human patients affected by hypoPP and in the K–deficient diet rats in which the RPs range between −60 and −50 mV. It has been reported that some hypoPP patients are resistant to acetazolamide action, however.[9–11] This discrepancy can be explained by the fact that the effects of acetazolamide can also be dependent on the intracellular Ca^{2+} ion concentration. Recently, Morril and Cannon[29] proposed that the three point mutations of the α_1S subunit of the L-type Ca^{2+} channel causing hypoPP led to a reduced current density and slower activation properties compared with wild-type channels. This can be associated with a reduction in the intracellular availability of Ca^{2+} ion depending on the severity of hypoPP phenotypes and may also influence the response of patients to acetazolamide treatment.

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