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# The Biophysical and Pharmacological Characteristics of Skeletal Muscle ATP-Sensitive $K^+$ Channels Are Modified in $K^+$-Depleted Rat, an Animal Model of Hypokalemic Periodic Paralysis

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## ABSTRACT

We evaluated the involvement of the sarcolemmal ATP-sensitive $K^+$ channel in the depolarization of skeletal muscle fibers occurring in an animal model of human hypokalemic periodic paralysis, the $K^+$-depleted rat. After 23–36 days of treatment with a $K^+$-free diet, an hypokalemia was observed in the rats. No difference in the fasting insulinemia and glycemia was found between normokalemic and hypokalemic rats. The fibers of the hypokalemic rats were depolarized. In these fibers, the current of sarcolemmal ATP-sensitive $K^+$ channels measured by the patch-clamp technique was abnormally reduced. Cromakalim, a $K^+$ channel opener, enhanced the current and repolarized the fibers. At channel level, two open conductance states blocked by ATP and stimulated by cromakalim were found in the hypokalemic rats. The two states could be distinguished on the basis of their slope conductance and open probability and were never detected on muscle fibers of normokalemic rats. It is known that insulin in humans affected by hypokalemic periodic paralysis leads to fiber depolarization and provokes paralysis. We therefore examined the effects of insulin at macroscopic and single-channel level on hypokalemic rats. In normokalemic animals, insulin applied *in vitro* to the muscles induced a glybenclamide-sensitive hyperpolarization of the fibers and also stimulated the sarcolemmal ATP-sensitive $K^+$ channels. In contrast, in hypokalemic rats, insulin caused a pronounced fiber depolarization and reduced the residual currents. Our data indicated that in hypokalemic rats, an abnormally low activity of ATP-sensitive $K^+$ channel is responsible for the fiber depolarization that is aggravated by insulin.

The familial HOPP is an inherited muscle disease characterized by episodes of flaccid paralysis and muscle weakness accompanied by lowering of serum $K^+$ concentration (Lehmann-Horn *et al*., 1994). The HOPP is transmitted as an autosomal dominant inheritance with a reduced penetrance in the woman (Fouad *et al*., 1997). For many years, HOPP has been classified as a metabolic disease. However, recently, it has been included in the muscle diseases caused by an abnormal functionality of ion channels and therefore defined as a channelopathy (Lehmann-Horn and Rüdel, 1996). Linkage studies have recently shown that the HOPP gene is colocalized with the gene encoding the $\alpha$1 subunits of the skeletal muscle L-type $Ca^{2+}$ channel (Fontaine *et al*., 1994; Jurkat-Rott *et al*., 1994; Ptacek *et al*., 1994). Three points mutations were found within the coding sequence of the $\alpha$1 subunit of muscular L-type $Ca^{2+}$ channel of patients with HOPP (Lehmann-Horn and Rüdel, 1996). However, these mutations do not affects the macroscopic $Ca^{2+}$ current of myotubes cultured from muscle of patients with HOPP or $Ca^{2+}$ current of channels expressed in cell line (Lapie *et al*., 1997). Furthermore, the $Ca^{2+}$ channel mutations found in HOPP patients do not correlate with the depolarization of the fibers, the characteristic muscle paralysis induced by insulin (Tricarico *et al*. 1997b; Lehmann-Horn *et al*., 1994), and the lowering of serum $K^+$ concentration occurring during the attacks (Cannon, 1996; Fouad *et al*., 1997). These observations suggest that the phatophysiological mechanisms responsible for HOPP are complex, possibly involving different factors other than the $Ca^{2+}$ channel.

Preliminary data showed that in an animal model of HOPP, the $K^+$-depleted rats (Dengler *et al*., 1979; Bond and Gordon, 1993), the activity of the sarcolemmal $K_{ATP}$ channels

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**ABBREVIATIONS:** $K_{ATP}$, ATP-sensitive $K^+$ channel; EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; AMP-PNP, adenylylimidodiphosphate; EDL, extensor digitorum longus; FDB, flexor digitorum brevis; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HOPP, hypokalemic periodic paralysis.

is abnormally reduced (Tricarico *et al.*, 1997b). In these animals as in the humans affected by HOPP, insulin depolarizes the fibers and provokes muscle paralysis (Dengler *et al.*, 1979; Bond and Gordon, 1993; Lehmann-Horn *et al.*, 1994; Tricarico *et al.*, 1997b). In contrast, in normokalemic rats, the hormone stimulates the sarcolemmal $K_{ATP}$ channels and leads to a glybenclamide-sensitive hyperpolarization of the fibers (Iannaccone *et al.*, 1989; Lehmann-Horn *et al.*, 1994; Tricarico *et al.*, 1997a). These findings suggest the possible involvement of this type of channel in the human HOPP. This is supported by the observations that the $K^+$ channel openers, cromakalim, and pinacidil are, respectively, capable of repolarizing the skeletal muscle fibers from humans affected by HOPP and restoring the muscle strength in the same patients (Spuler *et al.*, 1989; Grafe *et al.*, 1990; Links *et al.*, 1993; Ligtenberg *et al.*, 1996).

In the current work, we investigated the properties of the $K_{ATP}$ channel of skeletal muscle fibers of $K^+$-depleted rats. In particular, we measured the macropatch current, the single-channel conductance, and its sensitivity to the specific blockers ATP and glybenclamide and to the agonist cromakalim. Experiments were devoted to evaluation of the effects of insulin on resting potentials and on sarcolemmal $K_{ATP}$ channels of hypokalemic and normokalemic rats.

## Materials and Methods

**Rat housing and diet.** Male Wistar rats (280 ± 20 g of body weight, 3 months old) were divided into two groups and housed in three rats per cage. The rats were fed 30 g of pellets/day based on different recipes for 23–36 days of treatment. The first group of rats was made hypokalemic by feeding them with a special food free of $K^+$ (Mucedola, Settimo Milanese, Milan, Italy) composed of 21.3% casein, 15% sucrose, 3% grain, 2% multivitamin mixture, 3% mineral water free of $K^+$, 15% DL-methionine, 0.25% choline, 5% corn oil, 5% lard, and 43.35% dextrin. The normokalemic rats were fed with food containing a normal concentration of $K^+$ (0.8%).

For the evaluation of the serum concentration of $K^+$ and $Na^+$, blood samples were collected from the tail vein of the animals on randomly selected rats from each group at the beginning and throughout the period of the treatment. At the time of death, intracardiac blood samples were collected from the rats after an overnight fast for the evaluation of the levels of the two ions and for serum glucose and insulin determinations. The rats were considered hypokalemic when the serum $K^+$ level was ≤ 3.2 mEq/liter. In our condition, ≥ 18 days of treatment were needed to induce measurable hypokalemia in the rats.

**Spectrophotometry and radioimmunoassay.** The blood samples were centrifuged at 2500 rpm at 7° for 15 min. After this time, a few microliters of clear serum samples were collected and used for the analysis. Only serum without evident emolysis was used for the analysis. Standard flame spectrophotometry (EEL 450 flame photometer; Corning Glassworks, Corning, NY) was used for detection of the serum $K^+$ and $Na^+$ levels. The values were expressed as mEq/liter concentration of ions. The glucose levels were measured on serum samples using a spectrophotometric method (Shimadzu 7000 Poli CL, Tokyo, Japan) based on enzymatic reaction (Tricarico and Conte Camerino, 1994b). The activity of the antibodies directed against insulin was evaluated by radioimmunoassay using a standard kit (Ct-Cis Bio International; CIS Diagnostici, Padova, Italy).

**Muscle preparations and single fiber isolation.** The EDL and FDB muscles were dissected from the bones with the animals under urethane anesthesia (1.2 g/kg). Resting potential measurements were performed mainly on EDL muscles, whereas patch-clamp data were obtained from single fibers of FDB and EDL muscles. After dissection, the muscles were immersed in Ringer's solution. Single fibers were obtained by enzymatic treatment of the muscles (Tricarico and Conte Camerino, 1994a).

**Solutions.** The pipette solution contained 150 mM KCl, 2 mM $CaCl_2$, 10 mM MOPS, pH 7.2. The bath solution contained normal Ringer's, 145 mM NaCl, 5.5 mM KCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5 mM glucose, and 10 mM MOPS, pH 7.2. The low $K^+$ solution contained 145 mM NaCl, 0.5 mM KCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5 mM glucose, and 10 mM MOPS, pH 7.2. The symmetrical $K^+$ solution contained 150 mM KCl, 0.5 mM EGTA, and 10 mM MOPS, pH 7.2.

Other solutions were prepared by lowering the concentration of the KCl in the bath from 150 to 30 mM with the addition of a small amount of sucrose as needed to obtain a final osmolarity of 298 mOsM on both sides of the membrane. Stock solutions (5 mM) of the nucleotide tested, $Na_2$ATP, AMP-PNP, MgATP, and MgADP, were prepared by dissolving the chemicals in the bath solution (symmetrical $K^+$).

Cromakalim (Sigma Chemical, St. Louis, MO) and glybenclamide (Sigma) were first dissolved in dimethylsulfoxide at concentrations of 0.28 M and 4.05 mM. In the range of the cromakalim and glybenclamide concentrations tested, the corresponding dimethylsulfoxide concentrations did not mimic the effects of the drugs on $K_{ATP}$ channels and on resting potentials (solvent control). Microliter amounts of the stock solutions of the nucleotides and drugs tested then were diluted in the bath solutions as needed. Insulin (bovine pancreas; Sigma) was dissolved at 4 units/liter concentration in the low $K^+$ solution and the normal Ringer's solution.

**Microelectrode technique.** The resting potentials of EDL muscle fibers were measured by using one intracellular microelectrode in current clamp mode (Tricarico *et al.*, 1994b). The fibers were impaled by an intracellular voltage electrode filled with KCl (3 mM) of resistance of 10–15 MΩ and connected to an holder/amplifier (WPI Instruments, New Haven, CT). To evaluate the effects of insulin and of cromakalim on resting potentials, the muscles were incubated for 30 min at 30° with low $K^+$ solution or normal Ringer's solution enriched with the compounds under study.

**Patch pipettes.** Pipettes were prepared as described previously (Tricarico and Conte Camerino, 1994a). The tip opening area of the pipettes were measured by scanning electron microscopy (Cambridge Instruments). Measurements of micro- and macropatch conductance and tip opening area were performed on the same pipettes according to the method of Sakmann and Neher (1983). A linear correlation between the pipette conductance and the tip opening area has been found in the range of conductance of 50–1600 nS. The slope of the straight line was 0.00698, the intercept was 0.302, and the coefficient of correlation was 0.778. Macropipettes with an average tip opening area of 5.2 ± 1 $\mu m^2$ (number of macropatches, 340) were used to measure the current sustained by multiple channels (25–35 channel/patch area) and the pharmacological properties of $K_{ATP}$ channels. However, the single-channel conductance and the channel open probability ($P_{open}$) were measured using micropipettes having a tip opening area of 0.9 ± 0.1 $\mu m^2$ (number of micropatches, 64). Using this type of pipette, no more than two or three open channels were observed in the patches. A few micropatches (3 of 41 excised from normokalemic rat fibers and 3 of 23 patches excised from hypokalemic rats) contained only single units. This was tested by observing the single-channel transitions for long periods of time (123 sec–257 sec) in the presence of internal 50 $\mu$M Mg ADP, a physiological stimulator of the $K_{ATP}$ channels, in the bath condition that ensures the maximum stimulation of the channel open probability (Allard and Lazdunski, 1992).

**Recordings of macropatch currents and single-channel currents.** Experiments were performed in cell-attached and inside-out configurations using standard patch-clamp techniques. Recordings of $K_{ATP}$ current were performed during voltage step of 53 sec from 0 mV of holding potential to different voltages (from −60 to +40 mV) after 20 sec from patch excision in the presence of 150 mM KCl on both sides of the membrane patches at 20°.

Recordings of single-channel current were performed under constant voltage, at 20°, in the presence of 150 mM KCl on both sides of the membrane. The current of single channel also was measured at various potentials (from −70 to +70 mV). The macropatch current and single-channel current were recorded at 20 kHz of sampling rate and filtered at 2 kHz using Axon Instruments (Burlingame, CA) hardware and the pClamp software package (Tricarico and Conte Camerino, 1994a).

The effects of the cromakalim on the current were evaluated at −60 mV (Vm), at 20°, in the presence of 150 mM KCl on both sides of the membrane. The $K^+$ channel opener was tested in the presence or in the absence of ATP in the bath. Before recordings, the macropatches were exposed to the agonists for ~20 sec.

To evaluate the effect of insulin on $K_{ATP}$ currents, the FDB and EDL muscles were incubated at 30° with a normal Ringer's solution enriched with the hormone (4 units/liter) for 45 min. After this time, the muscles were exposed to the enzyme solution containing insulin for single fiber dissociation.

**Analysis of the macropatch current and single-channel current.** The currents flowing through the macropatches were calculated subtracting the base-line level of the current defined as the closed state of the channels, measured in the presence of ATP, from the open-channel level. Two methods were used to evaluate the rundown of the current. First, after patch excision, the time-dependent decay of the current was followed during voltage steps from 0 to −60 mV (Vm) of 16 min at 20°. We found that no time-dependent decay of the current of the normokalemic (number of observations, 12) rat fibers (number of observations, 11) was observed during the first 3 min after excision. Therefore, the patch-clamp data of hypokalemic and normokalemic rat channels were collected during this period of time. Second, we evaluated whether in normokalemic and hypokalemic rats the maximum amplitude of the current recorded in the presence of 50 μM MgADP matched those recorded in the absence of the nucleotide.

The single-channel current was measured at various potentials (from −70 to +70 mV), using the cursor method provided by the Fetchan program (pClamp software package). The single-channel conductance was calculated as the slope of the voltage-current relationship of the channel in the range of potentials from −70 to −10 mV and in the range of potentials from +10 to +70 mV. No correction of liquid junction potential was made, estimated to be <+1.8 mV in our experimental conditions.

The $P_{open}$ was measured as the ratio between the time spent by the channel in an open state over the total time of recording.

**Statistics.** The data are expressed as mean ± standard error unless otherwise specified. The frequency of finding $K_{ATP}$ channels in the macropatches was calculated as reported previously (Tricarico *et al.*, 1997c).

The open and close time distributions could be fitted with the equation $f(t) = P_1(1/\tau_1)\exp(-t/\tau_1) + P_2(1/\tau_2)\exp(-t/\tau_2)$, where $P_1$ and $P_2$ are the fractional contributions for the respective components to the area under the curves, $\tau_1$ and $\tau_2$ are the time constants of each component, and $t$ is the time. This type of analysis was performed within the bursts of openings. The close time interval between bursts was calculated testing different time intervals of variable durations to find the minimum one at which the number of closing events were relatively insensitive to further increase of this parameter. In the patches from normokalemic rats containing only one open conductance level, we calculated a mean time interval between bursts of 5.3 ± 0.6 msec (three patches). This parameter was assumed to be the same in the hypokalemic rats.

The current distributions of normokalemic rats could be fitted with the Gaussian function $f(A) = P\cdot\exp\{[-(A-\mu)^2][2(\sigma^2)]\}/[\sqrt{(2\Pi)}\sigma]$, or with the sum of two terms, where $P$ is the fractional contribution of the component(s) to the area under the curves, $A$ is the bin center amplitudes of the component or components, $\mu$ is the mean of the component, and $\sigma$ is the standard deviation of the component or components.

The agonist effect of cromakalim on the channel current was evaluated as the ratio between the current in the presence of drug + ATP and the current in the presence of ATP alone ($I_{control}$). The concentration-response relationship could be fitted with the equation $I_{drug}/I_{control} = (1 - k)/[1 + (ED_{50}/[drug])^n]$, where $I_{drug}/I_{control}$ is the ratio between the current measured in the presence of the drug and that measured in the absence of drug, $ED_{50}$ is the concentrations of the drug needed to enhance the current by 50%, [drug] is the concentration of the drug tested, $n$ is the slope of the curves, and $k$ is the maximum change observed in the current. However, the antagonist effect of ATP and glybenclamide was described by an equation in which the factor $(ED_{50}/[drug])^n$ was replaced by $([drug]/IC_{50})^n$, where $IC_{50}$ is the concentration of the compound needed to reduce the current by 50%.

The algorithms of the fitting procedures used were based on Marquardt least-squares fitting routine. Significant differences between individual pairs of mean values were determined by Student's $t$ test.

## Results

***In vivo* observations.** After 23–36 days of treatment with the $K^+$-free diet, the serum $K^+$ concentration significantly decreased from 5.1 ± 0.3 mEq/liter (nine rats) in the normokalemic rats to 2.5 ± 0.2 mEq/liter (nine rats) ($p < 0.001$) in the hypokalemic rats. The serum $Na^+$ level was unaltered by hypokalemia with 142 ± 3 mEq/liter (nine rats) and 144 ± 4 mEq/liter (nine rats) in the normokalemic and hypokalemic rats, respectively. No significant differences between hypokalemic and normokalemic rats were observed in the serum fasting insulinemia and glycemia states. The insulin concentration was 166 ± 12 pM (five rats) in normokalemic animals and 170 ± 17 pM (five rats) in hypokalemic rats. The glucose levels were 168 ± 11 mg/ml (five rats) in normokalemic animals and 164 ± 12 mg/ml (five rats) in hypokalemic rats.

**Resting potentials of skeletal muscle fibers of normokalemic and hypokalemic rats and effects of insulin.** In normal Ringer's solution (5.5 mEq/liter $K^+$ ion), the EDL muscle fibers of hypokalemic rats were depolarized compared with the normokalemic rat fibers (Table 1). The perfusion of the muscles with low $K^+$ solution (0.5 mEq/liter $K^+$ ion) caused a more pronounced depolarization that was enhanced further after the addition of insulin. The exposure of the muscle to normokalemic $K^+$ solution containing insulin did not reverse the depolarization (Table 1), indicating that this effect was due to insulin action rather than to changes in $K^+$ concentrations. In contrast, the *in vitro* administration of the hormone to normokalemic rats (Table 1) caused a marked hyperpolarization of the muscle fibers (Table 1). In agreement with *in vivo* study (Tricarico *et al.*, 1997a), this effect was antagonized dose-dependently by *in vitro* application of glybenclamide (100 nM to 1 μM).

Macropatch currents of the $K_{ATP}$ channels of skeletal muscle fibers of normokalemic and hypokalemic rats. In FDB and EDL muscle fibers, cell-attached recordings, made by macropatches at potential similar to the resting potential with 150 mM KCl in the bath and in the pipette solutions, revealed inward currents. As expected for currents sustained by $K_{ATP}$ channel, the excision of the patches from the fibers into ATP-free solution increased the currents. The cytosolic MgATP (100 μM to 500 μM), $Na_2ATP$ (100 μM), or AMP-PNP (100 μM) reduced the macropatch currents of hypokalemic and normokalemic rats, indicating that these currents were

sustained by $K_{ATP}$ channels. However, when macropatches were excised from fibers of hypokalemic rats in ATP-free solution, the increase in the current was less pronounced, and in some macropatches, it was not observed. Although the decrease in the $K_{ATP}$ current with hypokalemia was observed in a wide range of voltages (Fig. 1A), this effect was more pronounced at negative membrane potentials. $K_{ATP}$ channels were detected in 55% and 100% of the macropatches excised from the hypokalemic and normokalemic rat fibers, respectively.

In hypokalemic rats, a certain patch to patch variability was observed. This phenomenon was due to the presence of two current populations that differ in their amplitudes (Fig. 2, B, D, and E). The $\mu \pm \sigma$, at $-60$ mV (Vm), calculated by the fitting routine was $-4 \pm 2.1$ pA/$\mu$m$^2$ (54/9 macropatches/rats) and $-11.1 \pm 2.5$ pA/$\mu$m$^2$ (goodness of fit, 1.9) (98/9 macropatches/rats) for the first and second populations, respectively (Fig. 2, B, D, and E). In contrast, only one type of current was found in the normokalemic rat fibers showing $\mu \pm \sigma$ of $-21.5 \pm 3$ pA/$\mu$m$^2$ (goodness of fit, 2) (170/9 macropatches/rats) (Fig. 2, A and C).

No significant differences were observed in the sensitivity of the currents of hypokalemic and normokalemic rats to ATP and glybenclamide. The $IC_{50}$ value of ATP was $28 \pm 5$ $\mu$M (Hill coefficient, 1.1), $30 \pm 5$ $\mu$M (Hill coefficient, 1.2), and $32 \pm 7$ $\mu$M (Hill coefficient, 1.2) for the first and second current populations of hypokalemic rats and for that of normokalemic animals, respectively (Fig. 3A). The $IC_{50}$ value of glybenclamide was $54 \pm 4$ nM (Hill coefficient, 0.9), $56 \pm 5$ nM (Hill coefficient, 0.8), and $63 \pm 9$ nM (Hill coefficient, 0.75) for the first and second current populations of hypokalemic rats and for that of normokalemic rats, respectively (Fig. 3B).

In normokalemic and hypokalemic rats, the internal perfusion of the macropatches with low concentrations of MgADP (50 $\mu$M) only slightly enhanced the current amplitudes living unaltered the distribution of the currents.

**Single $K_{ATP}$ channel currents of skeletal muscle fibers of normokalemic and hypokalemic rats.** At the channel level, at least two open conductance states (O1 and O2) that were blocked by ATP and opened by cromakalim were recorded routinely by micropatches in the hypokalemic rat fibers (Fig. 4B), but only one conductance level blocked by ATP and opened by cromakalim was detected in the normokalemic rat fibers (Fig. 4A).

In hypokalemic rats, the O1 level had a slope conductance of $73 \pm 3$ and $46 \pm 3$ pS in the negative and positive ranges of potentials, respectively (28/6 patches/rats) (Fig. 5B). O2 had a slope of $29 \pm 4$ and $52 \pm 6$ pS in the negative and positive ranges of potentials (7/6 patches/rats) (Fig. 5B). In normokalemic rats, only one level was counted showing slope conductance of $71 \pm 1.4$ and $47 \pm 1.6$ pS (26/6 patches/rats) in the negative and positive range of potentials, respectively (Fig. 5, A and C). The lowering of the internal concentration of $K^+$ ion shifted the reversal potentials of the single-channel currents of hypokalemic and normokalemic rats on the voltage axis from 0 mV to positive values, indicating that the channels under study selected $K^+$ versus $Cl^-$.

Although in most of our recordings the two conductance levels, O1 and O2, seemed to act independently, we also observed simultaneous closures of both levels (Fig. 5D).

The two open conductance states present in hypokalemic rats also showed a reduced channel open probability. The $P_{open}$ measured over the total recording time, at $-60$ mV (Vm), was $0.18 \pm 0.08$ (3 patches) and $0.13 \pm 0.07$ (3 patches) for O1 and O2, respectively, and $0.437 \pm 0.06$ (3 patches) in normokalemic rats.

The duration of the bursts of openings of the two conductance states present in the hypokalemic rats was significantly reduced compared with that of the normokalemic rats. The mean $\pm$ standard deviation of the burst duration was $9.5 \pm 9$ msec, $8.3 \pm 8$ msec for O1 and O2 levels, and $246.5 \pm 21$ msec ($p < 0.001$) for the normokalemic rat channel, respectively. Kinetic analysis performed within the bursts of openings revealed that the mean open time did not differ significantly being $2.31 \pm 1.5$ msec (three patches), $2.22 \pm 1.9$ msec (three patches), and $2.36 \pm 2.1$ msec (three patches) for O1 and O2 levels and for that of the normokalemic rat channel, respectively. The distribution of the open dwell-time of hypokalemic and normokalemic rat channels could be fitted by the sum of two exponential functions (Fig. 6, A, C, and E) (goodness of fit, 1.8–2.05). The $\tau 1$ was $0.311 \pm 0.08$ and $0.321 \pm 0.08$ msec for O1 and O2, respectively, and $0.351 \pm 0.09$ msec for the channel of normokalemic rats, where $\tau 2$ was $2.5 \pm 0.8$ and $2.3 \pm 0.6$ msec for O1 and O2, respectively, and $2.1 \pm 0.9$ msec for the channel of the normokalemic rats. No significant changes were observed in the mean close time of $2.01 \pm 1.7$ msec (three patches), $1.99 \pm 1.8$ msec (three patches), and $2.26 \pm 2.0$ msec (three patches) for O1 and O2 levels and for that of the normokalemic rat channel, respectively. Also, the distribution of the close dwell-time of hypokalemic and of the normokalemic rat channels could be fitted by the sum of two exponential functions (Fig. 6, B, D, and F) (goodness of fit, 1.9–2.1). The $\tau 1$ was $0.211 \pm 0.06$ and $0.198 \pm 0.08$ msec for O1 and O2 levels, respectively, and

TABLE 1

Effects of *in vitro* administration of insulin on resting potentials of skeletal muscle fibers of $K^+$-depleted rats.

Serum $K^+$ concentrations and resting membrane potentials of extensor digitorum longus muscle fibers of normokalemic and hypokalemic rats under control conditions and after *in vitro* incubation of the muscles with insulin (4 units/liter)

Before recording, the preparations (each from a different rat) were incubated with the solutions tested for 30 min. Blood intracardiac samples were collected at the time of death for serum $K^+$ level measurement.

| Experimental condition | Normal Ringer's | Low $K^+$ | Low $K^+$ + insulin | Normal Ringer's + insulin |
|---|---|---|---|---|
| | | | *mV* | |
| Mean of nine normokalemic rat preparations | $-78 \pm 1$ (n = 135) | $-90 \pm 3^a$ (n = 96) | $-87 \pm 2^a$ (n = 85) | $-89 \pm 3^a$ (n = 55) |
| Mean of nine hypokalemic rat preparations | $-61 \pm 1^b$ (n = 221) | $-57 \pm 3$ NS (n = 241) | $-51 \pm 2^c$ (n = 368) | $-52 \pm 3^c$ (n = 101) |

Data are expressed as mean $\pm$ standard error. The numbers of fibers sampled are given in parentheses.

[ab] Significantly different from the corresponding control (normal Ringer's) ($p \leq 0.001$).

[c] Significantly different from the corresponding normokalemic value ($p \leq 0.001$).

0.195 ± 0.09 msec for that of the normokalemic rats channel, whereas $\tau 2$ was 2.4 ± 0.8 and 2.3 ± 0.6 msec for O1 and O2 levels, respectively, and 2.1 ± 0.9 msec for that of the normokalemic rat channels. In our experiments, it was not possible to accurately evaluate the long close times separating the bursts because longer recording periods were needed to collect sufficient data and the channel activity often decreased with time, leading to a considerable patch-to-patch variability (Ashcroft and Ashcroft, 1990).

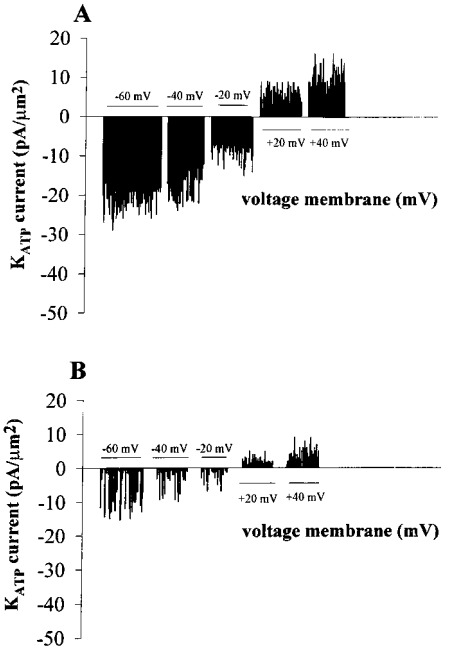


**Fig. 1.** $K_{ATP}$ currents of FDB and EDL muscle fibers of normokalemic and hypokalemic rats at different voltages. *Each bar*, current per patch of normokalemic rats (A) and hypokalemic rats (B). The macropatch currents were recorded after 20 sec from macropatch excision during voltage steps from 0 to −60 mV at 20° in the presence of 150 mM KCl on both sides of the membrane.

Effects of cromakalim on resting potential and $K_{ATP}$ currents of the skeletal muscle fibers of normokalemic and hypokalemic rats. The $K_{ATP}$ currents of hypokalemic rats were activated by cromakalim (10–100 μM). The $K^+$ channel opener enhanced the currents in presence of ATP in the bath with an $ED_{50}$ value of 17 ± 3 μM (6/3 macropatches/rats) and 21 ± 4 μM (5/3 macropatches/rats) for the first and second current populations, respectively (Fig. 7, A and B). In normokalemic rat fibers, cromakalim enhanced the $K_{ATP}$ current but with an $ED_{50}$ of 72 ± 3 μM (12/4 macropatches/rats). In the absence of internal ATP, cromakalim did not affect either type of $K_{ATP}$ currents of the hypokalemic rats or the normokalemic counterparts.

At the macroscopic level, we found that cromakalim (10–100 μM) in normal Ringer's solution repolarized the fibers of hypokalemic rats. This compound also caused a significant repolarization of the fibers previously depolarized by low $K^+$ solution and insulin (Table 2). These effects were antagonized dose-dependently by glybenclamide (60–100 nM). Concentrations of cromakalim lower than 10 μM did not stimulate the current and did not produce any beneficial effect on the resting potential of the hypokalemic rat fibers. No significant hyperpolarization of the fibers of normokalemic rats was observed after incubation of EDL muscles with different concentrations of cromakalim (Table 2).

**Effects of insulin on $K_{ATP}$ currents of the skeletal muscle fibers of normokalemic and hypokalemic rats.**

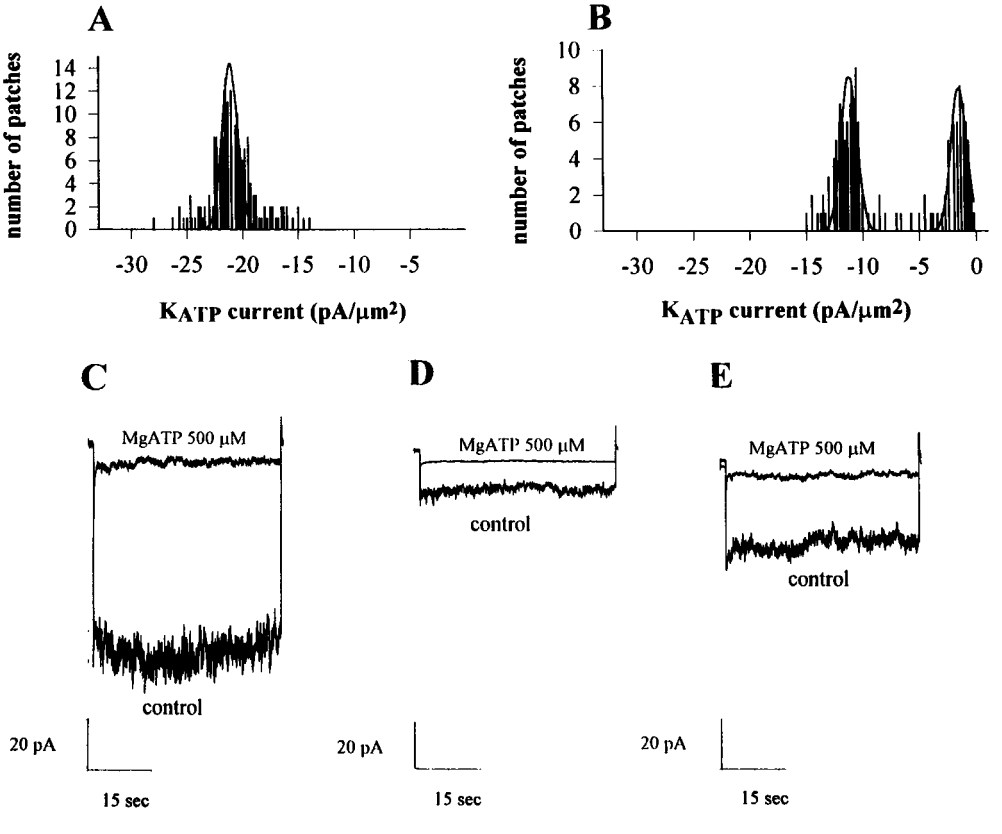


**Fig. 2.** Distribution of $K_{ATP}$ currents in FDB and EDL muscle fibers of normokalemic and hypokalemic rats. Currents were recorded after 20 sec from macropatch excision in presence of 150 mM KCl on both sides of the membrane during voltage steps from 0 to −60 mV (Vm) at 20°. *Downward deflections in the current records*, inward currents. In normokalemic rats (A), the current distribution was fitted by one Gaussian function, indicating the presence of only one current population. In hypokalemic rats (B), the current distribution was fitted by the sum of two Gaussian functions, indicating the presence of two current populations. C, Sample trace of $K_{ATP}$ current of a normokalemic rat. The exposure of the macropatch (pipette area, 5.1 μm²) to MgATP reduced the currents. The background current present in this macropatch was due to leak current + inward rectifier $K^+$ current. D, Sample trace of $K_{ATP}$ current (first population) of an hypokalemic rat. Also, the exposure of the macropatch (pipette area, 5.0 μm²) to MgATP reduced the current, whereas the background current was due to leak current. E, Sample trace of $K_{ATP}$ current (second population) of an hypokalemic rat. The exposure of the macropatch (pipette area, 4.9 μm²) to MgATP reduced the current. The background current present in this macropatch was due to leak current + inward rectifier $K^+$ current.

In hypokalemic rats, the incubation of FDB and EDL muscles with insulin reduced the residual $K_{ATP}$ current. In these fibers, only one population of $K_{ATP}$ current was detected with $\mu \pm \sigma$ calculated by fitting routine of $-3.6 \pm 2$ pA/$\mu m^2$ at $-60$ mV (Vm) (101/17 macropatches/rats) (goodness of fit, 1.89) (Fig. 8B). In contrast, in normokalemic rats, insulin en-

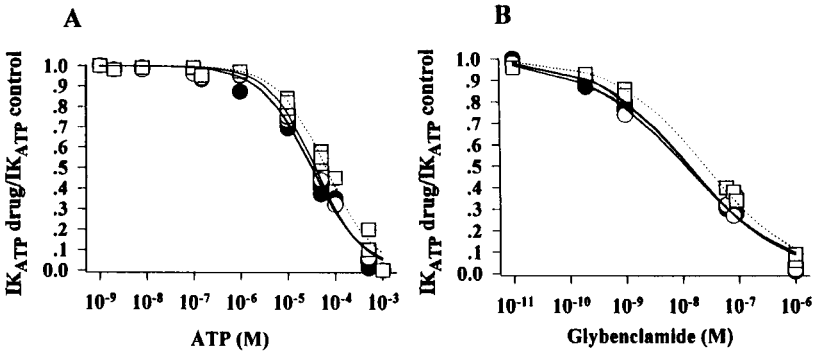


**Fig. 3.** Dose-response curves of $K_{ATP}$ currents in the presence of drug relative to control ($IK_{ATP\ drug}/IK_{ATP\ control}$) of skeletal muscle fibers of normokalemic and hypokalemic rats versus ATP (A) and glybenclamide (B) concentrations. *Dotted lines* (□), responses of the normokalemic rat currents to the blockers. *Continuous lines*, responses of the two components, of lower (●) and higher amplitude (○), of the hypokalemic rat currents to the blockers. The macropatch currents were recorded after 20 sec from macropatch excision during voltage steps from 0 to $-60$ mV at 20° in the presence of 150 mM KCl on both sides of the membrane. The macropatches were exposed to the blockers for 70 sec. The data were pooled from three or four patches.

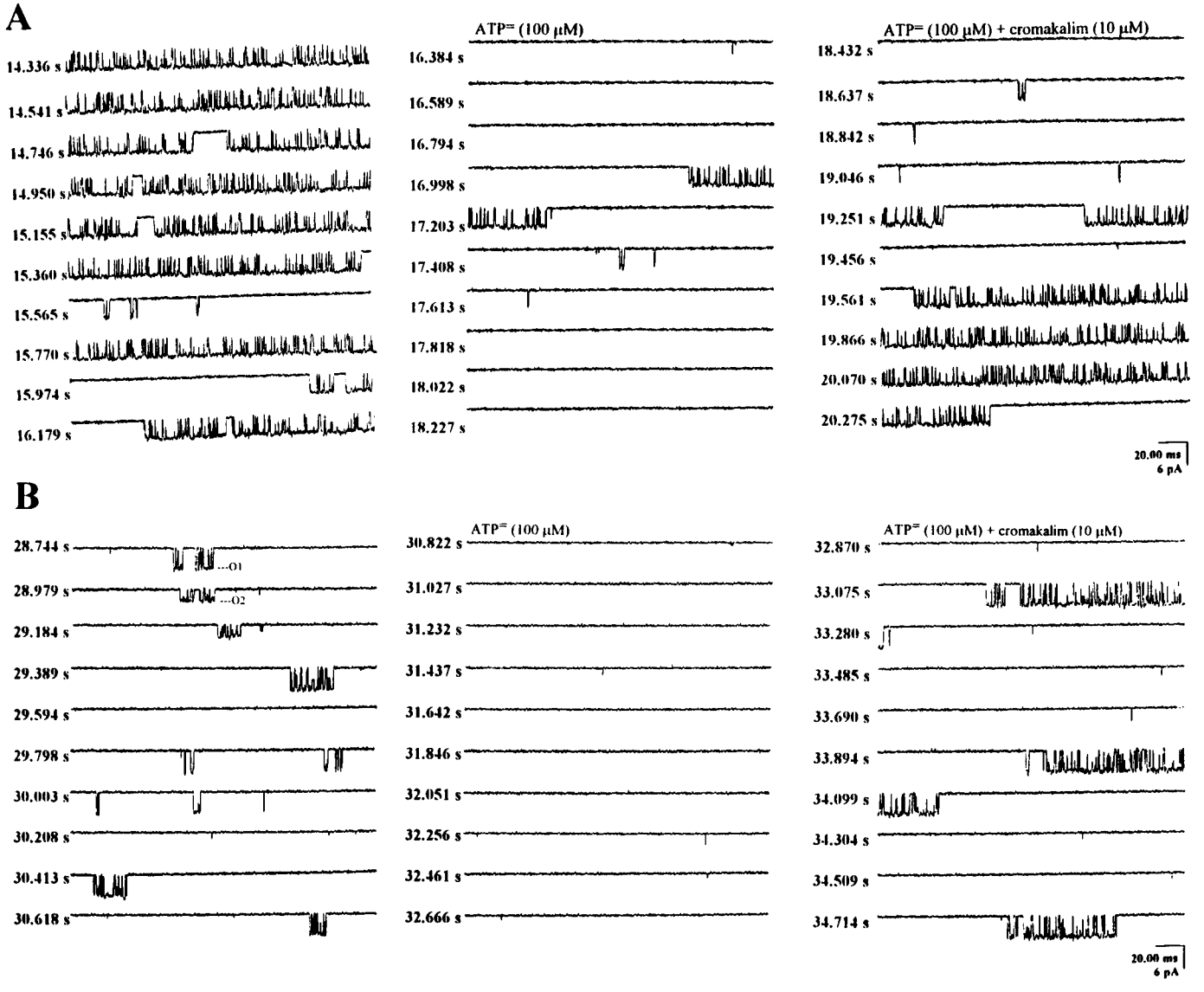


**Fig. 4.** Single $K_{ATP}$ channels of skeletal muscle fibers of normokalemic and hypokalemic rats and effects of ATP and cromakalim. The single-channel transitions were recorded by micropatches (pipette area, $1.1 \pm 0.2$ $\mu m^2$) after 20 sec from excision in the presence of 150 mM KCl on both sides of the membrane patches at $-60$ mV (Vm) at 20°. The cromakalim was tested in presence of $ATP^=$ in the bath. *Downward deflection of the current records*, inward currents. In normokalemic rat (A), the $K_{ATP}$ channel showed a unique open conductance level of $-4.15$ pA and $P_{open}$ of 0.41 that was blocked by $ATP^=$ and opened by cromakalim. In hypokalemic rats (B), two open conductance levels can be distinguished on the basis of their conductance and $P_{open}$. The first (O1) had a current amplitude of $-4.5$ pA and $P_{open}$ of 0.19. The second (O2) had a current amplitude of $-2.01$ pA and $P_{open}$ of 0.12. Both open conductance levels were blocked by $ATP^=$ and opened by cromakalim.

hanced the currents from $-21.5 \pm 3$ pA/$\mu$m$^2$ (170/9 macropatches/rats) to $-29 \pm 5$ pA/$\mu$m$^2$ (139/7 macropatches/rats) (goodness of fit, 2.1) (Fig. 8A).

The incubation of EDL and FDB muscles of hypokalemic rats with insulin did not alter the sensitivity of the current to ATP and glybenclamide in respect to that of the untreated hypokalemic rats; the corresponding $IC_{50}$ values calculated by fitting routine were $37 \pm 7$ $\mu$M and $62 \pm 8$ nM. In normokalemic rats, in agreement with previous reports (Tricarico *et al*., 1997a), ATP and glybenclamide inhibited the $K_{ATP}$ current of muscle fibers preincubated with insulin but with less efficacy; the corresponding $IC_{50}$ values were $380 \pm 10$ $\mu$M and $110 \pm 11$ nM.

## Discussion

**$K_{ATP}$ channel in hypokalemic rats.** We report the presence of an abnormal $K_{ATP}$ channel in the skeletal muscle fibers of the hypokalemic rats. This channel fluctuates between at least two open conductance states that could be distinguished on the basis of their slope conductance (at negative membrane potential) and open probability. None of these states were detected on fibers from EDL and FDB muscles of normokalemic rats. Low conductance channel states may have originated either from substates of a single population of channel or from separate channel populations. The frequent fluctuations observed between the fully open (O1 level) and the low conductance state (O2 level) and the occurrence of direct transitions from a fully open to a closed state crossing the intermediate O2 level (Fig. 5, *arrows*) suggest that the two levels arise from the same pore. The fact that the kinetic parameters of the two levels found in the hypokalemic rats measured within the bursts of openings were not different in respect to that of the normokalemic rat channel supports this view.

Subconductance states in $K_{ATP}$ channels are observed rarely, and the molecular mechanisms that explain the appearance of these states in $K_{ATP}$ channel are not known. Fan *et al*. (1993) and Vivaudou and Forestier (1995) have shown that the lowering of internal pH below 6.5 leads to the appearance of several subconductance states in cardiac and skeletal muscle $K_{ATP}$ channels, respectively. This phenomenon is reverted by rising the internal pH to physiological values (Fan *et al*., 1993). Weik *et al*. (1989) have shown that the internal block by high concentration of adenine produces subconductance states in the skeletal muscle $K_{ATP}$ channel (Weik *et al*., 1989). Both situations imply reversible modifications of the channel pore, such as changes in the surface charge distribution or internal block of the pore. Although it is unlikely that a low internal pH or adenine is responsible for the appearance of subconductance states in our experiments (subconductance states were observed in excised patches on which the effects of endogenous factors can be excluded), it is still possible that a small molecule or ion directly plugs the conduction pathway and, by shuttling in and out rapidly, reduces the unitary current. This is supported by the fact that the subconductance state (O2) disappeared when the patches were depolarized.

From our experience, an homogeneous distribution of $K_{ATP}$ current can be detected in the normokalemic rats of 3–4 months of age. No silent macropatches were detected in these animals. In contrast, in hypokalemic rats of the same age, two populations of currents of lower amplitude were detected. The reduced $K_{ATP}$ currents that we recorded in the fibers of the hypokalemic rats seem to be the result of the lower $P_{open}$ rather than of the two open conductance states, although an additional mechanism is required to explain the presence of the two current populations. Recently, Inagaki *et al*. (1997) and Bryan *et al*. (1997) showed that an optimal molar ratio of the $\alpha$ and $\beta$ monomers that compose the $K_{ATP}$ channel complex of 1:1 is required to have functional $K_{ATP}$ channel in cell line (Clement *et al*., 1997; Inagaki *et al*., 1997). These authors also showed that the coexpression of $(\alpha\beta) + \alpha$

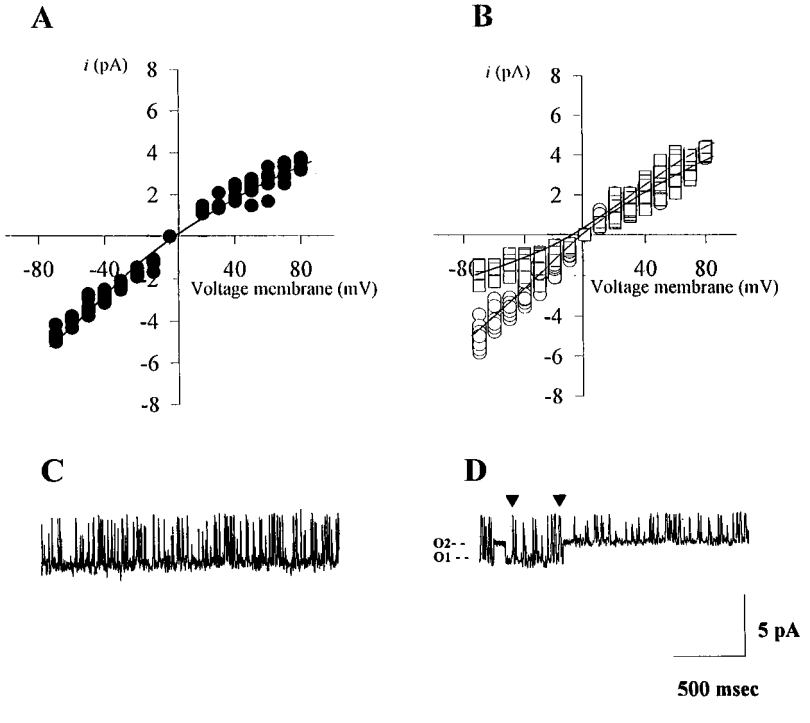


**Fig. 5.** Current-voltage relationships of $K_{ATP}$ channels of skeletal muscle fibers of normokalemic and hypokalemic rats. The single-channel currents were recorded during voltage steps from 0 mV of holding potential to different voltages in inside-out configuration in the presence of 150 mM KCl on both sides of the membrane at 20°. In normokalemic rats (A), only one open conductance level was routinely detected showing a slope conductance of 71 pS (●). Two open conductance levels were observed in the hypokalemic rats (B). One had a slope conductance of 29 pS in the negative range of potential (□). The other had a slope conductance of 73 pS in the negative range of potential (○). C, Transitions from a single $K_{ATP}$ channel of a normokalemic rat. *Downward deflection of the current records*, inward currents. No evident fluctuations between subconductance states are visible in this trace. D, Transitions from a single $K_{ATP}$ channel of an hypokalemic rat. *Downward deflection of the current records*, inward currents. In this trace, frequent fluctuations are visible between the fully open (O1 level) and the low conductance state (O2 level), as are the occurrence of direct transitions from fully open to closed state crossing the intermediate O2 level (*arrows*). This suggest that the two levels came from a common pore.

results in a sort of negative dominant effects of the $\alpha$ monomer on the complex so that a very low $K_{ATP}$ current can be recorded (Inagaki *et al.*, 1997). The coexpression of the $\alpha\beta$ alone elicits a high $K_{ATP}$ current of well known properties. On the basis of these findings, we can hypothesize that in hypokalemic rats an erroneous assembly occurring in same fractions of the membrane of the $\alpha$ and $\beta$ monomers results in a complex responsible for the first current population of lower amplitude.

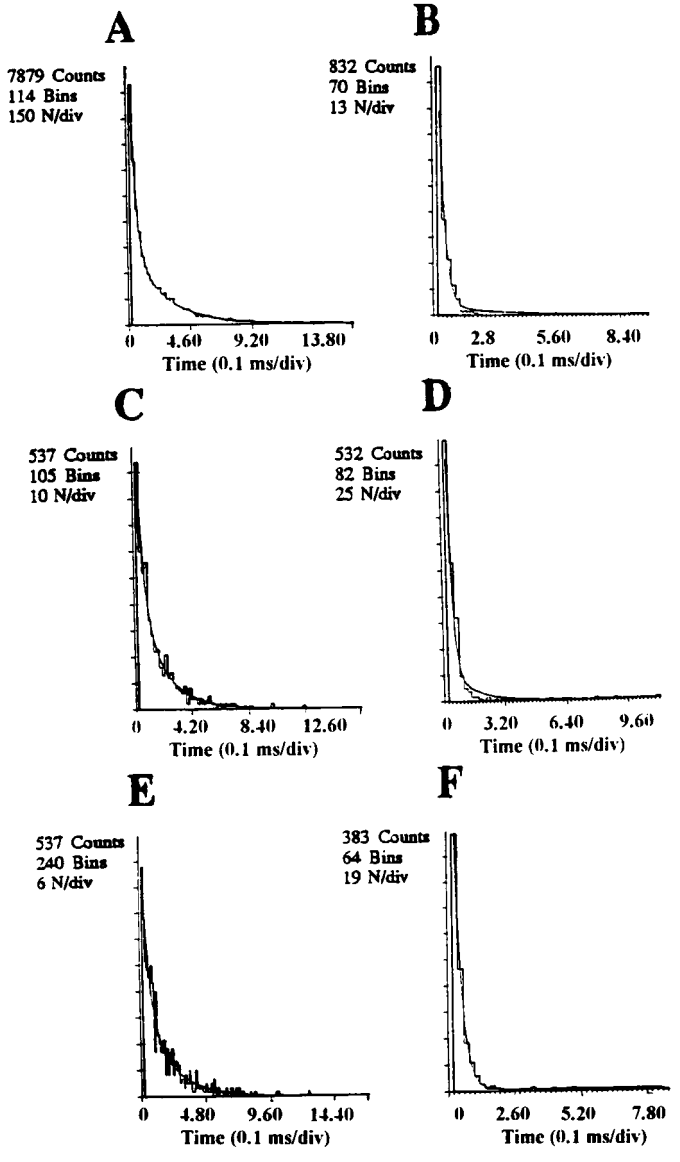


**Fig. 6.** Distribution of open time (A, C, and E) and close time (B, D, and F) for the normokalemic rat $K_{ATP}$ channel (A and B) and for the two conductance levels of hypokalemic rats, O1 (C and D) and O2 (E and F). The recordings were performed in the presence of 150 mM KCl on both sides of the membrane at −60 mV at 20° using a patch in which only single unit was observed. Sampling rate was 20 kHz; filter, 2 kHz. This type of analysis was performed within bursts of openings. The distribution of the open and close dwell-times of hypokalemic and of the normokalemic rat channels could be fitted by the sum of two exponential functions. In these patches, the fitted parameters were normokalemic rat channel, open-time distribution (A) $\tau 1 = 0.27$ msec and $\tau 2 = 2.01$ msec, close-time distribution (B) $\tau 1 = 0.195$ msec and $\tau 2 = 2.3$ msec; hypokalemic rat channel, O1 level, open-time distribution (C) $\tau 1 = 0.29$ msec and $\tau 2 = 2.4$ msec, close-time distribution (D) $\tau 1 = 0.198$ msec and $\tau 2 = 2.1$ msec; O2 level, open-time distribution (E) $\tau 1 = 0.31$ msec and $\tau 2 = 2.1$ msec, close-time distribution (F) $\tau 1 = 0.211$ msec and $\tau 2 = 2.2$ msec.

There was a good correlation between the effects of cromakalim at single-channel and macroscopic levels. This is supported by two findings. First, the concentrations of cromakalim effective on $K_{ATP}$ channels also were effective on the resting potentials of the hypokalemic rat fibers. Second, the repolarization of the hypokalemic rat fibers induced by the $K^+$ channel opener was abolished by nanomolar concentrations of glybenclamide. However, the agonist effects of the compound seemed to be more pronounced on the hypokalemic rat channels than on the normokalemic one, suggesting a state-dependent effect of the drug. The lack of effects of cromakalim on the resting potential of fibers of normokalemic rats was due to the fact that this parameter was close to the equilibrium potential for $K^+$, so there was no favorable electrical gradient allowing an efflux of $K^+$ from the fibers.

**Effects of insulin on resting potentials and on $K_{ATP}$ channels of the skeletal muscle fibers of normokalemic and hypokalemic rats.** The relevance of our findings resides in the fact that insulin exerted opposite electrical effects in the hypokalemic and normokalemic rat fibers at macroscopic and single-channel levels. In normokalemic rats, insulin enhanced the current and induced a glybenclamide-sensitive hyperpolarization of the fibers. In hypokalemic rats, the hormone almost completely abolished the current and strongly depolarized the fibers. The relationship among the $K_{ATP}$ channel, resting potential, and the effects of insulin is consistent with the existence of a functional coupling between $K_{ATP}$ channels and $Na^+/K^+$-ATPase. There is evidence that in epithelial cells (Hurst *et al.*, 1993) and pancreatic $\beta$ cells (Ding *et al.*, 1996), the $K_{ATP}$ channels are functionally coupled to $Na^+/K^+$-ATPase so a stimulation of the pump promotes different effects; for example, it leads to an activation of the outward $K_{ATP}$ current that sustains the hyperpolarization of the cell membrane and controls the duration of the spike burst intervals (Hurst *et al.*, 1993; Ding *et al.*, 1996). An inhibition of the pump down-regulates the $K_{ATP}$ channels (Hurst *et al.*, 1993). In skeletal muscles, the existence of a functional coupling between these two macrocomplexes is supported by the fact that the *in vivo* or *in vitro* administration of insulin to the normokalemic rats caused an hyperpolarization of the fibers that is antagonized by glybenclamide and ouabain (Iannaccone *et al.*, 1989; Bond and Gordon, 1993; Tricarico *et al.*, 1997a). We believe that the functional coupling between $Na^+/K^+$-ATPase and $K_{ATP}$ channels helps to explain the mechanism by which insulin produces depolarization and possibly the paralysis in hypokalemic rats. It is known that insulin hyperpolarizes the skeletal muscle fibers of normokalemic rats by a direct action on the $Na^+/K^+$-ATPase, producing an influx of $K^+$ into the muscle and transient hypokalemia (Iannaccone *et al.*, 1989; Lehmann-Horn *et al.*, 1994). We propose here that in normokalemic rats, the effects of insulin are buffered by an activation of the sarcolemmal $K_{ATP}$ channel that sustains the hyperpolarization of the fibers, whereas in hypokalemic rats, insulin stimulates the influx of $K^+$ into the muscles but no efflux of $K^+$ occurs due to the abnormally low basal activity of the $K_{ATP}$ channels. This precipitates the hypokalemia leading to depolarization of the fibers and possibly to the paralysis. An additional contribution to the depolarization of the fibers came from the inhibitory effects exerted by insulin on the residual $K_{ATP}$ current and from the fact no significant

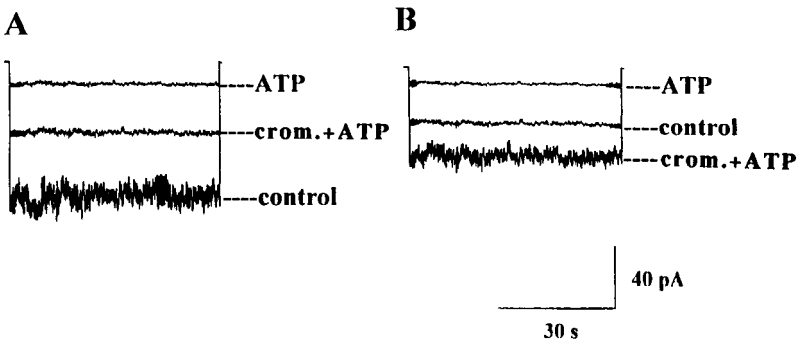


**Fig. 7.** Effects of cromakalim on $K_{ATP}$ current of skeletal muscle fibers of normokalemic and hypokalemic rats (higher current component). Currents were recorded by macropatches (pipette area, 4.1 and 4.0 $\mu m^2$ for normokalemic and hypokalemic rats, respectively) in inside-out configuration after 20 sec from macropatch excision during voltage steps from 0 to −60 mV (Vm) in the presence of 150 mM KCl on both sides of the membrane at 20°. The current was measured in three different experimental conditions: in the absence of ATP and drug (*control*), in the presence of 100 $\mu$M concentration of ATP (*ATP*), or in the presence of 100 $\mu$M concentration of cromakalim and ATP (*Crom.+ATP*). The $K^+$ channel opener enhanced the current in both normokalemic (A) and hypokalemic (B) macropatches but with different efficacies.

change in the sensitivity of the channel to ATP was found after insulin treatment.

**Relationship between the $K^+$-depleted rats and human HOPP.** A point of interest is the relationship between the effects of the hypokalemia tested in the rats and patients with HOPP. In normokalemic solution, the fibers of $K^+$-depleted rats and of humans affected by HOPP are already depolarized, although in patients, a less pronounced depolarization occurs (Rudel *et al*., 1984; Lehmann-Horn *et al*., 1994). In both $K^+$-depleted rats and patients, further depolarization occurs after *in vitro* exposure of the muscles to low $K^+$ solution, insulin, or both (Dengler *et al*., 1979; Lehmann-Horn *et al*., 1994). Muscle fiber depolarization and flaccid paralysis occur in $K^+$-depleted rats and in other secondary forms of the disorder after *in vivo* administration of insulin and glucose (Lehmann-Horn *et al*., 1994; Tricarico *et al*., 1997b). This also represents a useful diagnostic test in humans with HOPP (Lehmann-Horn *et al*., 1994). The fact that insulin causes depolarization and paralysis in the primary and secondary forms of the disorder is consistent with the hypothesis that this phenomenon is not directly related to the mutations of the $Ca^{2+}$ channel detected in humans with HOPP.

The main difference between the $K^+$-depleted rats and patients with HOPP remains the serum $K^+$ level that in the rats, after 18 days of $K^+$-free diet, is constantly below 3.2 mEq/liter ranging (2.2–2.9 mEq/liter), whereas in humans, a decrease to <3.2 mEq/liter during the attacks leads to a transient hypokalemia. However, we should stress that in same patients with HOPP, the hypokalemia can be severe, lasting several days (Lehmann-Horn *et al*., 1994). This often is observed in young male patients (9–18 years of age) in whom the attacks of weakness and paralysis last 6–7 days.

We found in the hypokalemic rats that the abnormalities are limited to muscular defects (abnormal $K_{ATP}$ channel complex, altered insulin pathway involved in the regulation of the channel, or both) rather than changes in the serum insulin and glucose levels. This is supported by the finding

TABLE 2

Effects of cromakalim on the resting membrane potentials of the extensor digitorum longus muscle fibers of normokalemic and hypokalemic rats

In normokalemic rat preparations (each from a different rat), the recordings were performed in normal Ringer's solution in the absence or presence of cromakalim. In hypokalemic rat preparations (each from a different rat), the drug was tested in normal Ringer's solution and in low $K^+$ solution (0.5 mEq/liter) enriched with insulin (4 units/liter). The numbers of fibers sampled are given in parentheses.

| Experimental condition | Normal Ringer's | Low $K^+$ + insulin |
|---|---|---|
| | *mV* | |
| Mean of three normokalemic rat preparations | −77 ± 4 (n = 31) | |
| Cromakalim 10 $\mu$M | −78 ± 2 NS (n = 19) | |
| Cromakalim 100 $\mu$M | −80 ± 4 NS (n = 21) | |
| Mean of four hypokalemic rat preparations | −63 ± 2 (n = 39) | −53 ± 2 (n = 123) |
| Cromakalim 10 $\mu$M | −66 ± 2 NS (n = 68) | −65 ± 2[a] (n = 111) |
| Cromakalim 100 $\mu$M | −71 ± 3[a] (n = 40) | −74 ± 2[a] (n = 129) |

Data are expressed as mean ± standard error.
[a] Significantly different from the corresponding controls ($p \leq 0.001$).

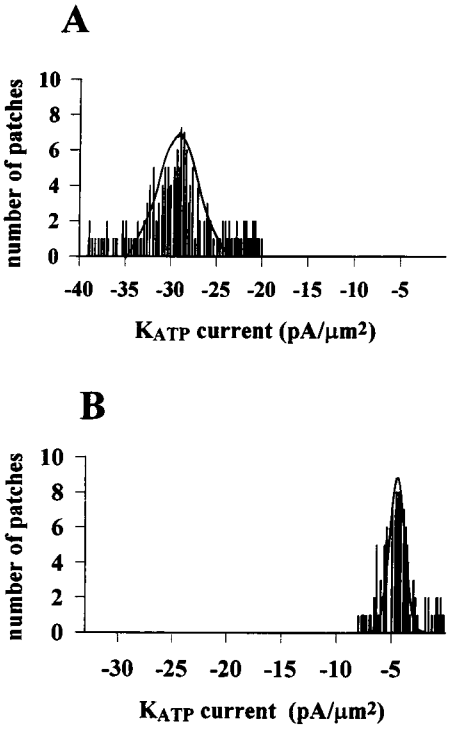


**Fig. 8.** Effects of insulin on the distribution of the $K_{ATP}$ currents of normokalemic and hypokalemic rats. The EDL and FDL muscles of the normokalemic and hypokalemic rats were incubated at 30° under 95% $O_2$/5% $CO_2$ atmosphere with normal Ringer's solution enriched with insulin (4 units/liter) for 30 min. After this time, the muscles were incubated with the enzyme solution for isolation of single fibers. Currents were recorded after 20 sec from macropatches excision in the presence of 150 mM KCl on both sides of the membrane during voltage steps from 0 to −60 mV (Vm), at 20°. In normokalemic rat fibers (A), the pretreatment of the muscles with insulin shifted to the left the distribution on the current axis in respect to that of the untreated muscles. In hypokalemic rat fibers (B), insulin pretreatment led to the disappearance of the current population of higher amplitude.

that no significant differences in the fasting insulinemia and glycemia were found between normokalemic and hypokalemic rats. Similar conclusions have been drawn from studies in humans (Ligtenberg *et al*., 1996). In addition to these observations, we showed that cromakalim, through openings of $K_{ATP}$ channels, repolarized the skeletal muscle fibers of the $K^+$-depleted rats. Similarly, the $K^+$ channel opener is effective in producing a significant repolarization and in increasing *in vitro* the force of contraction of skeletal muscle from humans with HOPP (Spuler *et al*., 1989; Grafe *et al*., 1990).

In conclusion, even with the knowledge that HOPP is linked to mutations of the $\alpha$1 subunit of skeletal muscle $Ca^{2+}$ channel, the lack of sarcolemmal $K_{ATP}$ channel activity helps to explain most of the symptoms of the primary and secondary forms of these disorders.

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