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Different sulfonylurea and ATP sensitivity characterizes the juvenile and the adult form of K_{ATP} channel complex of rat skeletal muscle

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Abstract

We have described here the changes of the biophysical and pharmacological properties of the sarcolemmal ATP-sensitive K^+ channels (K_{ATP}) of rat skeletal muscle fibres, occurring from an early postnatal period (5 days) to adulthood (210 days). The age-dependent changes of the mean current of the K_{ATP} channel (channel activity) and the effects of the blockers, ATP and glybenclamide, were examined by using the patch-clamp technique. Measurements of the single channel conductance, open probability and channel density were also performed. Excision of cell-attached patches into an ATP-free solution dramatically increased the K_{ATP} channel activity; however, the intensity of this activity was age dependent. The relative activity was low at 5–6 days of postnatal life, increased to a plateau at 12–13 days, then declined toward adult values after 37 days. Two distinct types of the K_{ATP} channel complex could be distinguished. The early developmental period (5–6 days) was dominated by a K_{ATP} channel having a conductance of 66 pS, a high open probability of 0.602, and an IC_{50} for ATP and glybenclamide of 123.1 μM and 3.97 μM, respectively. This type of channel disappeared with maturation of the muscle to be replaced by the adult form of the K_{ATP} channel. The later developmental period (from 56 days) was dominated by a K_{ATP} channel having a 71 pS conductance, but a low open probability of 0.222. This adult channel was also 3.2 and 73.5 times more sensitive to ATP and glybenclamide, respectively. We have also observed that the sensitivity of the K_{ATP} channel to ATP and glybenclamide develops differently. Indeed, the greater increase in the sensitivity of the channel to ATP was observed between 5 and 12 days of age. Conversely, the greater enhancement of the sensitivity of the channel to glybenclamide occurred between 12 and 37 days. A further increase of this parameter was also observed between 37 and 56 days of age. The differential age-dependent acquisition of the sensitivity of K_{ATP} channels to ATP and glybenclamide poses the hypothesis that in rat skeletal muscle the ATP regulatory site and sulfonylurea site are located on different subunits of the K_{ATP} channel complex. The intense K_{ATP} channel activity recorded between 12 and 37 days of postnatal life sustains the high resting macroscopic K^+ conductance characteristic of the early postnatal development.

Keywords: K_{ATP} channel; Skeletal muscle; Glybenclamide receptor; Patch clamp

1. Introduction

The ATP-sensitive K^+ channel is the most abundant K^+ channel present in different types of muscle, including skeletal muscle (Ashcroft and Ashcroft, 1990; Davies et al., 1991; Tricarico and Conte Camerino, 1994; Hussain and Wareham, 1994). It has been extensively reported that the activity of the K_{ATP} channel is strictly dependent on the cellular metabolism and on the state of the muscle (Davies et al., 1991; Allard and Lazdunski, 1992; Terzic et al., 1994; Cameron and Baghdady, 1994). Open and close states of the channel are indeed modulated by the redox potential of the cells (Tricarico and Conte Camerino, 1994), by the intracellular ATP and ADP concentrations or by the internal pH (Davies et al., 1991; Allard and Lazdunski, 1992; Vivaudou and Forestier, 1995). Further, in striated muscle the stretch of the sarcolemma (Van Wagoner and Lamorgese, 1994) as well as the selective disruption of actin filaments (Terzic and Kurachi, 1995) enhances the K_{ATP} channel openings even in the presence of internal ATP suggesting that the gating of the channel is related to the contractility function of the muscle.

Very recently, the K_{ATP} channel has been cloned and sequenced from pancreatic β-cells (Inagaki et al., 1995; Sakura et al., 1995). Functional expression studies have revealed that the K_{ATP} channel is composed by an assembly of at least two subunits with unknown stochiometry (Inagaki et al., 1995). One subunit is the receptor for the sulfonylureas (Aguilar-Bryan et al., 1995) which is a class

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of drugs that block the K_{ATP} channels in different tissues (Ashcroft and Ashcroft, 1990), including skeletal muscle (Tricarico and Conte Camerino, 1994). The second represents the conductive subunit strongly selective for K^+ ion that belongs to a subfamily of weak inward rectifier K^+ channels (Ammala et al., 1996). This novel channel showed biophysical properties corresponding to those of the native K_{ATP} channel (Sakura et al., 1995). The mRNA is strongly expressed in different tissues, including skeletal muscle indicating that it can form part of the K_{ATP} channel complex (Sakura et al., 1995). However, the functional relationship existing between the sulfonylurea receptor and the K^+ ionophore is still under debate. Some authors have proposed that the sulfonylurea receptor of pancreatic β-cells confers ATP sensitivity to the ionophore by virtue of its nucleotide binding domains and regulates the channel function (Inagaki et al., 1995). Conversely, some others have shown that sulfonylurea receptor couples to different types of inward rectifier K^+ channels endowing them sulfonylurea sensitivity but not necessarily ATP sensitivity (Ammala et al., 1996).

Our previous experiments have shown that the density, the open probability and the sensitivity to ATP and to glybenclamide of K_{ATP} channels of the adult skeletal muscle fibres undergo profound changes as a result of the aging process over the period from 7 to 24 months (Tricarico and Conte Camerino, 1994; Tricarico et al., 1994). Little, however, is known of the condition of the K_{ATP} channels in the period before the muscle fibre matures, and especially in the period immediately after birth when the fibres are still developing.

Profound modifications of cellular metabolism, gene expression and muscle structure occur during postnatal development (Jones and Rolph, 1985). Some of these changes involve the progressive acquisition by the muscle of the oxidative or glycolytic capacity as well as the coordinated development of the transverse tubular system and sarcoplasmic reticulum (Schiaffino and Margreth, 1969; Jones and Rolph, 1985). The insulin resistance already develops after about 30 days of postnatal life (Gulve et al., 1993). Further reduction of the sensitivity of the skeletal muscle to insulin occurs during aging (Gulve et al., 1993). These modifications are parallel to the changes of the electrical properties of skeletal muscle fibres. Macroscopic recordings have shown that the resting K^+ conductance (g_K) is high in the first days of postnatal life then tends to decrease toward adult values after 30 days of age (Conte Camerino et al., 1989; Gonoi and Hasegawa, 1991). To date, there has also been no satisfactory explanation for the presence of this high g_K in the muscle membrane during the neonatal period. One hypothesis is that there is a single form of the K_{ATP} channel which is present at birth and which undergoes gradual continuous changes during life. An alternate hypothesis is that there exist at least two distinct molecular forms, a juvenile form and an adult form. There is precedence for early and late developmental forms for several channel types. For example, in the case of muscle Na^+ channels the tetrodotoxin-insensitive juvenile form completely disappears shortly after birth to be replaced by the tetrodotoxin-sensitive adult form (Weiss and Horn, 1986; Gonoi et al., 1989). Ca^{2+} currents with different kinetics (transient and sustained types) have been observed in muscle from neonatal mice (Bean and Knudson, 1988). The transient type, which is insensitive to the dihydropyridine class of Ca^{2+} channel inhibitors, disappears during postnatal development, while the sustained current increases (Bean and Knudson, 1988).

To test these ideas we have used the patch-clamp technique to survey the K_{ATP} channels present on the surface membrane of skeletal muscle fibres from 5- to 210-day-old rats. We evaluated the mean currents, as indicator of channel activity, the open probability, the single channel conductance and the density of the channels we observed, and examined the age-dependent effects of the specific blockers, ATP and glybenclamide.

2. Materials and methods

2.1. Muscle fibre preparations

Single muscle fibres were prepared from flexor digitorum brevis muscles of newborn and adult Wistar male rats (Tricarico and Conte Camerino, 1994). Newborn and adult rats were killed by CO_2 atmosphere replacement after which both hind limbs were removed, skinned, and immersed in Ringer solution. The Ringer solution was then enriched with collagenase (3.3 units/mg, type XI-S; Sigma, St. Louis, MO, USA) at concentrations of 0.7–3 mg/ml depending on the muscle mass. The muscles were then incubated for 20–60 min at 30°C under 95% O_2/5% CO_2 atmosphere in a Dubnoff shaking incubator. Only fibres that appeared to be intact after dissociation under high optical magnification ($400\times$) and showing clear sarcomere cross-striations were patched.

2.2. Patch pipettes

Pipettes were pulled from Corning 7052 glass capillary tubing (Garner Glass, Claremont, CA, USA) on an electrode puller (DMZ-Universal Puller, Zeitz-Instrumente, Augsburg, Germany). The electrodes were then coated with Sylgard and fire polished (MF-83 Pipette Microforge, Narishighe, Tokyo, Japan). Measurements of electrode conductance and tip opening area were made on sample pipettes by measuring the direct-current resistance and by scanning electron microscopy (Cambridge Instruments), respectively (Tricarico and Conte Camerino, 1994). Patches having a tip opening area of 6.1 ± 0.4 μm^2 (number of patches = 193) were used to measure the mean current and the pharmacological properties of the K_{ATP} channels, whereas the single channel conductance, the selectivity of

the channel to different ions, the channel open probability and the channel density were measured using micropatches having a tip opening area of 1.1 ± 0.1 μm^2 (number of patches = 165). With the newborn and adult muscle fibre preparations, high-resistance seals of 20–30 GΩ were readily formed by applying a small amount of suction (10 mmHg) to the electrode with a syringe (1-ml capacity).

2.3. Solutions

The solutions had the following compositions: pipette, 150 mM KCl, 2 mM $CaCl_2$, 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.2; bath, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5 mM glucose, 10 mM MOPS, pH 7.2 (normal Ringer); 150 mM KCl, 5 mM, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*-tetraacetic acid (EGTA), 10 mM MOPS, pH 7.2 (symmetrical K^+). Other solutions were prepared by lowering the concentration of the KCl (150 mM) in the bath to 100 mM, 70 mM or 30 mM, or replacing it with an equimolar amount of RbCl, NaCl and CsCl with addition of a small amount of sucrose as needed to obtain a final osmolarity of 298 mOsmol on both sides of the membrane. Stock solutions (5 mM) of the nucleotide tested, Na_2ATP and AMP-PNP (adenylylimidodiphosphate), were prepared by dissolving the chemicals in the bath solution containing 150 mM KCl, while MgATP (100 μM), MgADP (100 μM) and MgUDP (100 μM) were dissolved in the symmetrical solution free of EGTA. Glybenclamide was first dissolved in dimethyl sulfoxide (DMSO) at a concentration of 2 mg/ml and then diluted in the symmetrical solution to obtain a stock concentration of glybenclamide of 0.5 mM. In the range of the glybenclamide concentrations tested, the corresponding DMSO concentrations (2.47×10^{-8}–1.23%) did not mimic the effects of glybenclamide on K_{ATP} channels (solvent control).

2.4. Recordings of mean currents and single channel currents

Experiments were performed in cell-attached and inside-out configurations using the standard single channel patch-clamp technique (Hamill et al., 1981). Continuous recording of the mean current and single channel current was performed under constant voltage, at 20°C, in the presence of 150 mM KCl on both sides of the membrane patches. The current of a single channel was also measured at various potentials (from −70 mV to +70 mV). In the inside-out configuration, before recording, the patches were exposed to 100 μM of MgATP to prevent channel rundown (Findlay and Dunne, 1986). Only patches with no evident channel rundown were selected for the analysis. To distinguish between channel rundown and the effects of the blockers, we have plotted the mean current, measured by macropatches, vs. time using a time slice of 512 ms, under control conditions, during the exposure of the patches to drugs, and during washout. Similar analysis was performed to distinguish between channel rundown and the slow gating mode in the adult rat fibres. However, in this case, we have plotted the $N \cdot P_{open}$, measured in patches having a tip opening area of about 1 μm^2, vs. time using a time slice of 102 ms. Using this type of pipettes no more than 2–3 open channels were observed in the patches of 5–6- and of 56–210-day-old rats. Continuous recording was then started after replacement of MgATP solution with the usual ATP-free solution or with bath solution enriched with the test compounds.

2.5. Hardware and software

Isolated skeletal muscle fibres were patch clamped in an RC-13 recording chamber (Warner instrument, Hamden, CT, USA). The channel current was recorded using an Axopatch 1D amplifier equipped with a CV-4 headstage. Currents were sampled at 94.6 kHz at 14-bit resolution by a pulse code modulation interface (Instrutech VR-10B), filtered at 2 kHz (4-pole Bessel low-pass filter, −3 dB) and then stored on a videotape for later analysis. The data points were then sampled at 20 kHz by a TL-1 interface (Axon Instruments, Foster City, CA, USA) and stored on the hard disk of a 80486/66 personal computer. The data acquisition hardware was driven by the Fetchex data acquisition program (pClamp software package, Axon Instruments). Data were analyzed using pClamp version 5.5.1 and our own software (Tricarico and Conte Camerino, 1994).

2.6. Analysis of the mean current

The mean current flowing through the patch, $I = (N \cdot P_{open} \cdot i)$ where N is the number of functional channel, P_{open} is the open probability and i is the single channel current, was calculated subtracting the baseline level of the current defined as closed state of the channels from the open channel level, sampling the data points every 512 ms for 4 min of time recording and then averaging them (Horie and Irisawa, 1989).

The dose-response relationships of I of the K_{ATP} channels vs. ATP and glybenclamide concentrations were constructed at a membrane potential of −60 mV, at 20°C, in the presence of 150 mM KCl at both sides of the membrane.

Before recordings, the macropatches were exposed to the blockers for about 60 s.

The analysis of the mean current was performed only when the patches containing K_{ATP} channels were not contaminated by inward rectifier K^+ channels or other types of K^+ channels such as calcium activated K^+ channels (Tricarico and Conte Camerino, 1994).

2.7. Single channel analysis

The channel current was measured at various potentials (from −70 mV to +70 mV), using the cursor method

provided by the program Fetchan (pClamp software package). The single channel conductance (γ) was calculated as the slope of the voltage-current relationship for the channel in the range of potentials from -70 mV to -10 mV. No correction for liquid junction potential was made, estimated to be less than $+2$ mV in our experimental conditions (Barry and Lynch, 1991).

The P_{open} was measured as ratio between the time spent by the channel in open state over the total time of recordings. Own software was developed to measure the time spent by the channel in open state when multiple channels of identical amplitude were present in the patches (Tricarico and Conte Camerino, 1994). The P_{open} was then calculated fitting the experimental data to a binomial distribution. If the experimental data matched the theoretical data the P_{open} was obtained as one of the parameters of the fit. Other parameters of the fit were the number of functional channels (N) and the time spent by the channel in open state.

The N was evaluated by a direct counting of the simultaneous openings, at membrane potentials of -60 mV and of $+40$ mV, observed for a period of time of 4 min. We found that at these voltages, the P_{open} of the juvenile and adult forms of the channel reaches the maximum values. This procedure was performed after the exposure of the patches to MgADP (100 μM) and MgUDP (100 μM) that activates the run-down channels (Hussain and Wareham, 1994). The value of N, derived from the direct counting of the channels, was then compared with that calculated by binomial distribution.

The $N \cdot P_{open}$ was measured for 4 min of time recording. Before this time, we did not observe decay in the $N \cdot P_{open}$ in the adult rat fibres.

2.8. Statistics

The data are expressed as mean ± standard error. The frequency of finding channels in the patch was calculated according to the following expression:

$$\text{Freq.} = \frac{\text{number of active patches}}{\text{number of total patches}} \cdot 100$$

where Freq. is the frequency of finding K_{ATP} channel in the patches; n. active patches is the number of patches containing K_{ATP} channels; n. total patches is the total number of patches performed.

The permeability ratio, P_{X^+}/P_{K^+}, has been calculated using the following bi-ionic expression (Hille, 1992):

$$\frac{P_{X^+}}{P_{K^+}} = \frac{[K^+]_o}{[X^+]_i} \exp \frac{E_{rev}\, zF}{RT}$$

where E_{rev} is the observed reversal potential of the single channel current in presence of different ions into the bath; R, T, z and F are the gas constant, the absolute temperature, the valence of the ion and the Faraday constant, respectively; $[K^+]_o$ is the external concentration of K^+ ion, whereas $[X^+]_i$ indicates the internal concentration of tested ions.

To fit the curves of I versus ATP and glybenclamide concentrations, a Marquardt, nonlinear, least-squares fitting routine was used based on the following equation:

$$\frac{I \text{ blocker}}{I \text{ control}} = \frac{\text{Max} - \text{Min}}{1 + \left(\frac{[\text{blocker}]}{IC_{50}}\right)^n}$$

The fitted parameters were: n, the slope of the curves; IC_{50}, the concentrations of the blockers required to produce a 50% decrease of I; Min, is the minimum normalized value of I; whereas Max, the maximum normalized values of I, was constrained to 1. The goodness of fit was calculated as described in Press et al. (1986). The significance of IC_{50} values of the blockers was calculated by comparing the 95% confidence intervals of the IC_{50} according to the Litchfield-Wilcoxon test (Tallarida and Murray, 1986). The IC_{50} values were considered significantly different when the 95% confidence intervals did not overlap. Significant differences between individual pairs of means were determined by Student t-test.

3. Results

3.1. K_{ATP} channels of newborn and adult rat skeletal muscle fibres

3.1.1. Recordings of mean currents

In flexor digitorum brevis muscle fibres of newborn and adult rats, cell-attached recordings made at -60 mV (ΔV_m) with 150 mM KCl in the bath and in the pipette solutions, revealed inward currents flowing through multiple channels. The reversal potential of the channel current was near 0 mV as predicted for current carried by K^+ ion. When macropatches were excised in inside-out configuration in ATP-free solution, an increase of K^+ channel activity was observed whose amplitude varied with the age of the animal from whom the muscle fibre was obtained (Fig. 1A,B). The application of 100 μM of MgATP, Na_2ATP and AMP-PNP, the non-hydrolizable analogue of ATP, to the cytoplasmic face of the channels drastically reduced their mean current, indicating that the K^+ channel activity was sustained by K_{ATP} channels. The frequency of finding channels in the patches, calculated as described in Section 2, also changed with development; indeed, K_{ATP} channels were present in 40% at 5–6 days, 70%, 91% and 90% at 12–13, 20 and 35–37 days, respectively, and in 60%, 52% and 55% of the inside-out patches at 56, 80–86 and 210 days, respectively.

A visual inspection of the current traces over a 4 min period revealed that the quantity of the channel current recorded from different macropatches showed a biphasic

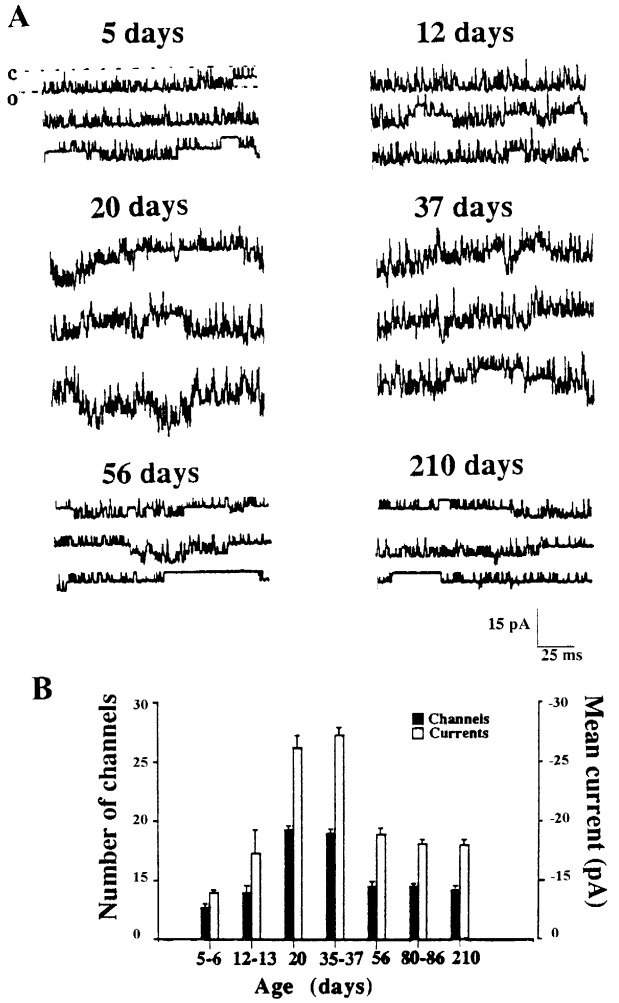

Fig. 1. K_{ATP} channel activity of skeletal muscle fibres of rats during postnatal development. (A) Traces of channel activity recorded at 20°C in the presence of 150 mM KCl on both sides of the membrane from inside-out patches held at −60 mV (membrane potential) (sampling rate, 20 kHz; filter, 2 kHz). Downward deflection on the current records indicates open state of the channels. The mean current/μm^2 of the patch from a 5-day-old rat was -9.1 ± 0.3 pA, while it was -16 ± 0.8 pA, -25 ± 3 pA, and -26 ± 4 pA in the patches from 12-, 20-, and 37-day-old rats, respectively. The patches from 56-, 80-, and 210-day-old rats showed a mean current/μm^2 of -18 ± 4 pA, -16 ± 3 pA and -17 ± 3 pA, respectively. (B) Age-dependent changes of the mean current/μm^2 of the macropatches and the number of functional K_{ATP} channels/μm^2 present in the micropatches are compared. A minimum of 7 and a maximum of 41 patches were performed to measure the number of functional channels, while a minimum of 9 and a maximum of 26 macropatches were performed to measure the mean current of the K_{ATP} channels. The data are expressed as mean ± standard error.

distribution which corresponded with the age of the animals (Fig. 1A); the *I* flowing through the patches were low at 5–6 days of age, then increased at 12–13 days reaching a plateau value between 20–37 days (Fig. 1B). After this time, the K_{ATP} channel activity declined toward the adult values (Fig. 1A,B). The number of functional channels present in the patches parallels the changes of K_{ATP} channel activity, indicating that the intensity of *I* is mainly a function of the local channel density (Fig. 1B).

The analysis of the long-term (30 min) gating properties of the channels revealed that the *I* recorded at 5 days of age did not show rundown. Conversely, from 12 days of age, a time-dependent decline of *I* was observed 8–10 min after patch excision. After rundown, the exposure of the macropatches to MgUDP and MgADP completely restored the *I* at all ages under study.

3.1.2. Recordings of single channel currents

To evaluate the possible presence of different forms of K_{ATP} channel in skeletal muscle of developing rats, we formed patches using micropipettes having an average tip opening area of 1.1 ± 0.1 μm^2 (see Section 2). However, due to the high number of channels per unit area present in the fibres from 12- and 37-day-old rats, even using these small pipettes we could not isolate single unit or few channels in the patches. As opposite, single channel transitions were clearly visible at 5–6, 56 and 210 days of age. We therefore compared the properties of the 5–6-day-old rat channel with those of the 56- and 210-day-old rat channels. The direct inspection of the current traces showed that the amplitude of the single channel currents measured at −60 mV (V_m) in the presence of 150 mM KCl on both sides of the membrane increases with development being -3.7 ± 0.2 pA, -4.2 ± 0.4 pA and -4.3 ± 0.5 pA, respectively, for the K_{ATP} channels of 5–6-, 56- and 210-day-old rats. The corresponding slope conductances, calculated by the current-voltage relationships were, respectively, 66.2 ± 1 pS (n. patches/n. rats = 20/4), 71.5 ± 2 pS (n. patches/n. rats = 19/4) and 71.3 ± 1pS (n. patches/n. rats = 41/4) (Fig. 2). Student *t*-test revealed

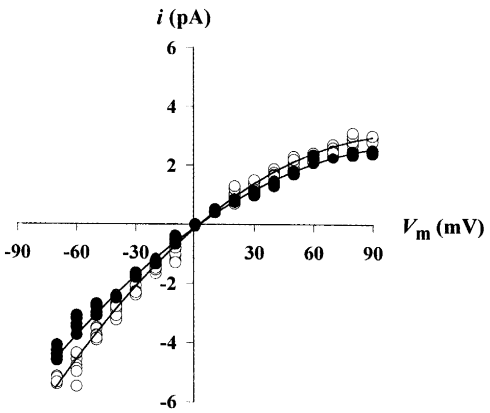

Fig. 2. Current–voltage relationships of the K_{ATP} channels of skeletal muscle fibres of 5–6- and 56-day-old rats. The channel currents were measured in inside-out configuration at different voltages at 20°C. The current–voltage (I/V) relationships of the K_{ATP} channels were constructed in the presence of 150 mM KCl on both sides of the membrane. The corresponding slope conductances calculated between −10 mV and −70 mV (V_m) were 66 pS for the 5–6-day-old (continuous line, ●) and 71 pS for the 56-day-old (dotted line, ○) rat channels, respectively. The data were pooled from a minimum of 19 and a maximum of 41 patches.

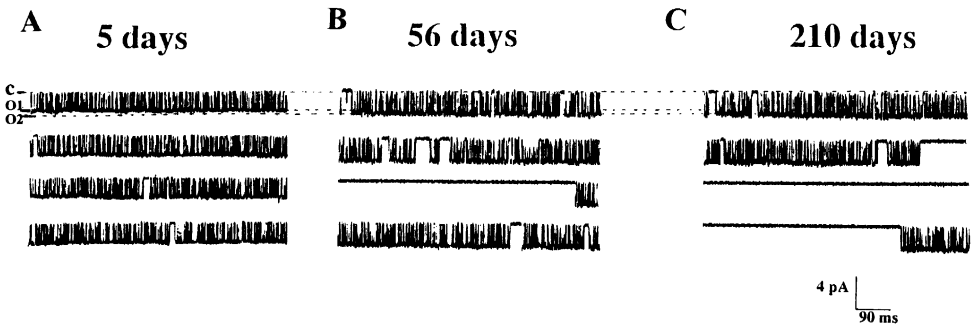

Fig. 3. Single channel transitions of the K_{ATP} channels of skeletal muscle fibres of 5-, 56-, and 210-day-old rats. Short segments (3.5 s) of channel activity recorded at 20°C from inside-out patches held at −60 mV (membrane potential) are shown (sampling rate, 20 kHz; filter 2 kHz). Downward deflection on the current records indicates open state of the channels. The fluctuations from single units were recorded using micropipettes having an average tip opening area of 0.8 ± 0.04 μm^2 (number of micropatches = 3). (A) Typical fast transitions between open (O1) and close (c) conductance levels from a K_{ATP} channel of a 5-day-old rat. At this age, the K_{ATP} channel does not show the slow gating mode. In this patch, the P_{open} of the channel was 0.612. (B) Typical transitions between open (O2) and close (c) conductance levels from a K_{ATP} channel of a 56-day-old rat. At this age, the K_{ATP} channel shows both fast and slow gating modes. In this patch, the P_{open} was 0.223. (C) Typical transitions between open (O2) and close (c) conductance levels from a K_{ATP} channel of a 210-day-old rat. As observed for the K_{ATP} channel of a 56-day-old rat, the K_{ATP} channel of a 210-day-old rat also shows fast and slow gating modes. In this patch, the P_{open} was 0.232.

that the conductance values of the 56- and 210-day-old rat channels are significantly different from that of the 5–6-day-old rat channel. The K_{ATP} channels of 5–6-, 56- and 210-day-old rats showed weak inward rectification properties (Fig. 2).

Open probability analysis performed in the range of potentials from −60 mV to +40 mV revealed that the P_{open} of the channels detected at 5–6 days of age was about 3 times higher than that measured at 56 and 210 days of age (Fig. 3A,B,C). The P_{open}, at −60 mV (V_m),

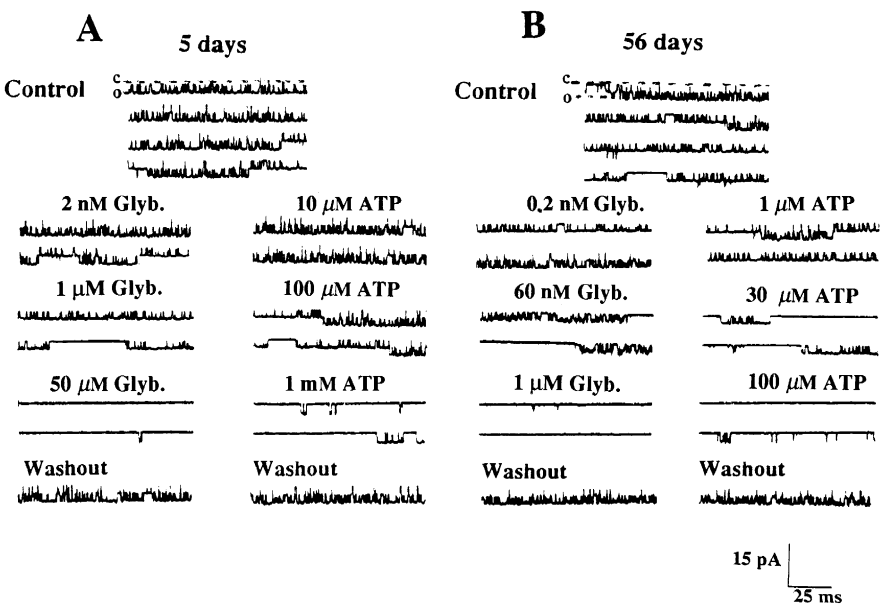

Fig. 4. Current–voltage relationships of the K_{ATP} channels of skeletal muscle fibres of 5–6- and 56-day-old rats. The channel currents were measured in inside-out configuration at different voltages at 20°C. (A) Current–voltage (I/V) relationships of 5-day-old rat K_{ATP} channel in the presence of 150 mM KCl on both sides of the membrane and after application of decreasing concentrations of K^+ ion to the bath from 150 mM (continuous line, ●) to 100 mM (continuous line, ■), 70 mM (continuous line, ▲) and 30 mM (continuous line, ▼). (B) Current–voltage (I/V) relationships of 56-day-old rat K_{ATP} channel in the presence of 150 mM KCl on both sides of the membrane and after application of decreasing concentrations of K^+ ion to the bath from 150 mM (dotted line, ○), to 100 mM (dotted line, □), 70 mM (dotted line, △) and 30 mM (dotted line, ▽). This procedure shifted the I/V curves of the currents of 5- and 56-day-old rat channels on the voltage axis to the right indicating that both channel types selected K^+ against Cl^- ion. (C) I/V relationships of 5-day-old rat K_{ATP} channel after replacement of internal K^+ ion (continuous line, ●) with an equimolar concentration of Rb^+ (dotted line, ■), Na^+ (dotted line, ▲) or Cs^+ (dotted line, ▼) ions. (D) I/V relationships of 56-day-old rat K_{ATP} channel after replacement of internal K^+ ion (continuous line, ○) with an equimolar concentration of Rb^+ (dotted line, □), Na^+ (dotted line, △) or Cs^+ (dotted line, ▽) ions. The reversal potentials of the currents of the 5- and 56-day-old rat channels recorded in the presence of Rb^+ ion in the bath were shifted to the right indicating that both channel types were highly selective for K^+ ion over Rb^+ ion. No outward current was recorded in the presence of Na^+ and Cs^+ ions in the bath. The data were pooled from a minimum of 2 to a maximum of 5 patches.

was 0.602 ± 0.04 (n. patches/n. rats = 20/4), 0.222 ± 0.03 (n. patches/n. rats = 19/4) and 0.201 ± 0.06 (n. patches/n. rats = 41/4), respectively, at 5–6, 56 and 210 days of age. This finding indicates that the K_{ATP} channels present in the 5–6-day-old rat muscles are mainly in the open state (Fig. 3A) as compared to the 56- and 210-day-old rat channels that instead show bursts of openings interrupted by long gaps of closures (Fig. 3B,C). No change with time was observed in the $N \cdot P_{open}$ of 56- and 210-day-old rat channels indicating that the long gaps of closures interrupted by burst of openings characteristic of the adult rat channels were due to the slow gating mode of the channel rather than to a fast rundown.

We also observed that the K_{ATP} channels of 5–6-, 56- and 210-day-old rats gate independently. This was done by comparison of the theoretical and experimental values of the fractions of time spent by the channel in the open state, calculated either by the binomial distribution or by our own program. The two methods were in agreement.

The different gating behaviour and the different single channel conductance showed by the channel of 5–6-day-old rats compared to the adult rat channel led us to believe that a different form of K_{ATP} channel was present in the early stage of postnatal development. To better characterize the channel types present in developing rat muscles we compared the selectivity properties of K_{ATP} channels to various ions at 5–6 days with those at 56 days. For this, we replaced the 150 mM KCl of the bath solution with lower concentrations of KCl or with solutions containing Rb^+, Na^+ or Cs^+ ions (150 mM) (see Section 2). As expected for a K^+-selective channel, the lowering of the internal concentration of K^+ ion shifted the reversal potentials of the currents on the voltage axis from 0 mV to positive values (Fig. 4A,B). When the internal K^+ ion was replaced with Rb^+ ion, the reversal potentials of the currents were $+32 \pm 3$ mV and $+26 \pm 4$ mV, respectively, giving a permeability ratio (see Section 2) Rb^+/K^+ of 0.28 and of 0.35 for 5–6- and 56-day-old rat channels, respectively (Fig. 4C,D), which were not significantly different. Moreover, the outward currents carried by Rb^+ ion were much smaller than those carried by K^+ ion (Fig. 4C,D). These findings indicate that Rb^+ ion may enter both types of K_{ATP} channels from the internal mouth of the pore, but traverses the channel much more slowly than K^+ ion does. No outward currents were detected in the presence of internal Na^+ or Cs^+ ions, suggesting that these ions do not

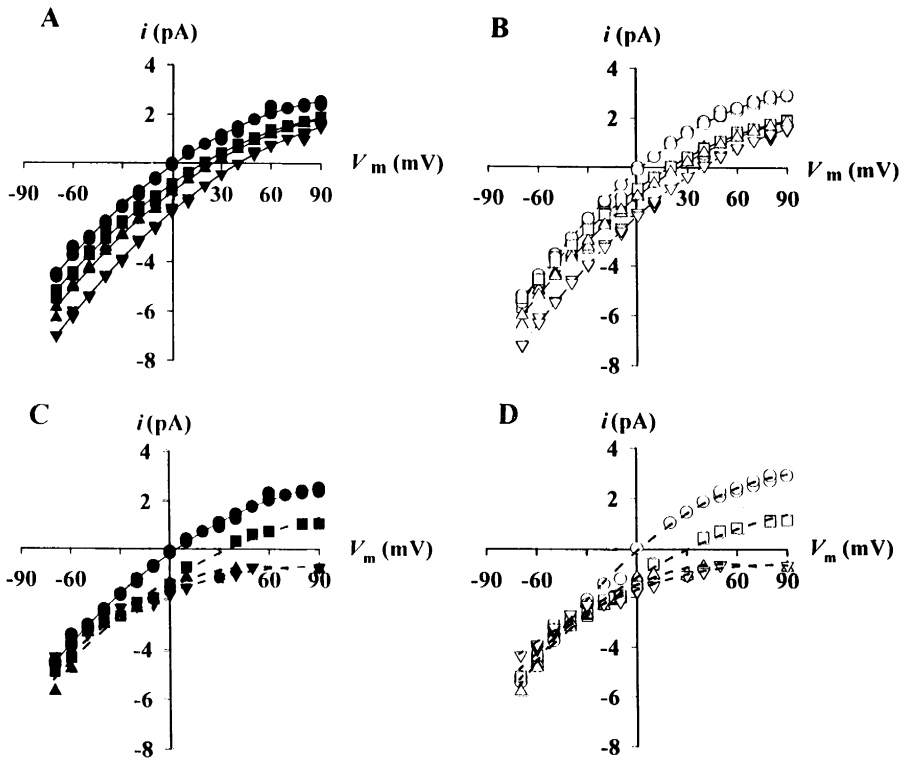

Fig. 5. Effects of ATP and glybenclamide on K_{ATP} channel activity of skeletal muscle fibres of 5- and 56-day-old rats. The channel activity was recorded at 20°C in the presence of 150 mM KCl on both sides of the membrane from inside-out patches held at −60 mV (membrane potential) (sampling rate, 20 kHz; filter, 2 kHz). Downward deflection on the current records indicates open state of the channels. (A) In this particular patch of a 5-day-old rat the threshold concentrations of glybenclamide and ATP were 2 nM and 10 μM, respectively; while a 50% decrease of channel activity was achieved with 1 μM and 100 μM of glybenclamide and ATP, respectively. The maximal inhibitory responses were reached with 50 μM and 1 mM of glybenclamide and ATP, respectively. (B) In this particular patch of a 56-day-old rat the threshold concentrations of glybenclamide and ATP were 0.2 nM and 1 μM, respectively, while a 50% decrease of channel activity was achieved with 60 nM and 30 μM of glybenclamide and ATP, respectively. The maximal inhibitory responses were reached with 1 μM and 100 μM of glybenclamide and ATP, respectively. However, at both ages, high concentrations of ATP and glybenclamide did not completely shut the K_{ATP} channels. Washout of the compounds restored the channel activity in both 5- and 56-day-old rats.

permeate either types of K_{ATP} channels (Fig. 4C,D). Therefore, the following permeability sequence for both types of K_{ATP} channels can be proposed: $K^+ > Rb^+ \gg Na^+ \geq Cs^+$, which is not different from permeability sequences previously reported (Quayle et al., 1988; Davies et al., 1991).

3.2. Effects of ATP and glybenclamide on K_{ATP} channels of newborn and adult rat skeletal muscle fibres

In order to establish if differences in the pharmacological profile of the K_{ATP} channels exist during development, we tested the effects of ATP and glybenclamide on *I* flowing through multiple K_{ATP} channels contained in a large patch area. The application of different concentrations of both glybenclamide and ATP to the cytoplasmic face of the channels dose and age dependently reduced the *I* of the K_{ATP} channels (Fig. 5); indeed, during development, we observed a leftward shift of the dose–response relationships of both blockers versus *I* on the log-dose axis.

The corresponding IC_{50} values calculated by the non-linear least squares fitting routine (see Section 2) were progressively decreased by development (Table 1). However, the sensitivity of the channel to ATP and glybenclamide develops differently. The largest leftward shift of the dose-response relationship for ATP was observed between 5 and 12 days of age; during this time the sensitivity of the channel to ATP increased by a factor of 2.5. After 12 days, no further change in this parameter was revealed. Conversely, the largest leftward shift of the dose-response relationship for glybenclamide was observed between 12 and 37 days of age; during this time the sensitivity of the K_{ATP} channel to the sulfonylurea increased by a factor of 6.2. A further leftward shift of the dose-response relationship for glybenclamide occurred in the period from 37 and 56 days of age; during this time the sensitivity of the channel to the blocker increased by a factor of 3.1. After 56 days, no further change in this parameter was revealed. The inhibitory effects exerted by glybenclamide and ATP were almost completely reversible at all ages under study (Fig. 5). In both developing and adult rats, the two blockers even at high concentration did not completely shut the channels. No significant change was observed in the slope of the dose-response curves of either blockers (Table 1) suggesting that the stoichiometry of the reaction was not modified by the postnatal development. Neither the nucleotide nor the sulfonylurea significantly affected the single channel current at different ages.

4. Discussion

4.1. There are two forms of K_{ATP} channel

Two forms of K_{ATP} channel showing different biophysical and pharmacological properties are present in rat skeletal muscle fibres. One form of K_{ATP} channel was exclusively found at 5 and 6 days of age being the only K_{ATP} channel type detectable at this time. It represents the 'juvenile' channel type having a higher P_{open} and lower single channel conductance in respect to the adult form. This juvenile channel was also less sensitive to the blockers showing an IC_{50} for ATP and glybenclamide of 123.1 μM and 3.97 μM, respectively. This type of channel disappeared with maturation of the muscle. A K^+ channel resembling the adult K_{ATP} channel but lacking the ATP sensitivity has been reported in a myoblast cell line (Fan and Makielski, 1995). These authors suggested that the K_{ATP} channel acquires ATP sensitivity during the first days of postnatal life (Fan and Makielski, 1995). In contrast, other types of K^+ channels present in muscle culture and myotubes show similar properties to those described in the adult muscle (Ohmori et al., 1981; Trautmann et al., 1986; Matsuda and Stanfield, 1989). The second form of K_{ATP} channel was detected at 56 days of age and later, and it represents the 'adult' channel type having a lower P_{open} and higher single channel conductance in respect to the

Table 1
Pharmacological properties of the K_{ATP} channels of skeletal muscle fibres of newborn and adult rats

Parameters	Age (days)				
	5–6	12–13	35–37	56	210
IC_{50} (μM) of ATP	123.1 ± 7	49.5 ± 3.0	42.7 ± 4.0	38.0 ± 5.0	36.3 ± 3.0
Slope	0.93 ± 0.03	1.20 ± 0.3	1.25 ± 0.4	1.22 ± 0.6	1.30 ± 0.5
IC_{50} (μM) of glybenclamide	3.97 ± 0.6	1.05 ± 0.7	0.170 ± 0.02	0.054 ± 0.8	0.0654 ± 0.001
Slope	0.91 ± 0.04	1.10 ± 0.4	1.000 ± 0.6	0.970 ± 0.03	0.99 ± 0.05

The IC_{50} and the slopes of the dose-response curves for ATP and glybenclamide vs. the mean currents of the K_{ATP} channels were calculated by a fitting routine (see Section 2). The IC_{50} for ATP calculated at 12–13 days was significantly different from that at 5–6 days of age; the corresponding 95% confidence intervals were 34–63 μM and 108–149 μM. The IC_{50} for ATP at the other ages were significantly different from that of 5–6 days, but not different among those. The 95% confidence intervals were: 28–53 μM, 23–54 μM and 22–51 μM for 35–37, 56 and 210 days, respectively. The IC_{50} for glybenclamide calculated at 12–13 days was not significantly different from that calculated at 5–6 days of age; the corresponding 95% confidence intervals were 0.5–1.6 μM and 0.97–6.97 μM. The IC_{50} for glybenclamide at 35–37 days was significantly different from that at 5–6 days and 12–13 days showing a 95% confidence interval of 0.08–0.4 μM. No significant differences were observed between the IC_{50} for glybenclamide at 56 and 210 days in respect to that at 35–36 days, while significant differences were found in respect to that at 5–6 days of age. The 95% confidence intervals were: 0.01–0.1 μM and 0.01–0.15 μM at 56 and 210 days of age, respectively.

juvenile form. This adult channel was also more sensitive to the blockers showing an IC_{50} for ATP and glybenclamide of 38 μM and 54 nM, respectively. All the macropatches and micropatches excised from the fibres of 56-day-old rats contained exclusively the adult form of the channel.

In spite of the significantly lower single channel conductance of the juvenile channel in respect to the adult one, the selectivity to different ions of both types of channels did not differ significantly. This finding may reflect an age-dependent expression of different types of the ionophore selective for K^+ ion that couples to sulfonylurea receptor (Ammala et al., 1996).

4.2. Effects of ATP and glybenclamide

We previously showed that the application of either ATP or glybenclamide on the cytosolic face of adult rat skeletal muscle K_{ATP} channels dose dependently reduces the channel activity (Tricarico and Conte Camerino, 1994). We have now shown that the effects of these specific blockers are also age dependent. The fact that the patches excised from the 5–6-day rat fibres were sensitive to both glybenclamide and ATP indicates that at that age the sulfonylurea receptor already interacts with the K^+ ionophore to form a K_{ATP} channel complex. However, the pharmacological profile of K_{ATP} channel to ATP and glybenclamide develops differently. The largest leftward shift of the dose-response relationships for ATP was observed between 5 and 12 days of postnatal life. After 12 days of age, the sensitivity of the channel to ATP did not change significantly suggesting that at this time the channel has already acquired an adult-like sensitivity to ATP.

Differently, the K_{ATP} channel acquires an adult-like sensitivity to glybenclamide later. The largest leftward shift of the dose-response relationship to this blocker was observed between 12 and 37 days of age; a further increase in the sensitivity to the sulfonylurea occurred between 37 and 56 days. After 56 days of age, the sensitivity of the channel to glybenclamide did not change significantly indicating that the complex composed by the K_{ATP} channel and by the sulfonylurea receptor reaches the adult-like behaviour at this age. Our previous experiments have shown that the sensitivity of K_{ATP} channel to glybenclamide increases even further as the rats age (Tricarico and Conte Camerino, 1994). Therefore, it seems feasible to conclude that there appear to be either three stages in the efficacy of the sulfonylurea receptor or a continuous increase of the sensitivity of the channel with age.

On the basis of our findings two main hypothesies can be proposed: first, apart from the presence of two distinct types of K_{ATP} channel complex which are present, respectively, at 5–6 days and 56 days of age, in the period from 12 and 37 days of age the complex can also exist in intermediate states which are difficult to investigate from a biophysical point of view. Indeed, this period of time is characterized by the presence of a high number of channels per unit area which do not allow to isolate the single unit in the patches even using a micropatch having a tip opening area less than 1 μm^2. Second, in line with the expression cloning study of Ashcroft and co-workers (Sakura et al., 1995; Ammala et al., 1996), our results suggest that the ATP and glybenclamide binding sites of K_{ATP} channel of rat skeletal muscle fibres are located on different subunits affecting the channel function. Our conclusion is supported by the fact that also in the skeletal muscle fibres of aged rats the sensitivity of K_{ATP} channel to ATP and glybenclamide is differently modified (Tricarico and Conte Camerino, 1994; Tricarico et al., 1994). Further, other factors may also contribute to the observed differential changes in the sensitivity of the K_{ATP} channel complex to ATP and glybenclamide during development. For example, an age-dependent expression of different sulfonylurea receptors or a differential regulation by phosphorylation during development may alter the response of the K_{ATP} channel complex to the blockers. It has been recently shown that skeletal muscle expresses a new isoform of the sulfonylurea receptor which appears to be a result of different genes (Inagaki et al., 1996). This isoform contains also several phosphorylation sites for cAMP-dependent protein kinase A and protein kinase C-dependent sites (Inagaki et al., 1996).

4.3. A putative function of the K_{ATP} channel during postnatal development

Our experiments confirm that the K_{ATP} channel is the most common K^+ channel present on the surface membrane of both developing and adult rat skeletal muscle fibres (Tricarico and Conte Camerino, 1994). We now report that the K_{ATP} channel activity increases in the first days of postnatal life and tends to decline toward adult values with maturation of the muscle. This behaviour parallels the resting g_K of rat and mouse skeletal muscle fibres, suggesting that K_{ATP} channels produce the high g_K detected during the neonatal life (Conte Camerino et al., 1989; Gonoi and Hasegawa, 1991). A high g_K capable of sensing changes in cellular metabolism may have an important role during the first days of postnatal development in which the oxygen demand of the muscle is particularly intense due to different factors such as the rapid increase in the oxidative capacity of the muscle or to the progressive acquisition of the contractility function (Schiaffino and Margreth, 1969; Jones and Rolph, 1985; Davies et al., 1991). Therefore, we propose here that the local rise of external concentration of K^+ ion, possibly mediated by K_{ATP} channels, assures the oxygen supply to the developing muscle in particular during muscle contraction (Medbo and Sejersted, 1990; Davies et al., 1991). It is known that the rise in plasma K^+ level in the appropriate range (≥ 6 mM) depolarizes and activates the carotid body chemoreceptors leading to the increase of the muscle ventilation (Band and Linton, 1986; Burger et al., 1988).

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