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ATP-Sensitive K^+ Channels of Skeletal Muscle Fibers from Young Adult and Aged Rats: Possible Involvement of Thiol-Dependent Redox Mechanisms in the Age-Related Modifications of Their Biophysical and Pharmacological Properties

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SUMMARY

In the present work, we have investigated whether thiol-dependent redox mechanisms play a role in the regulation of ATP-sensitive K^+ (K_{ATP}) channels present on the surface membrane of skeletal muscle fibers from 5–7-month-old ("young adult") and 24–26-month-old ("aged") rats. The K_{ATP} channels were surveyed by using patch-clamp techniques. Continuous recordings of channel activity were performed in the inside-out configuration at a constant voltage at 20°, in the presence of 150 mM KCl on both sides of the membrane. As expected, the excision of cell-attached patches from young adult rat fibers, into ATP-free solution, dramatically increased K_{ATP} channel activity. In contrast, when patches were excised from aged rat fibers no increase of channel activity was detected. Open probability (P_{open}) analysis in the range of potentials from −70 mV to +60 mV revealed that the P_{open} of the channels of aged rat fibers was about 7.5 times lower than that of young adult rat fibers. Moreover, a decrease in the number of functional channels present in the patches of aged rat fibers was also observed. No change with aging was found in the single-channel conductance, which was 60 pS. The application of increasing concentrations of the sulfhydryl group-reducing agents L-cysteine (5 μM to 5 mM) and *N*-acetyl-L-cysteine (0.5–5 mM) restored the P_{open} of the channels of aged rat fibers without increasing the number of functional channels. Thimerosal, a sulfhydryl group-oxidizing agent, and glybenclamide applied to the cytoplasmic face of K_{ATP} channels from fibers of either young adult or aged rats dramatically abolished channel openings. However, the K_{ATP} channels of aged rat fibers were 30–200 times more sensitive to the inhibitory effects of these chemicals. In both young adult and aged rat fibers the effect of thimerosal was reversed only by addition of L-cysteine. In contrast, the effect of glybenclamide was fully reversible. Moreover, after preincubation of aged rat channels with 1 mM L-cysteine, the blocking effect of glybenclamide was reduced and was similar to that observed in young adult rat fibers. These observations lead us to conclude that, in rat skeletal muscle, the K_{ATP} channel proteins contain thiol groups essential for channel activity. Oxidation of these groups occurs during aging and prolonged channel closure. This modification may explain the altered pharmacological response to both thimerosal and glybenclamide observed in aged rat skeletal muscle fibers.

K_{ATP} channels are the most abundant K^+ channels in some tissues. They were first observed by Noma (1) in the membrane of heart muscle fibers. Subsequently, they were also detected in a wide variety of nonexcitable and excitable tissues, such as vascular smooth muscle (2), insulin-secreting cells (3), cortical rat brain (4), and sarcolemma blebs of human (5) and frog skeletal muscle (6), as well as on the surface membrane of young adult mouse skeletal muscle fibers (7). In the last few years, the role of K_{ATP} channels in various pathophysiological situations has become more clear (8). In particular, in mammalian and amphibian skeletal muscles K_{ATP} channels open in response to various metabolic insults; in ischemic and reperfused rat skeletal muscle the lowering of intracellular ATP levels causes an increase of resting K^+ conductance mainly due to an increase of glybenclamide-sensitive K^+ conductance (9). The observed increase of K_{ATP} channel conductance is believed to be responsible also for shortening of the action potential and for the reduced excitability of the ischemic and reperfused fibers (9). In exhausted skeletal muscle as well as during severe muscle exercise, a decrease of intracellular pH concomitant with an increase of ADP concentration greatly sustains the activation of K_{ATP} channels, thus producing hyperpolarization and a decrease in muscle contraction and saving the residual intracellular ATP (10–12). More recently it has been hypothesized that K_{ATP} channels are involved in the pathogenesis of myopathy

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ABBREVIATIONS: K_{ATP} channel, ATP-sensitive K^+ channel; P_{open}, open probability; *N*, number of functional channels present in the patches; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DMSO, dimethylsulfoxide.

such as hypokalemic periodic paralysis (13). Several reports indicate that the molecular mechanisms underlying the activation of K_{ATP} channels in skeletal muscle appear to be complex, involving binding of nucleotide diphosphates and a G protein-dependent process (14, 15). Recent studies suggest that closure of K_{ATP} channels of mouse pancreatic β-cells (16) and skeletal muscle fibers (7) can be induced by sulfhydryl group-oxidizing agents or thiol group inhibitors such as thimerosal or *N*-ethylmaleimide. This finding suggests that the channel protein may contain thiol groups, which sense changes in the metabolism and in the redox potential of the cells (16). Profound alterations of the metabolism and redox potential of the cells have been found during aging processes (17–19). These phenomena have been related to an age-dependent increase of oxygen free radicals, which have been proposed as one of the multiple causes of aging (17–19). Oxygen free radicals may react with various thiol-containing proteins, including channel proteins, producing a switch of the thiol groups from the reduced to the oxidized form (19).

Although many reports support the important roles of K_{ATP} channels in muscle physiology and pathology, at present not much is known about their protein structure or the molecular regulation of their activity under pathophysiological conditions such as aging. Single-channel recordings have shown that the P_{open} and the density of K_{ATP} channels of rat skeletal muscle fibers are reduced by aging (20), whereas the sensitivity of the same channels to ATP is slightly increased. Indeed, the EC_{50} values of ATP needed to reduce the P_{open} of K_{ATP} channels are 28 μM and 20 μM for young adult and aged rat fibers, respectively (20). Preliminary experiments also indicated that the sulfhydryl group-reducing agent L-cysteine may have some effect on the P_{open} of K_{ATP} channels of aged rats, increasing it (20). In the present work, by using patch-clamp techniques, we have investigated the presence of critical thiol groups on K_{ATP} channels of skeletal muscle and their possible involvement in the modulation of channel activity in 5–7-month-old ("young adult") and, in particular, in 24–26-month-old ("aged") rat skeletal muscle fibers. With this aim we have tested the effects of the sulfhydryl group-oxidizing agent thimerosal and the sulfhydryl group-reducing agents L-cysteine and *N*-acetyl-L-cysteine on K_{ATP} channels of young adult and aged rat fibers. Differences in the inhibitory effects of glybenclamide on the P_{open} of K_{ATP} channels of young adult and aged rat fibers have also been evaluated. Indeed, the molecular basis of the interaction of the sulfonylureas with their receptor is still an open question, although one of the hypotheses proposed involves an interaction of the compounds with a membrane protein that is associated with K_{ATP} channels and contains critical thiol residues (16, 21). The results we present here support the presence of thiol groups on K_{ATP} channels of rat skeletal muscle fibers. We also show that the alteration of the P_{open} of the channel during the aging process could be due to changes in the oxidation state of the thiol groups of the channel protein. This modification may lead to altered pharmacological sensitivity of K_{ATP} channels to glybenclamide.

Materials and Methods

Muscle fiber preparations. Single muscle fibers were prepared from flexor digitorum brevis muscles of the hind feet of young adult and aged Wistar Kyoto male rats, using a modification of the method of Bekoff and Betz (22). In particular, young adult and aged rats were sacrificed by CO_2 atmosphere replacement, after which both hind limbs were removed, skinned, and immersed in Ringer solution. The feet were then pinned, dorsal side up, in a Sylgard-coated dish filled with Ringer solution. The Ringer solution in the dish was then replaced with 2 ml of enzyme solution containing 3 mg/ml collagenase (3.3 units/mg, type XI-S; Sigma Chemical Co., St., Louis, MO) dissolved in Ringer solution. The muscle was incubated for 1.5–2 hr at 30° under a 95% 0_2/5% CO_2 atmosphere, in a Dubnoff shaking incubator. Most of the fibers dissociated from young adult and aged rat muscles appeared to be intact. These cells were washed several times with fresh Ringer solution and transferred to a Ringer solution-filled recording chamber. The average lengths of the fibers, measured with a high-power (400×) Zeiss (Axiovert 10) inverted microscope, were 901 ± 31 μm (n = 79 fibers) and 1100 ± 112 μm (n = 67 fibers) for young adult and aged rats, respectively. The diameters of the same fibers were also measured and were 49 ± 7 μm (n = 79 fibers) and 33 ± 9 μm (n = 67 fibers) for young adult and aged rat fibers, respectively. Only fibers with clearly visible sarcomere cross-striations were patched.

Patch pipette fabrications. Patch pipettes were pulled from Corning 7052 glass (Garner Glass Co., Claremont, CA), in two stages, on a patch electrode puller (DMZ universal puller; Zeitz Instruments, Augsburg, Germany). The electrodes were then coated with Sylgard to within 50–150 μm of the pipette tip and fire polished (MF-83 pipette microforge; Narishighe, Tokyo, Japan). The tip opening area of the pipettes was measured by scanning electron microscopy (Cambridge Instruments). Measurements of membrane resistance and tip opening area were performed with the same pipettes according to the method of Sakmann and Neher (23). A inverse relationship between tip opening area and pipette resistance has been found for the range of resistances from 0.21 MΩ to 5 MΩ (coefficient correlation, 0.9). The slope of the straight line was −1.8 μm^2/MΩ. The micropipettes used to form patches on young adult rat fibers, with a tip opening area of 4.3 ± 0.2 μm^2, showed direct-current resistances of 3.41 ± 0.3 MΩ (n = 61 pipettes) at 20° when filled with our usual pipette solution. With this type of micropipette, we never observed <3 simultaneously open channels in the patches excised from young adult rat fibers. Patches containing multiple channels were always observed, even using micropipettes having a tip opening area of about 0.5 μm^2. Therefore, in young adult rat fibers, the effects of channel blockers were tested on patches containing multiple K_{ATP} channels. In contrast, in aged rat fibers, using the same type of pipettes, no more than 1 open channel/patch area was found. In light of these observations, several patches were formed on aged rat fibers using macropipettes showing a resistance of 2.04 ± 0.4 MΩ (n = 66 pipettes) and a tip opening area of 6.7 ± 0.3 μm^2. The use of macropipettes increased the N value in the patches, thus providing the possibility to test the effects of blockers on fibers having a low channel density and reduced channel P_{open}, such as aged rat fibers.

With both young adult and aged rat fibers, high-resistance seals of 20–60 GΩ were readily formed by pressing the patch pipettes lightly against the membrane, releasing the positive pressure in the pipettes, and applying a small amount of suction (15 mm of Hg) to the electrode with a syringe (1-ml capacity). Before recording, the patches excised from either young adult and aged rat muscle fibers were exposed to 100 μM MgATP for several seconds, to prevent channel rundown (24). Moreover, we have plotted the $N \cdot P_{open}$ versus time, using a time slice of 512 msec, under control conditions, during the exposure of the patches to drugs, and during washout. This analysis allowed us to follow the long term gating properties of the channels and to distinguish between channel rundown and the pharmacological effects of drugs. Continuous recording was then started after replacement of MgATP solution with the usual ATP-free bath solution or with bath solution enriched with the test compounds. All of the dose-response relationships for the activators and blockers of K_{ATP} channels tested here were constructed at a membrane potential of −60 mV. Furthermore, the effects of the same compounds were also tested with a wide range of potentials (from −70 mV to +60 mV).

Solutions. The pipette solution contained 150 mM KCl, 2 mM $CaCl_2$,

and 10 mM MOPS, pH 7.2. The bath solution contained 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5 mM glucose, and 10 mM MOPS, pH 7.2 ("normal Ringer"); 150 mM KCl, 5 mM EGTA, and 10 mM MOPS, pH 7.2 ("symmetrical potassium"); or 400 mM KCl, 5 mM EGTA, and 10 mM MOPS, pH 7.2 ("high potassium").

Stock solutions of the compounds tested were prepared by dissolving the chemicals in symmetrical potassium solution or in pipette solution. Na_2ATP (5 mM), L-cysteine (5 mM), and *N*-acetyl-L-cysteine (5 mM) (Sigma) were dissolved in symmetrical potassium solution, whereas MgATP (5 mM) was dissolved in symmetrical potassium solution free of EGTA. Thimerosal (5 mM) was dissolved in pipette solution or in symmetrical potassium solution. Glybenclamide (Sigma) was first dissolved in DMSO at a concentration of 2 mg/ml, which corresponds to the maximal solubility of the drug in this solvent. This solution was then diluted in symmetrical potassium solution to obtain a final glybenclamide concentration of 0.5 mM (DMSO concentration, 12.3%). Microliter amounts of the stock solutions of the chemicals were then added to symmetrical potassium or pipette solutions as needed. In the range of concentrations tested, DMSO (2.47×10^{-8} to 1.23%) did not mimic the effect of glybenclamide on the P_{open} of K_{ATP} channels from young adult or aged rat fibers. However, we could not test glybenclamide at concentrations higher than 50 μM (DMSO concentration, 1.23%), because DMSO at concentrations higher than 3% affected the channel properties.

Hardware and software. Isolated skeletal muscle fibers were patch clamped in an RC-13 recording chamber (bath volume, 0.7 ml; Warner instrument Corp., Hamden, CT), which allows complete exchange of bath solution in <10 sec. Single-channel activity was recorded using an Axopatch 1D patch amplifier with a CV-4 headstage. Single-channel currents were recorded under constant voltage at 20° in the presence of 150 mM KCl on both sides of the membrane patches. Currents were filtered at 2 kHz (four-pole Bessel low-pass filter, −3 dB), sampled at 20 kHz at 12-bit resolution, and stored on the hard disk of a 80386/33 personal computer. The data acquisition hardware (TL-1 interface; Axon Instruments, Foster City, CA) was driven by the Fetchex data acquisition program (pClamp software package; Axon Instruments). Data were analyzed using pClamp and our own software. Experiments were performed using standard single-channel patch-clamp technique (25). In the cell-attached configuration, the bath contained symmetrical potassium solution. Inside-out patches were rapidly formed by withdrawing the patch electrode from the cell while in the cell-attached configuration (25). Direct inspection of the recorded current records was used to measure the single-channel current at various potentials, using the cursor method provided by the program Fetchan (pClamp software package). The single-channel conductance was then calculated as the slope of the voltage-current relationship for the channel.

***P*$_{open}$ analysis.** Software was developed by Prof. S. H. Bryant and Dr. R. Wagner (Department of Pharmacology and Cell Biophysics, University of Cincinnati, OH) to analyze Fetchex data files containing multiple channels or subconductance levels of identical amplitude. This software determines the fraction of an "episode" spent at every conductance level for each episode in the file and outputs these data to a tab-delimited ASCII file for further analysis. Channel (or sublevel) independence and the *N* value (or the number of subconductance states) in the record can be determined by summing the fraction of time spent at each conductance level and fitting the data to an appropriate binomial distribution. If the observed fraction of time spent by the channels is not significantly different from that calculated by binomial distribution, the channels gate independently, and the P_{open} of a single channel (or subconductance state) is obtained as one of the parameters of the fit. Further insight into the gating process may be obtained by examining graphs of the output data in various forms. Details about the program are available directly from the authors.

Statistics. The data are expressed as mean ± standard error. The normalized values of P_{open} and $N \cdot P_{open}$ were determined by dividing the P_{open} and $N \cdot P_{open}$ values obtained in the presence of the test compounds by the same parameters measured in control solution. The standard error estimates for the normalized values of the aforementioned parameters were obtained as described by Green and Margerison (26). To fit the curves of normalized P_{open} values versus glybeclamide concentrations, a Marquardt-type, nonlinear, least-squares fitting routine was used. The curves were drawn according to the equation

$$P_{open} = \frac{\text{Max} - \text{Min}}{1 + \left(\frac{[\text{drug}]}{EC_{50}}\right)^n}$$

The fitted parameters were the slopes of the curves (n), the concentrations of glybenclamide required to produce a 50% decrease of P_{open} (EC_{50}), and the minimum normalized values of P_{open} (Min), whereas the maximum normalized values of P_{open} (Max) were constrained to 1. The goodness of fit was calculated from the minimum χ^2 values for each curve and the number of degrees of freedom and was considered acceptable when larger than 0.001 (27). The significance of the EC_{50} values of glybenclamide were calculated by comparing the 95% confidence intervals of the EC_{50} values according to the Litchfield-Wilcoxon test (28).

Results

K_{ATP} channels of young adult and aged rat skeletal muscle fibers. In flexor digitorum brevis muscle fibers of young adult and aged rats, cell-attached recordings made at +60-mV pipette potential with 150 mM KCl in the pipette and 150 mM KCl and 5 mM EGTA in the bath (symmetrical potassium) revealed inward currents flowing through single channels. Rare and brief openings of about −4 pA were observed. The reversal potential of single-channel current was near 0 mV, as predicted for currents carried by K^+ ions. As expected for K_{ATP} channels, the excision of cell-attached patches from fibers of 5–7-month-old rats, into ATP-free solution (inside-out configuration), dramatically increased channel activity (Fig. 1A). In contrast, when patches were excised from fibers of aged rats, no increase of channel activity was detected (Fig. 1B). In patches excised from both young adult and aged rat fibers, increasing the concentration of K^+ in the bath solution from 150 mM to 400 mM shifted the reversal potential of the current flowing through the channels from 0 mV to 22 ± 3 mV (n = 16 patches) and 23 ± 3 mV (n = 13 patches) for aged and young adult rat channels, respectively, confirming that the channels under study selected K^+ against Cl^- ions. The application of different concentrations of ATP to the cytoplasmic face of patches from fibers of both young adult and aged rats reduced the P_{open} of the channels, indicating that they were indeed ATP sensitive. With normal pipettes (see Materials and Methods), a minimum of 3 and a maximum of 6 simultaneously open channels were observed in the fibers from young adult rats, giving an average N value of 3.9 ± 0.7 (n = 61 patches) in an area of 4.3 ± 0.2 μm^2 (see Materials and Methods). In contrast, no more than 1 open channel was observed in the fibers from aged rats even during prolonged recording times (3 min) (n = 16 patches). However, the use of macropipettes improved the average N value in the patches isolated from aged rat fibers, increasing it to 2.8 ± 0.8 (n = 66 patches) in an area of 6.7 ± 0.3 μm^2 (see Materials and Methods). No significant difference in the single-channel conductance was observed between young adult and aged rat channels. The amplitudes of the currents flowing through K_{ATP} channels at −60 mV (membrane potential) were −3.9 ± 0.2 pA (n = 61 channels) in the young adult rat fibers and −3.8 ± 0.1 pA (n =

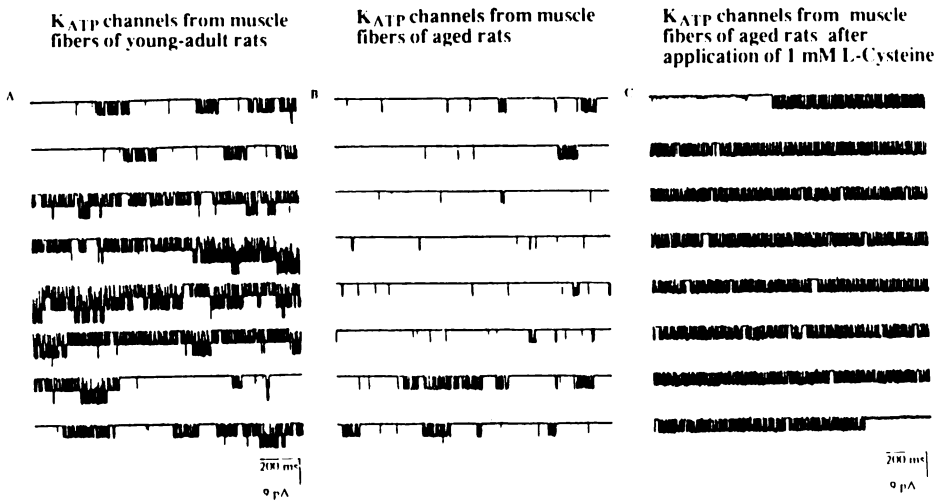

Fig. 1. Effects of the aging process on the P_{open} of K_{ATP} channels and on the *N* value in patches excised from muscle fibers. Short segments (1.3 sec) of channel activity recorded at 20° from inside-out patches held at −60 mV (membrane potential) are shown (sampling rate, 20 kHz; filter, 2 kHz). The direction of ionic currents follows the standard convention; downward deflections on the current records indicate the movement of positively charged ions from the extracellular side of the membrane to the intracellular side (inward current). A, Typical activity of K_{ATP} channels from muscle fibers of a young adult rat. At least 3 channels were present in the patch. In this particular patch the P_{open} value was 0.198. The tip opening area of the pipette used was 3.8 μm^2. B, Typical activity of K_{ATP} channels from muscle fibers of an aged rat. The P_{open} value was 0.0341. No more than 1 open channel was seen during 290 sec of recording. The tip opening area of the pipette used was 3.9 μm^2. C, Typical activity of K_{ATP} channels from muscle fibers of an aged rat (same patch as in B) after application of 1 mM L-cysteine to the cytoplasmic face of the channel. The P_{open} value was 0.098. Only 1 open channel was seen during 230 sec of recording. The tip opening area of the pipette used was 3.9 μm^2.

66 channels) in the aged rat fibers. The current-voltage relationships of the channels from both young adult and aged rat fibers showed a weak inward rectification. In symmetrical potassium solution, the slope conductances were 60.0 ± 1 pS (n = 61 channels) and 60.3 ± 2 pS (n = 66 channels) for channels from young adult and aged rat fibers, respectively. P_{open} analysis in the range of potentials from −70 mV to + 60 mV revealed that the P_{open} of the channels from aged rat fibers was about 7.5 times lower than the P_{open} of channels from young adult rat fibers. The P_{open} values measured at −60 mV (membrane potential) were 0.231 ± 0.01 (n = 61 patches) and 0.031 ± 0.02 (n = 66 patches) for young adult and aged rat fibers, respectively. A total of 122 and 208 patches were formed on muscle fibers from young adult (n = 7 rats) and aged (n = 8 rats) rats, respectively. However, only patches with no evident channel rundown over 120 sec of continuous recording at constant voltage were selected for the P_{open} and single-channel conductance analyses (see Materials and Methods). When multiple channels were present in the patches, the theoretical and experimental values of the fractions of time spent by the channels in the open state, calculated by binomial distribution and by our own program (see Materials and Methods), respectively, were compared. These analyses revealed that the two groups of values were close and not significantly different. As predicted by the binomial model, this observation indicates that the K_{ATP} channels in both young adult and aged rats gate independently.

Effects of L-cysteine and *N*-acetyl-L-cysteine on K_{ATP} channels of young adult and aged rat skeletal muscle fibers. The application of L-cysteine (0.005–5 mM) to the cytoplasmic face of patches from aged rat fibers containing K_{ATP} channels produced a dose-dependent and significant increase in the P_{open} (Fig. 2) without altering the single-channel current. However, L-cysteine did not cause appearance of multiple channel openings (Fig. 1C). The increase of P_{open} produced

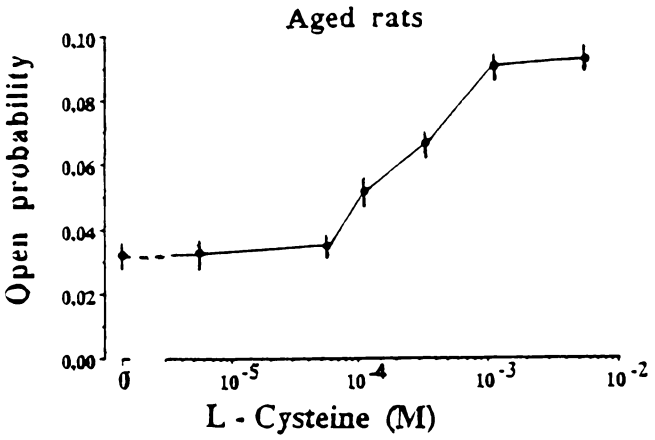

Fig. 2. Changes in the P_{open} of K_{ATP} channels from muscle fibers of aged rats produced by different concentrations of L-cysteine. Each *point* represents the mean ± standard error of P_{open}. A minimum of three and a maximum of seven patches were formed. The sulfhydryl group-reducing agent was applied to the cytoplasmic face of the channels. The experiments were performed at −60 mV (membrane potential) at 20°. The patches were exposed to each concentration of L-cysteine for about 250 sec.

by L-cysteine, especially at high concentrations, was stable over the usual time of recording (120 sec), as well as during long periods of recording (3 min). The effects of L-cysteine on the P_{open} were similar over the entire range of potentials studied (from −70 mV to +60 mV). However, the P_{open} values for K_{ATP} channels of aged rat fibers measured after application of 1 or 5 mM L-cysteine never reached the P_{open} values recorded for young adult rat fibers (Fig. 2). The effect of L-cysteine on aged rat channels was poorly reversible. In contrast to aged rats, the application of L-cysteine (up to 1 mM) to the patches excised from young adult rat fibers did not alter the single-channel current or the P_{open} of K_{ATP} channels, indicating that the effect of the amino acid was specific for K_{ATP} channels of aged rat

fibers. *N*-Acetyl-L-cysteine (0.5–5 mM) showed the same effects as L-cysteine on K_{ATP} channels of aged rat fibers; indeed, the application of *N*-acetyl-L-cysteine to the cytoplasmic face of patches excised from aged rat fibers increased the P_{open} of K_{ATP} channels from 0.037 ± 0.01 (n = 10 patches) under control conditions to 0.068 ± 0.02 (n = 4 patches) or 0.092 ± 0.03 (n = 6 patches) after 0.5 mM or 5 mM compound, respectively. *N*-Acetyl-L-cysteine did not produce any effect on the young adult rat channels.

Effects of thimerosal on K_{ATP} channels of young adult and aged rat skeletal muscle fibers. In both young adult and aged rats, the application of different concentrations of thimerosal (3 nM to 20 μM) to the internal side of patches containing K_{ATP} channels reduced both N and P_{open} (Fig. 3). The threshold concentrations needed to produce a measurable effect on $N \cdot P_{open}$ of K_{ATP} channels were 3 μM and 0.015 μM for channels from young adult and aged rats, respectively. A reduction of about 50% in the $N \cdot P_{open}$ of the K_{ATP} channels was achieved with 10 μM and 0.05 μM thimerosal for channels from young adult and aged rats, respectively (Fig. 3). At high concentrations the inhibitory action of thimerosal on $N \cdot P_{open}$ of K_{ATP} channels was rapid (<30 sec) and complete; indeed, after application of 20 μM and 0.1 μM to young adult and aged rat channels, respectively, no channel openings were observed, giving a residual P_{open} near 0 (Fig. 4). The switch to a thimerosal-free solution did not cause spontaneous return of channel openings even when the patches were observed for a prolonged period of time (up to 3 min). However, the inhibitory effect of thimerosal on $N \cdot P_{open}$ was reversed only by addition of L-cysteine in both young adult and aged rat fibers, after several seconds of application of the amino acid (Fig. 4). The blocking effect of thimerosal on $N \cdot P_{open}$ values for young adult and aged rat fibers was similar throughout the range of potentials studied (from −70 mV to +60 mV). Conversely, thimerosal applied at 20 μM on the extracellular surface membrane of patches from young adult and aged rat fibers did not produce measurable effects on $N \cdot P_{open}$.

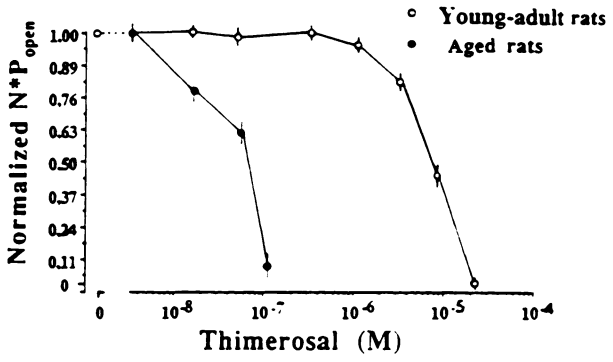

Fig. 3. Changes in the $N \cdot P_{open}$ of K_{ATP} channels of muscle fibers from young adult and aged rats produced by different concentrations of thimerosal. Each *point* represents the mean ± standard error of the normalized $N \cdot P_{open}$. A minimum of three and a maximum of six patches were formed for both young adult and aged rat fibers, using pipettes with tip opening areas of 3.9 ± 0.3 μm^2 and 7 ± 0.6 μm^2, respectively. The average N values in the patches were 3.4 ± 1 and 2.9 ± 1.1 for young adult and aged rat fibers, respectively. The sulfhydryl group-oxidizing agent was applied to the cytoplasmic face of the channels in both types of muscle preparations. The experiments were performed at −60 mV (membrane potential) at 20°. The patches from muscle fibers of young adult and aged rats were exposed to each concentration of thimerosal for about 130 sec.

Effects of glybenclamide on K_{ATP} channels of young adult and aged rat skeletal muscle fibers. The application of glybenclamide (1 pM to 50 μM) to the cytoplasmic face of K_{ATP} channels of young adult and aged rat fibers dose-dependently reduced the P_{open} of the channels (Fig. 5). In the range of concentrations tested, glybenclamide did not seem to affect N in either young adult or aged rat fibers. The dose-response relationships showed that glybenclamide was more potent in blocking K_{ATP} channels of aged rat fibers than young adult rat fibers; indeed, the EC_{50} values of the sulfonylurea were 60.1 ± 0.4 nM and 2.1 ± 0.2 nM (95% confidence interval, 56–65 nM and 1.46–3 nM) for K_{ATP} channels of young adult and aged rat fibers, respectively. No difference was observed in the calculated slopes of the curves, with these being 1.1 and 1.0 for channels of young adult and aged rat fibers, respectively (Fig. 6). In contrast to thimerosal, exposure to high concentrations of glybenclamide did not produce a full block of the channels; indeed, several openings of K_{ATP} channels were still observed after application of 50 μM glybenclamide, giving residual P_{open} values near 0.0085 and 0.00037 for channels of young adult and aged rat fibers, respectively. Washout of the compound rapidly reversed the inhibitory effect on K_{ATP} channels of both young adult and aged rat fibers (Fig. 5). Moreover, to evaluate the possible involvement of thiol groups in the reduction of the EC_{50} of glybenclamide to block the channels in aged rat fibers, we tested the effect of the sulfonylurea on K_{ATP} channels of aged rat fibers after preincubation of the patches with 1 mM L-cysteine. Under these experimental conditions, the EC_{50} of glybenclamide calculated by the fitting routine was 50 ± 0.8 nM (95% confidence interval, 43–58 nM), with a slope factor of 0.95 (Fig. 6), which was not significantly different from the EC_{50} of glybenclamide for young adult rat K_{ATP} channels (because the 95% confidence intervals overlap). Therefore, after exposure to L-cysteine, the decrease of P_{open} of K_{ATP} channels produced by glybenclamide in aged rat fibers was similar to that observed in young adult rat fibers.

Discussion

K_{ATP} channels of young adult and aged rat skeletal muscle fibers. As reported previously (20), the K_{ATP} channel was the most commonly observed K^+ channel on the surface membranes of both young adult and aged rat fibers. Our study shows that the properties of K_{ATP} channels of rat skeletal muscle fibers change with aging. Indeed, we found that the P_{open} of K_{ATP} channels from aged rats was about 7.5-fold lower than that of channels from young adult rats. The reduction of the P_{open} of K_{ATP} channels from aged rat fibers was observed in a wide range of potentials studied, suggesting that this effect is not voltage dependent. No more than 1 open channel/4.3 ± 0.2 μm^2 of patch area was detected in the aged rat fibers, whereas an average of 3.9 ± 0.7 channels were counted in young adult rat fibers. Even with pipettes having a tip opening area of 0.5 μm^2, no fewer than 3 open channels were detected in young adult rat fibers. A similar observation was made by Spruce *et al.* (6), who found multiple channels in frog skeletal muscle, using micropipettes of 0.3–0.4-μm tip diameter. We also emphasize that K_{ATP} channels were present in 50% and 30% of the patches formed in young adult and aged rat fibers, respectively. All of these observations suggest that in young adult rats the K_{ATP} channels are organized as clusters of channels, as has been proposed by Stanfield and co-workers (10); con-

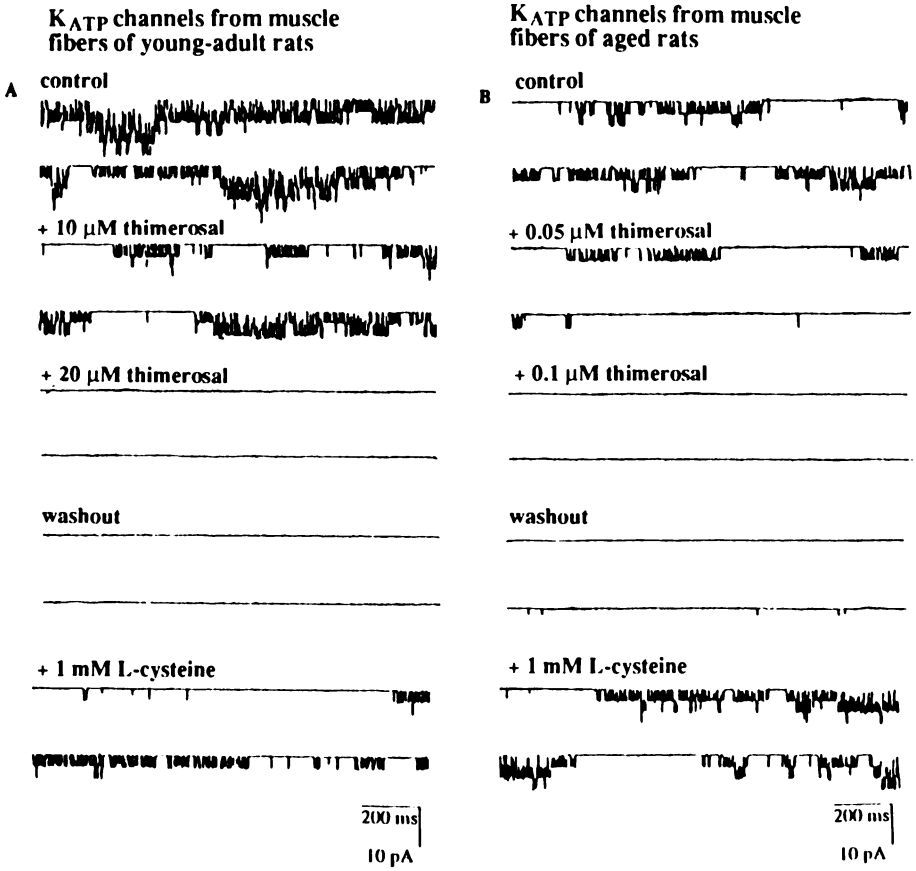

Fig. 4. Effects of thimerosal on the P_{open} value of K_{ATP} channels from muscle fibers of young adult and aged rats. Short segments (1.3 sec) of channel activity recorded at 20° from inside-out patches held at −60 mV (membrane potential) are shown (sampling rate, 20 kHz; filter, 2 kHz). The direction of ionic currents follows the standard convention; downward deflections on the current records indicate the movement of positively charged ions from the extracellular side of the membrane to the intracellular side (inward current). A, Typical activity of K_{ATP} channels from muscle fibers of a young adult rat. At least 5 channels were present in the patches. In this particular patch, the P_{open} values were 0.202 under control conditions and 0.111 and 0 after application of 10 μM and 20 μM thimerosal, respectively. After washout of thimerosal, the P_{open} value was still near 0. The application of 1 mM L-cysteine increased the P_{open} value from 0 to 0.096. The tip opening area of the pipette used was 4 μm^2. B, Typical activity of K_{ATP} channels from muscle fibers of an aged rat. The P_{open} values were 0.041 under control conditions and 0.023 and 0 after application of 0.05 μM and 0.1 μM thimerosal, respectively. Using a macropipette of 7-μm^2 tip opening area, 2 channels were observed during 230 sec of recording. After washout of thimerosal, L-cysteine increased the P_{open} value from 0 to 0.07.

versely, a dramatic decrease of channel density seems to occur with aging.

K_{ATP} channels of skeletal muscle fibers and thiol groups. Our results indicate that a thiol-dependent redox mechanism may regulate the activity of K_{ATP} channels of rat skeletal muscle fibers. The existence of such a regulatory mechanism has been proposed for various types of ion channels (29, 30), including K_{ATP} channels of mouse pancreatic β-cells, in which 10–100 μM thimerosal induces closure of the channel that is reversed only by addition of dithiothreitol (16); it has been suggested also for K_{ATP} channels of mouse skeletal muscle, where 2 mM *N*-ethylmaleimide inhibits channel activity (7). The presence of thiol groups on K_{ATP} channels of skeletal muscle fibers of young adult and aged rats is supported by the observation that thimerosal induced rapid and complete closure of the channels that was reversed only by addition of L-cysteine. In striated fibers, as in pancreatic β-cells (16), the thiol groups are probably located on the cytoplasmic face of the channels; indeed, the application of thimerosal on the extracellular face of the patches did not alter the single-channel properties. In young adult rats the application of L-cysteine by itself did not produce any effects; in contrast, in aged rats this amino acid and *N*-acetyl-L-cysteine were able to dose-dependently open K_{ATP} channels, supporting the hypothesis that an oxidation of thiol groups occurs in aged rat muscle fibers. Moreover, the K_{ATP} channels of aged rats were more sensitive to thimerosal, compared with those of young adult rats. This finding can be explained by assuming that the oxidation of a certain number of K_{ATP} channel thiol groups that occurs in aged rats may cooperatively favor the accessibility of oxidizing substances such as thimerosal to residual thiol groups, thus reducing the concentration of the chemical needed to block the channels. The oxidation of thiol groups could be due to an age-related increase of oxygen free radicals in the muscles, as has been well documented in other tissues (17, 18). These substances may act either by lowering the cytoplasmic concentrations of cofactors such as the reduced form of gluthatione or by directly oxidizing channel proteins, thus mediating cross-linking reactions between proteins and bioactive macromolecules (17–19). In aged rat muscle, this phenomenon could have favored an interconversion between the reduced and oxidized forms of the cytoplasmic thiol groups of K_{ATP} channels, thus inducing closure of the channels. An increase in openings of K_{ATP} channels after application of thiol-containing compounds has been observed also in myocardial ischemia, a situation in which the role of free radicals is well established (31, 32). Indeed, zofenopril, an inhibitor of angiotensin-converting enzyme, and *N*-acetyl-L-cysteine protect the heart from the ischemic insult by opening K_{ATP} channels (32). However, in our experiments L-cysteine and *N*-acetyl-L-cysteine, in spite of their ability to open K_{ATP} channels, were unable to restore the *N* value of aged rat skeletal muscle fibers. This finding raises the question of whether mechanisms other than free radical generation may be involved in the reduction of K_{ATP} channel activity in aged rat fibers. It has been reported that a change in Ca^{2+} homeostasis occurring in skeletal muscle fibers with aging results in an higher cytosolic Ca^{2+} concentration (33, 34). Moreover, it was demonstrated that the exposure of K_{ATP} channels of skeletal muscle to micromolar concentrations of Ca^{2+} reduced channel activity (35). In light of these observations, in our experiments

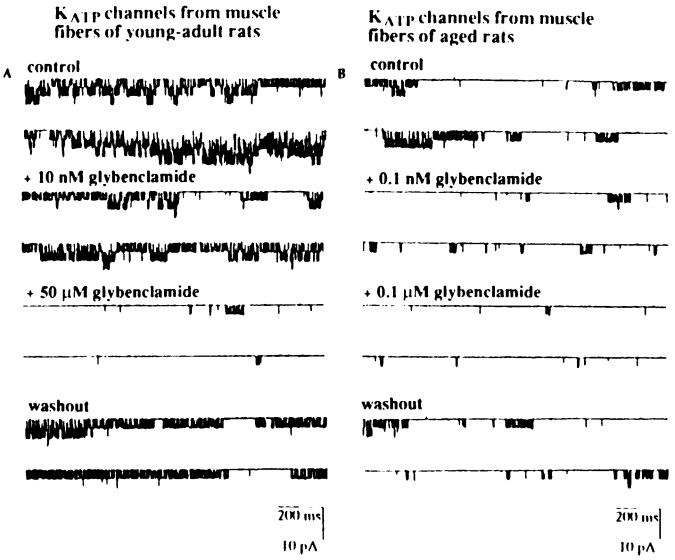

Fig. 5. Effects of glybenclamide on the P_{open} of K_{ATP} channels from muscle fibers of young adult and aged rats. Short segments (1.3 sec) of channel activity recorded at 20° from inside-out patches held at −60 mV (membrane potential) are shown (sampling rate, 20 kHz; filter, 2 kHz). The direction of ionic currents follows the standard convention; downward deflections in the current records indicate the movement of positively charged ions from the extracellular side of the membrane to the intracellular side (inward current). A, Typical activity of K_{ATP} channels from muscle fibers of a young adult rat. At least 5 channels were present in the patches. In this particular patch the P_{open} values were 0.251 under control conditions and 0.227 and 0.002 after application of 10 nM and 50 μM glybenclamide, respectively. After washout of the sulfonylurea, the P_{open} increased to 0.261. The tip opening area of the pipette used was 3.8 μm^2. B, Typical activity of K_{ATP} channels from muscle fibers of an aged rat. The P_{open} values were 0.03 under control conditions and 0.022 and 0.0006 after application of 0.1 nM and 0.1 μM glybenclamide, respectively. Using a macropipette of 6.8-μm^2 tip opening area, 2 channels were observed during 200 sec of recording. After washout of glybenclamide, the P_{open} value increased to 0.028.

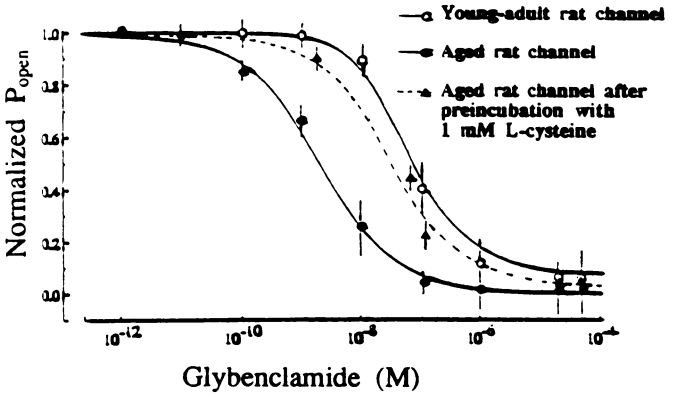

Fig. 6. Dose-response relationships for the normalized P_{open} values of young adult rat K_{ATP} channels, aged rat K_{ATP} channels, and aged rat K_{ATP} channels preincubated with 1 mM L-cysteine versus glybenclamide concentrations. The *curves* were drawn as described in Materials and Methods. The fitting procedure predicted EC_{50} values of 60.1 ± 0.4 nM (95% confidence interval, 56–65 nM) with a slope of 1.1, 2.1 ± 0.2 nM (95% confidence interval, 1.46–3 nM) with a slope of 1, and 50 ± 0.8 nM (95% confidence interval, 43–58 nM) with a slope of 0.95 for young adult rat channels, aged rat channels, and aged rat channels pretreated with L-cysteine, respectively (goodness of fit, 0.999, 0.996, and 0.922 for curves for young adult rat channels, aged rat channels, and aged rat channels pretreated with 1 mM L-cysteine, respectively). Each *point* represents the mean ± standard error of the P_{open} value for a minimum of three and a maximum of 11 patches. The experiments were performed using pipettes with tip opening areas of 4.1 ± 0.5 μm^2, 7 ± 0.2 μm^2, and 6.9 ± 0.5 μm^2 for young adult rat K_{ATP} channels, aged rat K_{ATP} channels, and aged rat K_{ATP} channels pretreated with 1 mM L-cysteine, respectively. The dose-response curves were constructed using average *N* values in the patches of 3.6 ± 1.1, 2.9 ± 0.7, and 3.1 ± 0.8 for young adult rat fibers, aged rat fibers, and aged rat fibers pretreated with 1 mM L-cysteine, respectively. The experiments were performed at −60 mV (membrane potential) at 20°. The patches from muscle fibers of young adult and aged rats were exposed to each concentration of glybenclamide for about 140 sec, whereas some patches excised from aged rat fibers were preincubated with 1 mM L-cysteine for 15–20 sec before glybenclamide exposure.

an increase in intracellular Ca^{2+} concentrations in aged rat fibers may have inhibited K_{ATP} channel activity by reducing *N*.

Effects of glybenclamide on K_{ATP} channels of skeletal muscle fibers. In inside-out patches, glybenclamide reduced the P_{open} of K_{ATP} channels of both young adult and aged rat skeletal muscle fibers, supporting the hypothesis that the sulfonylurea receptor in skeletal muscle can be strictly associated with the channel, as previously observed in other tissues (3, 36, 37). Recently, a 140-kDa sulfonylurea receptor was cloned from pancreatic α- and β-cells (38); however, whether this protein has K_{ATP} channel function is not yet established. In our experiments, the inhibitory action of glybenclamide on K_{ATP} channels was more pronounced in aged rat fibers, compared with young adult rat fibers. Indeed, the EC_{50} values for reducing the P_{open} of K_{ATP} channels were 60.1 ± 0.4 nM and 2.1 ± 0.2 nM in young adult and aged rats, respectively. In agreement with previous reports, young adult rat skeletal muscle appears to be less sensitive to glybenclamide than are heart (36) and β-cells (3), in which full inhibition of channel activity was achieved with concentrations lower than 20 nM. Moreover, in contrast to pancreas (3) and heart (36), in skeletal muscle the inhibitory effect of glybenclamide was reversible and the sulfonylurea, even at high concentrations (20–50 μM), did not completely shut the channels. In contrast to glybenclamide, the effects of thimerosal were irreversible and the compound completely shut the channels. Moreover, as has been proposed for pancreatic β-cells (21), our data suggest that in skeletal muscle the sulfonylurea receptor protein or a subunit closely associated with it may contain thiol groups. Indeed, the concentration of glybenclamide needed to produce a 50% block of K_{ATP} channels in aged rat fibers was increased by a factor of 24 after preincubation with L-cysteine, which opens aged rat channels (possibly by reducing thiol groups). This observation also indicates that the pharmacological properties of the aged rat K_{ATP} channels after treatment with L-cysteine were similar to the properties of the young adult rat channels. In support of the presence of thiol groups in the vicinity of the glybenclamide binding site, some authors have proposed that the sulfonylurea receptor is associated with nucleotide binding sites (39), which also contain thiol groups (7, 40, 41).

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References

1. Noma, A. ATP-regulated K^+ channels in cardiac muscle. *Nature (Lond.)* **305**:147–148 (1983).
2. Kovacs, R., and M. Nelson. ATP-sensitive K^+ channels from aortic smooth muscle incorporated into planar lipid bilayers. *Am. J. Physiol.* **261**:H604–H609 (1991).

3. Dunne, M. J., and O. H. Petersen. Potassium selective ion channels in insulin-secreting cells: physiology, pharmacology and their role in stimulus-secretion coupling. *Biochim. Biophys. Acta* **1071**:67–82 (1991).
4. Ashford, M. L. J., N. C. Sturgess, N. J. Trout, N. J. Gardner, and C. N. Hales. Adenosine-5′-triphosphate-sensitive ion channels in neonatal rat cultured central neurones. *Pflügers Arch.* **412**:297–304 (1988).
5. Burton, F., U. Dörstelmann, and O. F. Hutter. Single-channel activity in sarcolemmal vesicles from human and other mammalian muscles. *Muscle Nerve* **11**:1029–1038 (1988).
6. Spruce, A. E., N. B. Standen, and P. R. Stanfield. Voltage-dependent ATP-sensitive potassium channels of skeletal muscle membrane. *Nature (Lond.)* **316**:736–738 (1985).
7. Weik, R., and B. Neumcke. ATP-sensitive potassium channels in adult mouse skeletal muscle: characterization of the ATP-binding site. *J. Membr. Biol.* **110**:217–226 (1989).
8. Longman, S. D., and T. Hamilton. Potassium channel activator drugs: mechanism of action, pharmacological properties, and therapeutic potential. *Med. Res. Rev.* **12**:73–148 (1992).
9. Tricarico, D., and D. Conte Camerino. Effects of ischaemia and post-ischaemic reperfusion on the passive and active electrical parameters of rat skeletal muscle fibres. *Pflügers Arch.* **426**:44–50 (1994).
10. Davies, N. W., N. B. Standen, and P. R. Stanfield. ATP-dependent potassium channels of muscle cells: their properties, regulation, and possible functions. *J. Bioenerg. Biomembr.* **23**:509–535 (1991).
11. Castle, N. A., and D. G. Haylett. Effect of channel blockers on potassium efflux from metabolically exhausted frog skeletal muscle. *J. Physiol. (Lond.)* **383**:31–43 (1987).
12. Davies, N. W. Modulation of ATP-sensitive K^+ channels in skeletal muscle by intracellular protons. *Nature (Lond.)* **343**:375–377 (1990).
13. Links, T. P., A. J. Smit, H. J. G. H. Oosterhuis, and W. D. Reitsma. Potassium channels in hypokalaemic periodic paralysis: a key to the pathogenesis? *Clin. Sci.* **85**:319–325 (1993).
14. Allard, B., and M. Lazdunski. Nucleotide diphosphates activate the ATP-sensitive potassium channel in mouse skeletal muscle. *Pflügers Arch.* **422**:185–192 (1992).
15. Parent, L., and R. Coronado. Reconstitution of the ATP-sensitive potassium channel of skeletal muscle. *J. Gen. Physiol.* **94**:445–463 (1989).
16. Islam, S., P. O. Berggren, and O. Larsson. Sulfhydryl oxidation induces rapid and reversible closure of the ATP-regulated K^+ channel in the pancreatic β-cell. *FEBS Lett.* **319**:128–132 (1993).
17. Dice, J. F. Cellular and molecular mechanism of aging. *Physiol. Rev.* **73**:149–159 (1993).
18. Nagy, I. Z. S. A proposal for reconsideration of the role of oxygen free radicals in cell differentiation and aging. *Ann. N. Y. Acad. Sci.* **673**:142–153 (1992).
19. Stadtman, E. Protein oxidation and aging. *Science (Washington D. C.)* **257**:1220–1224 (1992).
20. Tricarico, D., R. Wagner, R. Mallamaci, and D. Conte Camerino. Cysteine restores the activity of ATP-sensitive potassium channels of skeletal muscle fibers of aged rats. *Ann. N. Y. Acad. Sci.* **717**:244–254 (1994).
21. Ammon, H. P. T., and M. Abdel-Hamid. Potentiation of the insulin-releasing capacity of tolbutamide by thiols: studies on the isolated perfused pancreas. *Naunyn-Schmiedebergs Arch. Pharmacol.* **317**:262–267 (1981).
22. Bekoff, A., and W. J. Betz. Physiological properties of dissociated muscle fibres obtained from innervated and denervated adult rat muscle. *J. Physiol. (Lond.)* **271**:25–40 (1977).
23. Sakmann, B., and E. Neher. Geometric parameters of pipettes and membrane patches, in *Single-Channel Recording* (B. Sakmann and E. Neher, eds.). Plenum, New York, 37–51 (1983).
24. Findlay, I., and M. J. Dunne. ATP maintains ATP-inhibited K^+ channels in an operational state. *Pflügers Arch.* **407**:238–240 (1986).
25. Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Nature (Lond.)* **391**:85–100 (1981).
26. Green, J. R., and D. Margerison. *Statistical Treatment of Experimental Data.* Elsevier, New York, 86–90 (1978).
27. Press, W. H., B. P. Flannery, S. A. Tenkolsky, and W. T. Vetterling. Modelling of data, in *Numerical Recipes* (W. H. Press and B. P. Flannery, eds.). Cambridge University Press, Cambridge, UK, 498–507 (1986).
28. Tallarida, R. J., and R. B. Murray. Methods of Litchfield and Wilcoxon: confidence limits of ED_{50}, in *Manual of Pharmacological Calculation with Computer Programs* (R. J. Tallarida and R. B. Murray, eds.), Ed. 2. Springer-Verlag, New York, 59–63 (1986).
29. Islam, S., P. Rorsman, and P. O. Berggren. Ca^{2+}-induced Ca^{2+} release in insulin-secreting cells. *FEBS Lett.* **296**:287–291 (1992).
30. Trimm, J. L., G. Salama, and J. J. Abramson. Sulfhydryl oxidation induces rapid calcium release from sarcoplasmic reticulum vesicles. *J. Biol. Chem.* **261**:16092–16098 (1986).
31. Das, D. K., and R. M. Engelman. Mechanism of free radical generation during reperfusion of ischaemic myocardium, in *Oxygen Radicals: Systemic Events and Disease Processes* (D. K. Das and W. B. Essman, eds.). Karger, Basel, 97–121 (1990).
32. Sargent, C. A., P. G. Sleph, S. Dzwonczyk, M. A. Smith, D. Normandin, M. J. Antonaccio, and G. J. Grover. Cardioprotection in ischemic rat hearts with the SH-containing angiotensin-converting enzyme inhibitor zofenopril: possible involvement of the ATP-sensitive potassium channel. *J. Pharmacol. Exp. Ther.* **265**:609–618 (1993).
33. Larsson, L., and G. Salviati. Effects of age on calcium transport activity of sarcoplasmic reticulum in fast- and slow-twitch rat muscle fibres. *J. Physiol. (Lond.)* **419**:253–264 (1989).
34. Gafni, A., and K. C. Yuh. A comparative study of the Ca^{2+}-Mg^{2+} dependent ATPase from skeletal muscles of young, adult and old rats. *Mech. Ageing Dev.* **49** (Suppl. 2):105–117 (1989).
35. Krippeit-Drews, P., and U. Lönnendonker. Dual effects of calcium on ATP-sensitive potassium channels of frog skeletal muscle. *Biochim. Biophys. Acta* **1108**:119–122 (1992).
36. Fosset, M., J. R. De Weille, R. D. Green, H. Schmid-Antomarchi, and M. Lazdunski. Antidiabetic sulfonylureas control action potential properties in heart cells via high affinity receptors that are linked to ATP-dependent K^+ channels. *J. Biol. Chem.* **17**:7933–7936 (1988).
37. Sturgess, N. C., M. L. J. Ashford, D. L. Cook, and C. N. Hales. The sulfonylurea receptor may be an ATP-sensitive potassium channel. *Lancet* **2,8453**:474–475 (1985).
38. Bryan, J., L. Aguilar-Bryan, and D. A. Nelson. Cloning of a cDNA for the high affinity sulfonylurea receptor from pancreatic α- and β-cells. *Biophys. J.* **66**:A349 (1994).
39. Bernardi, H., M. Fosset, and M. Lazdunski. ATP/ADP binding sites are present in the sulfonylurea binding protein associated with brain ATP-sensitive K^+ channels. *Biochemistry* **31**:6328–6332 (1992).
40. de Weille, J. R., M. Muller, and M. Lazdunski. Activation and inhibition of ATP-sensitive K^+ channels by fluorescein derivatives. *J. Biol. Chem.* **267**:4557–4563 (1991).
41. Lee, K., S. E. Ozanne, C. N. Hales, and M. L. J. Ashford. Effects of chemical modification of amino and sulfhydryl groups on K_{ATP} channel function and sulfonylurea binding in CRI-G1 insulin-secreting cells. *J. Membr. Biol.* **139**:167–181 (1994).

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