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Effects of ATP-sensitive Potassium Channel Regulators on Chloride Channels in the Sarcoplasmic Reticulum Vesicles from Rabbit Skeletal Muscle

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Abstract. The lipid bilayer technique was used to examine the effects of the ATP-sensitive K^+ channel inhibitor (glibenclamide) and openers (diazoxide, minoxidil and cromakalim) and Cl^- channel activators (GABA and diazepam) on two types of chloride channels in the sarcoplasmic reticulum (SR) from rabbit skeletal muscle. Neither diazepam at 100 μM nor GABA at 150 μM had any significant effect on the conductance and kinetics of the 75 pS small chloride (SCl) channel.

Unlike the 150 pS channel, the SCl channel is sensitive to cytoplasmic glibenclamide with K_i ~ 30 μM. Glibenclamide induced reversible decline in the values of current (maximal current amplitude, I_{max} and average mean current, I') and kinetic parameters (frequency of opening F_o, probability of the channel being open P_o and mean open time, T_o, of the SCl channel. Glibenclamide increased mean closed time, T_c, and was a more potent blocker from the cytoplasmic side (*cis*) than from the luminal side (*trans*) of the channel.

Diazoxide increased I', P_o, and T_o in the absence of ATP and Mg^{2+} but it had no effect on I_{max} and also failed to activate or remove the glibenclamide- and ATP-induced inhibition of the SCl channel. Minoxidil induced a transient increase in I' followed by an inhibition of I_{max}, whereas cromakalim reduced P_o and I' by increasing channel transitions to the closed state and reducing T_o without affecting I_{max}. The presence of diazoxide, minoxidil or cromakalim on the cytoplasmic side of the channel did not prevent $[ATP]_{cis}$ or $[glibenclamide]_{cis}$ from blocking the channel.

The data suggest that the action(s) of these drugs are not due to their effects on the phosphorylation of the channel protein. The glibenclamide- and cromakalim-induced effects on the SCl channel are mediated via a ''flicker'' type block mechanism. Modulation of the SCl channel by $[diazoxide]_{cis}$ and $[glibenclamide]_{cis}$ highlights the therapeutic potential of these drugs in regulating the Ca^{2+}-counter current through this channel.

Key words: ATP-sensitive chloride channel — Sarcoplasmic reticulum — Skeletal muscle — Cromakalim — Diazoxide — Glibenclamide

Introduction

The understanding of the pharmacological regulation of ATP-modulated Cl^- channels is important for the strategy of elaborating the role of Cl^- channels in normal cellular functions such as volume regulation (*see* Holevinsky et al., 1994; Ballatori et al., 1995) and physiopathologies, e.g., solute transport associated with Cl^- channelopathies such as cystic fibrosis and diarrhea (*see* Sheppard & Welsh, 1992). The modulatory effects of ATP-sensitive K^+ channel openers and inhibitors on the conductance and kinetic properties of ATP-activated Cl^- channels have been examined in recent studies (Sheppard & Welsh, 1992; Ballatori et al., 1995; Holevinsky et al., 1994; Hongre et al., 1994; Tominaga et al., 1995). These studies revealed that the ATP-activated Cl^- channels in transfected NIH 3T3 fibroblasts expressing recombinant cystic fibrosis transmembrane conductance regulator, CFTR, (Sheppard & Welsh, 1992), ATP-dependent-swelling-activated Cl^- conductance in hepatocytes (Ballatori et al., 1995) and in smooth muscle fibers (Holevinsky et al., 1994), and isoproterenol-forskolin-cAMP-activated Cl^- conductance in cardiac ventricular cells (Tominaga et al., 1995) are inhibited by both inhibitors and openers of the ATP-sensitive K^+ channels. In the above studies the effects of the ATP-sensitive K^+ channel regulators were examined (i) on ATP-activated Cl^- channels but not on ATP-sensitive

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Cl^- channels and (ii) either in whole-cell Cl^- current or Cl^- efflux experiments but not in single Cl^- channel experiments. Hence, the effects of the above-mentioned compounds on the single ATP-sensitive Cl^- channels are still not known. Chloride-selective channels that have been briefly mentioned to be inhibited by ATP are found in human platelet surface membrane (Manning & Williams, 1989) and in nuclear membranes isolated from both rat liver (Tabares, Mazzanti & Clapham, 1991) and sheep cardiac ventricular cells (Rousseau et al., 1996). Recently, it was found that SCl channels in SR vesicles from skeletal muscle reconstituted into bilayers are ATP-sensitive and share some properties with the ATP-sensitive K^+ channels (Kourie, 1997*b*). Firstly, the ATP-sensitivity of the single SCl channel is similar to that of the ATP-sensitive K^+ channel. The efficacy of [adenine nucleotides]$_{cis}$ in inhibiting the SCl channel is in the following order ATP^{4-}>ADP^{3-}>>>AMP^{2-} as has been previously suggested for the ATP-sensitive K^+ channel (Ashcroft & Kakei, 1989). Secondly, phosphorylation is not required for SCl channel inhibition and this is in agreement with the properties of the ATP-sensitive K^+ channel in pancreatic β-cell (Cook & Hales, 1984) and cardiac myocytes (*see* Terzic, Jahangir & Kurachi, 1995). The present study was undertaken to investigate modulatory effects of ATP-sensitive K^+ channel openers diazoxide, minoxidil and cromakalim as well as the inhibitor glibenclamide on the conductance and kinetic properties of single ATP-sensitive SCl channels.

Materials and Methods

Preparation of SR Vesicles

Terminal cisternae or longitudinal SR vesicles from rabbit skeletal muscle (Saito et al., 1984) were incorporated into lipid bilayers (Miller & Racker, 1976) as detailed previously (Kourie et al., 1996; Kourie, 1997*a*,*b*).

Solutions

Solutions contained choline Cl^- (250 mM *cis*/50 mM *trans*) plus 1 mM $CaCl_2$ and 10 mM HEPES (pH 7.4, adjusted with Tris). Stock solutions of glibenclamide and diazoxide were prepared in dimethyl sulfoxide, cromakalim in 70% (vol/vol) ethanol and minoxidil sulfate in methanol 0.02%. μl aliquots of Cl^- channel inhibitors and openers (Sigma) stock solutions, i.e., *cis* solution containing pharmacological agents, buffered to pH 7.4, were used to increment drug concentration in the *cis* and *trans* chambers. Appropriate control experiments using the vehicle solutions did not affect the SCl channel activity.

Lipid Bilayers and Vesicle Fusion

Lipid bilayers were formed across a 150 μm hole in the wall of a 1-ml delrin™ cup using a mixture of palmitoyl-oleoyl-phosphatidylethanolamine, palmitoyl-oleoyl-phosphatidylserine and palmitoyl-oleoyl-phosphatidylcholine (5:3:2, by volume) (Kourie, 1996), obtained in chloroform from Avanti Polar Lipids (Alabaster, AL). The side of the bilayer to which vesicles were added was defined as *cis*, and the other side as *trans*. The orientation of cytoplasmic side of the vesicle is thought to face the *cis* chamber (Miller & Racker, 1976). This was verified by using common ligands which are known to bind to the cytoplasmic domain of the ryanodine Ca^{2+} release channel protein (e.g., Ahern, Junankar & Dulhunty, 1994). This cytoplasmic orientation is also true for the Cl^- channel proteins (e.g., Kawano et al., 1992). The experiments were conducted at 20–25°C.

Recording Single-Channel Activity

The pClamp program (Axon Instruments) was used for voltage command and acquisition of Cl^- current families (*see* Fig. 1) with an Axopatch 200 amplifier (Axon Instruments). The current was monitored on an oscilloscope and stored on videotape using pulse code modulation (PCM-501; Sony). The *cis* and *trans* chambers were connected to the amplifier head stage by Ag/AgCl electrodes in agar salt-bridges containing the solutions present in each chamber. Voltages and currents were expressed relative to the *trans* chamber. Data were filtered at 1 kHz (4-pole Bessel, -3dB) and digitized via a TL-1 DMA interface (Axon Instruments) at 2 kHz. Unless stated otherwise, the ''optimal bilayer'' (Kourie, 1996) was held at −40 mV in asymmetrical choline-Cl (250 mM/50 mM; *cis/trans*).

Data Analysis

The criteria for defining ion currents as belonging to a ''single channel'' have been described elsewhere (Colquhoun & Hawkes, 1983). Episodes of up to 100 sec of single-channel activity were analyzed for overall characteristics using CHANNEL 2 (developed by P.W. Gage and M. Smith, JCSMR). CHANNEL 2 allows online analysis of the entire current record for computation of the average mean current I'. I' is defined as the integral of the current passing through the channel divided by the total time. The integral current is determined by computation of the area between a line set on the noise of the closed state and channel opening to various levels. The maximal current (I_{max}) is the current which passes through a fully open channel. The value of I_{max} is obtained by measuring the distance (in pA) between two lines, one set on the noise of the closed level where the current amplitude is 0 pA and the other set on the noise of the majority of distinct events which were in the open state. I_{max} was also obtained by measuring the distance (in pA) between the peak at 0 pA (representing the closed state) and the extreme peak on the left (representing the open state) in the all-points histogram generated using CHANNEL 2. Both methods were used and the results were generally in agreement. To obtain the open probability, P_o, i.e., the fraction of time that the channel was open, the threshold was set at 1 pA, approximately 18 to 22% of I_{max}, to include current transitions to substates less than 50% of the maximum conductance (*see* Kourie, 1997*b*). This low threshold was necessary since transitions to these substates contribute significantly to channel activity. This was particularly essential in the presence of agents, e.g., nucleotides or glibenclamide where transitions to substates were often prominent. All points exceeding the threshold current for 0.5 msec or longer were considered to be channel openings for CHANNEL 2 determination of P_o. Each SCl channel was used as its own control and the comparison was between conductance and kinetic parameters of the SCl channel recorded at −40 mV before and after the channel was subjected to any treatment. Data are reported as means ± SD, and the difference in means was analyzed by Student's *t*-test. Data were considered statistically significant when P values were ≤ 0.05.

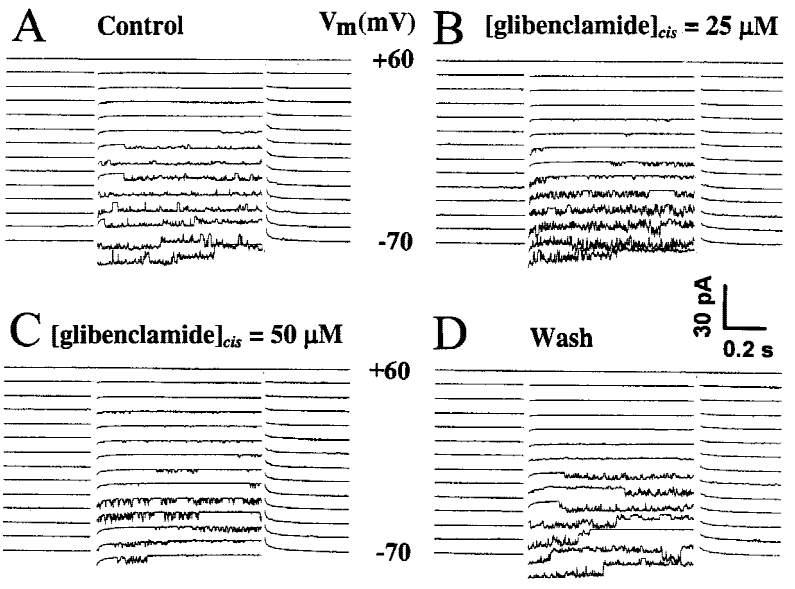

Fig. 1. Effects of $[glibenclamide]_{cis}$ on the voltage dependence of SCl channel activity. (*A*) Control current traces recorded in asymmetrical choline-Cl (250 mM/50 mM; *cis/trans*). (*B*) Current traces recorded after the addition of 25 μM and (*C*) 50 μM $[glibenclamide]_{cis}$ to the *cis* chamber. (*D*) Wash, recovery after perfusion with glibenclamide-free solution. Following convention the downward deflections denote activation of the inward Cl^- current, i.e., chloride ions moving from the *cis* chamber to the *trans* chamber. The baseline of each trace corresponds to the level of the leakage current that remains when no channels are open in the bilayer. The leakage conductance, g_{Lb}, for the optimal bilayer having a specific capacitance, Cb, value of 0.42 $\mu F/cm^2$ is < 12.5 pS. The current traces are filtered at $f_c = 0.2$ kHz, the fast transient capacitive current is removed and the traces are offset by 10 pA for a better display. This bilayer contains two overlapping SCl channels. The inhibitory effects of $[glibenclamide]_{cis}$ were observed on every single SCl channel that was examined ($n = 18$).

Abbreviations

ATP Adenosine-5′-triphosphate (magnesium salt); I' Average mean current; I_{max} Maximum current; SCl channel Small Cl^- channel; SR Sarcoplasmic reticulum.

Results

Effects of Glibenclamide on a Large Cl^- Permeant Anion Channel

It has been previously shown that the voltage-independent Cl^- channel which has P_o of ~1 and maximal conductance of 150 pS with two subconductance states of 107 and 71 pS is insensitive to the addition of 0.5 mM ATP to the cytoplasmic side of the channel facing the *cis* chamber. Similarly, this channel is insensitive to the sulfonylurea glibenclamide in the *cis* chamber. The kinetic and conductance properties of the channel are unaffected by 50 and 100 μM $[glibenclamide]_{cis}$. The values of P_o and I_{max} being approximately 1.0 and −10.7 pA, respectively, for the channel recorded at −20 mV in control and in $[glibenclamide]_{cis}$ solutions.

Effects of Glibenclamide on the SCl Channel

Unlike the large Cl^- channel the SCl channel is sensitive to the addition of 0.5 mM ATP to the cytoplasmic side of the channel facing the *cis* chamber (Kourie, 1997*b*). ATP induces channel inhibition either when the channel is in the long closed state or in the operative ''burst'' state. In a way similar to that described for the ATP-sensitive K^+ channels (*see* Edwards & Weston, 1993), the kinetic and conductance properties of this ATP-sensitive SCl channel are sensitive to $[glibenclamide]_{cis}$. At a holding potential of −40 mV the values of P_o and I_{max} for the total SCl channel activity of 16 episodes lasting 8 sec were 0.62 ± 0.14 and −5.25 ± 0.73 pA for control and 0.13 ± 0.06 and −2.1 ± 0.27 pA in *cis* solutions containing 50 μM glibenclamide.

The effects of glibenclamide on the conductance and kinetics of activation and inactivation of the SCl channel were also examined at different voltages. A voltage protocol was used to activate the voltage- and Ca^{2+}-dependent SCl channel currents. From an initial holding potential (V_h) of +60 mV lasting 500 msec the bilayer potential (V_m) was stepped to voltages between −70 and +60 mV, in steps of +10 mV, for periods lasting 500 msec. The steps are also separated by a 500 msec interval where the holding potential was also kept at +60 mV. Figure 1 shows typical families of single current traces recorded at voltages between −70 and +60 mV in glibenclamide-free control solution (Fig. 1*A*) and during exposure to 25 μM (Fig. 1*B*) and 50 μM $[glibenclamide]_{cis}$ (Fig. 1*C*). Channel activity which was characterized by bursts of openings with a maximum conductance of ~75 pS, and openings to several subconductance states, was affected by the micromolar additions of glibenclamide to the *cis* chamber. The glibenclamide-sensitivity of the SCl channel is characterized by an initial increase in the transitions to maximal and submaximal open states within bursts at 25 μM $[glibenclamide]_{cis}$ followed by a gradual decrease in I_{max} and in the number of events to high conductance levels and the appearance of many incomplete transitions at 50 μM $[glibenclamide]_{cis}$. However, bursts of channel activity and channel inactivation were still apparent, e.g., the last current traces at

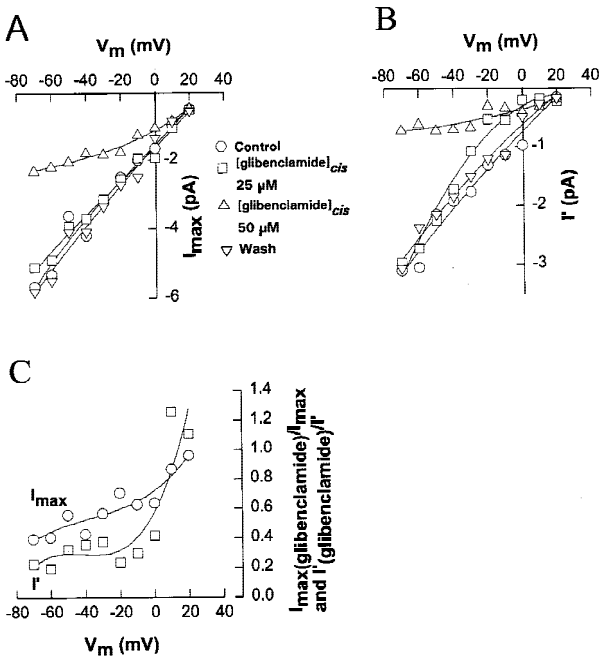

Fig. 2. Effects of $[glibenclamide]_{cis}$ on the voltage-dependence of SCl channel current parameters. (*A*) Maximal current, I_{max}, and (*B*) the average mean current, I', the integral of the current divided by the total time. (○) Control, (□) 25 μM, (△) 50 μM $[glibenclamide]_{cis}$ and (▽) wash. (*C*) voltage dependency of I_{max}(glibenclamide)/I_{max} (○) and I'(glibenclamide)/I' (□). The solid lines are drawn to a second (*A* and *B*) and a third order (*C*) polynomial fits.

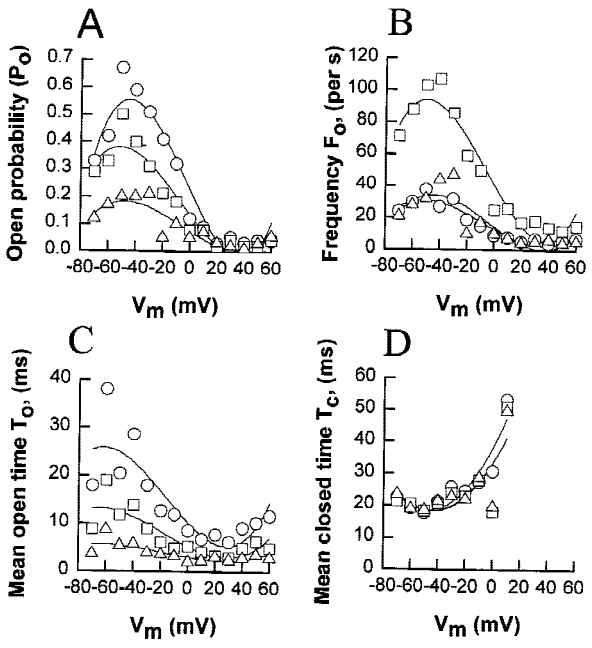

Fig. 3. Effects of $[glibenclamide]_{cis}$ on the voltage-dependence of SCl channel kinetic parameters (○) Control, (□) 25 μM, (△) 50 μM [glibenclamide]$_{cis}$. (*A*) Open probability (P_o) (*B*) frequency F_o, (*C*) mean open time T_o and (*D*) mean closed time T_c. Single-channel currents are recorded after an SR vesicle of skeletal muscle incorporated in an optimal bilayer held at −40 mV and in asymmetrical choline-Cl (250 mM/50 mM; *cis/trans*). The solid lines are drawn to a third order polynomial fit (*A, B* and *C*) and to a second polynomial fit (*D*).

−60 and −70 mV (Fig. 1*C*). Furthermore, the effects of $[glibenclamide]_{cis}$ on channel activity were fully reversible immediately after washing with glibenclamide-free solution (Fig. 1*D*).

Current-voltage relationships were constructed to examine the voltage-dependence of glibenclamide-induced changes in channel conductance (Fig. 2). The current-voltage relationship for the maximal current show that I_{max} is reduced significantly. For example, at −40 mV, I_{max} was reduced from −4.19 pA in control to −1.81 pA in 50 μM $[glibenclamide]_{cis}$. However, the I_{max} value recovered to −4.09 pA after wash with glibenclamide-free control solution. The effects of glibenclamide on the voltage-dependency of the average mean current is also shown in Fig. 2*B*. It is apparent that in the presence of 50 μM $[glibenclamide]_{cis}$, I' values were reduced to an approximately similar level at volt2ages between 0 and −70 mV. For example, I' values in the presence of 50 μM $[glibenclamide]_{cis}$ were −0.43, −.73 and −0.73 at voltages of 0, −50 and −70 mV, respectively. The parameter ratios I_{max}(glibenclamide)/I_{max} and I'(glibenclamide)/I' at different voltages show that at voltages between −70 and 0 mV, where channel activity is dominant, the reduction in I_{max} is voltage dependent whereas the reduction in I' is voltage independent (Fig. 2*C*). The glibenclamide-induced changes in the channel conductance and kinetics were reversible ($n = 5$) after perfusion with glibenclamide-free solution.

Analysis of the SCl channel activity, where the current level for detecting channel opening was set at 1 pA, reveals that the effects of $[glibenclamide]_{cis}$ on the voltage-dependent kinetic parameters of the SCl channel were mainly voltage independent at voltages between −70 and +60 mV (Fig. 3). The presence of 50 μM $[glibenclamide]_{cis}$ compressed the bell-shaped voltage dependency of P_o, and T_o (Fig. 3*A* and *C*). The bell-shaped voltage dependency of F_o was initially amplified at 25 μM $[glibenclamide]_{cis}$ and then compressed in the presence of 50 μM $[glibenclamide]_{cis}$ to levels close to that measured for glibenclamide-free solution (Fig. 3*B*). The mean closed time did not appear to change in the presence of 25 and 50 μM $[glibenclamide]_{cis}$ (Fig. 3*D*). These findings suggest that the glibenclamide-induced decrease in P_o is due to a reduction in T_o and not to changes in T_c.

Concentration-Dependency of Glibenclamide-Inhibition of the SCl Channel

The $[glibenclamide]_{cis}$ dependency of the conductance and kinetic properties of the SCl channel was also ex-

amined. Decreases in P_o and T_o in response to increases in $[glibenclamide]_{cis}$ are shown in Fig. 4*A* and *C*, respectively. Glibenclamide-induced channel flickering is manifested by slight increases in T_c and initial increase in F_o followed by a decrease at $[glibenclamide]_{cis}$ between 30 and 50 μM. The increase in F_o at $[glibenclamide]_{cis}$ between 0 and 30 μM (Fig. 4*B*) confirms that the decline in P_o is mainly due to a decrease in the mean open time T_o (Fig. 4*C*). At $[glibenclamide]_{cis}$ higher than 30 μM the decline in P_o is not due only to a decrease in T_o but also to a decrease in F_o and a slight increase in T_c.

The inhibitory effects of $[glibenclamide]_{cis}$ on the parameters I_{max}, I' and P_o expressed as fractions of that in the glibenclamide-free control solution were also obtained (*data not shown*). The estimated concentrations of the $[glibenclamide]_{cis}$ for the half inhibitory constant, K_i, were 33.15 and 31.05 μM for the parameters I_{max} and I', respectively. Similarly, K_i values were 30.73, 29.62 and 21.85 μM for the parameter ratios I_{max}(glibenclamide)/I_{max}, I'(glibenclamide)/I' and P_o(glibenclamide)/P_o, respectively.

Inhibitory Effects of Glibenclamide (Cytoplasmic Side vs. Luminal Side of the SCl Channel)

The effects of the luminal additions of glibenclamide on the SCl channel were recorded and corresponding all-points histograms were constructed (Fig. 5). Glibenclamide was less effective from the luminal side than from the cytoplasmic side of the channel (Fig. 5*B*). For example, compare current traces at 50 μM $[glibenclamide]_{trans}$ in Fig. 5*B* with those of the same channel at 50 μM $[glibenclamide]_{cis}$ in Fig. 1*C*. To eliminate possible differences in channel sensitivity to glibenclamide the effectiveness of $[glibenclamide]_{cis}$ in inhibiting the SCl channel from the cytoplasmic side over that from the luminal side was also reproduced on the same channel ($n = 3$).

At 50 μM $[glibenclamide]_{trans}$, channel transitions between the closed and the open states increased from 43 per sec to 57 per sec and the mean open time decreased from 12.47 msec to 9.13 msec (both obtained form the total channel activity in 16 episodes) causing only a small decline in I'. The ratio I'(glibenclamide)/I' is reduced on the average by only ~20% (*data not shown*).

For another SCl channel, the ratio I_{max}(glibenclamide)/I_{max} (mean of channel activity in 16 episodes) was not affected significantly ($P > 0.05$) by 50 μM $[glibenclamide]_{trans}$. The mean values of I_{max} and I' were −4.43 ± 0.37 and −1.46 ± 0.26 pA in control and −4.36 ± 0.75 pA and −1.71 ± 0.52 pA in 50 μM $[glibenclamide]_{trans}$, respectively. The inhibitory effects of $[glibenclamide]_{cis}$ (Fig. 1) or $[ATP]_{cis}$ (Kourie, 1997*b*) on the ATP-sensitive SCl channel were not prevented by the presence of 50 μM $[glibenclamide]_{trans}$. In Fig. 5*C* the SCl channel was typically inhibited by 2 mM $[ATP]_{cis}$. Both I' and I_{max} were affected significantly ($P < 0.01$) by 2 mM $[ATP]_{cis}$. The mean values of I_{max} and I' were reduced from −4.36 ± 0.75 pA and −1.76 ± 0.52 pA in 50 μM $[glibenclamide]_{trans}$ to −2.50 ± 0.22 and −0.34 ± 0.09 pA in 50 μM $[glibenclamide]_{trans}$ + 50 μM $[ATP]_{cis}$, respectively. Channel activity recovered fully after wash with ATP-free and glibenclamide-free *cis* and *trans* control solution, respectively (Fig. 5*D*), in a manner similar to that observed after wash with glibenclamide and ATP-free *cis* control solution (Fig. 1*D*).

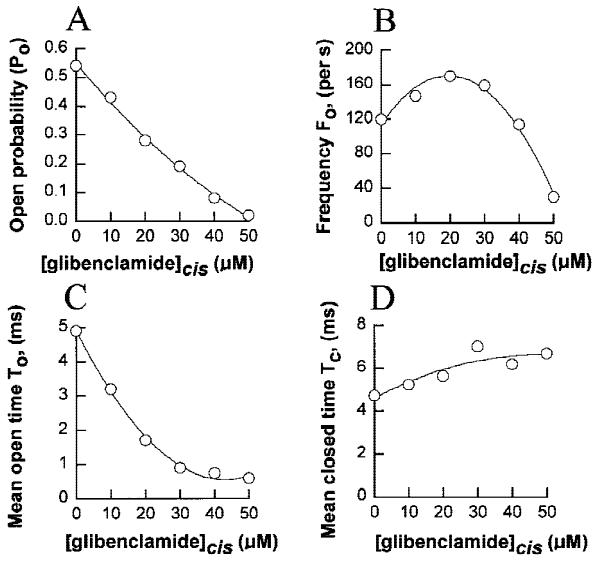

Fig. 4. Concentration-dependency of glibenclamide effects on the SCl channel kinetic parameters. (*A*) Open probability (P_o) (*B*) frequency F_o, (*C*) mean open time T_o and (*D*) mean closed time T_c. Single-channel currents are recorded after an SR vesicle of skeletal muscle incorporated in an optimal bilayer held at −40 mV and in asymmetrical choline-Cl (250 mM/50 mM; *cis/trans*). The solid lines are drawn to a second order polynomial fit.

Effects of ATP-Sensitive K^+ Channel Openers on the SCl Channel Effects of Diazoxide

The effects of $[diazoxide]_{cis}$ on the ATP-sensitive SCl channel were examined at −40 mV (Fig. 6) and analysis of the channel activity within bursts was conducted. The $[diazoxide]_{cis}$-sensitivity of the SCl channel kinetics, within bursts, is characterized by a progressive reduction in the transitions to the closed state so that the maximal open state is dominant after 25 sec from the time of the treatment with the saturating concentration of ≈0.4 mM $[diazoxide]_{cis}$. However, $[diazoxide]_{cis}$ had no effect on the conductance properties of the channel as indicated by maximal current, I_{max} (Fig. 6*A* and *B*). The all-points histograms constructed from burst activities in longer segments (13–15 episodes) of recording at −40 mV also

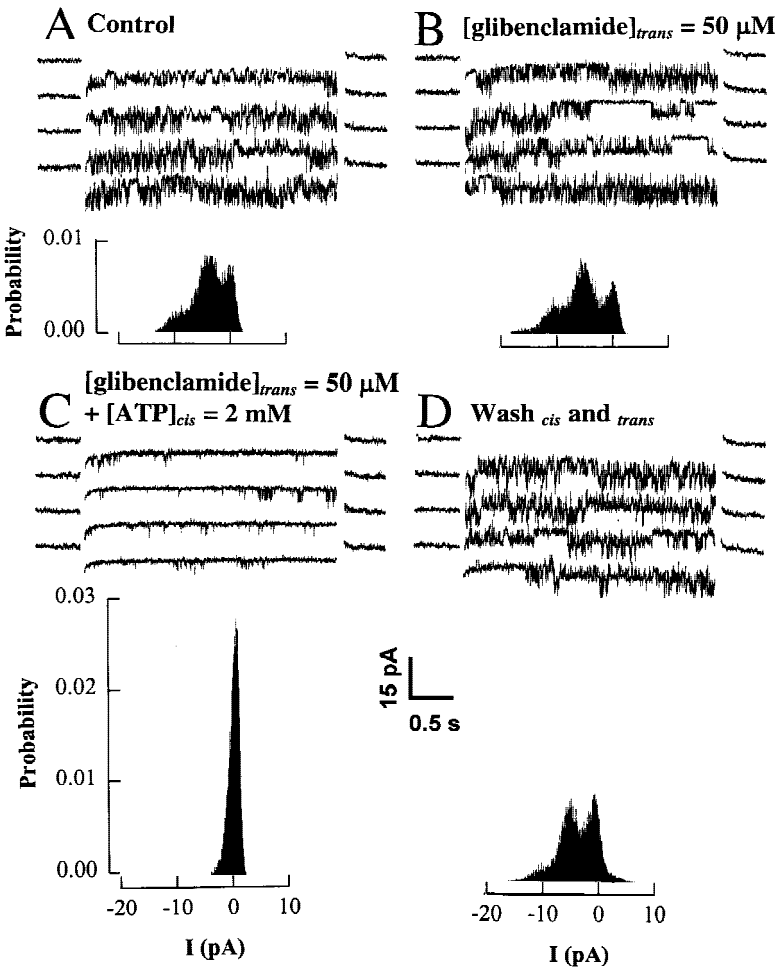

Fig. 5. Effects of $[ATP]_{cis}$ on the SCl channel activity recorded at −40 mV in the presence of $[glibenclamide]_{trans}$. Shown are four representative single-channel current traces and all-points histograms measured for (*A*) control, (*B*) 50 μM $[glibenclamide]_{trans}$, (*C*) 50 μM $[glibenclamide]_{trans}$ + 2 mM $[ATP]_{cis}$, and (*D*) wash, recovery after perfusion with ATP- and glibenclamide-free solution. This bilayer contains two overlapping SCl channels seen in (*A*), (*B*), and (*D*).

show that I_{max} (the distance between the two peaks) is not affected significantly by the saturating concentration of ≈0.4 mM $[diazoxide]_{cis}$ (Fig. 6*A* and *B*). Analysis of burst activities of current families consisting of 13 to 15 episodes confirms that $[diazoxide]_{cis}$ caused a significant ($P < 0.01$) increase in I' from −2.34 ± 0.37 pA in control to −2.94 ± 0.21 pA in the saturated concentration of ≈0.4 mM $[diazoxide]_{cis}$. I_{max} was not affected significantly being −4.58 ± 0.49 pA in control and −4.71 ± 0.49 pA in the saturated concentration of ≈0.4 mM $[diazoxide]_{cis}$. Analysis of the time course of diazoxide effect on an SCl channel shows that I' increased significantly within 70 sec whereas I_{max} changed only slightly. The values of P_o and T_o within the burst increased significantly ($P > 0.01$) from 0.43 ± 0.19 and 16.78 ± 1.88 msec in control to 0.75 ± 0.18 and 27.92 ± 2.11 msec in the saturated concentration of ≈0.4 mM $[diazoxide]_{cis}$.

In agreement with the pharmacological modulation of the ATP-sensitive K^+ channel, the diazoxide-increased I' of the ATP-sensitive SCl channel was significantly ($P < 0.01$) inhibited by the sulfonylurea glibenclamide (Fig. 6*C*). This effect was observed at voltages between −60 and 0 mV (*data not shown*). For example, for a bilayer that is clamped at −40 mV, I' was reduced significantly ($P < 0.01$) from −2.94 ± 0.21 pA in the saturated concentrations of ≈0.4 mM $[diazoxide]_{cis}$ to −1.42 ± 0.72 pA in the saturated concentration of ≈0.4 mM $[diazoxide]_{cis}$ + 50 $[glibenclamide]_{cis}$. I_{max} was also significantly ($P < 0.01$) reduced from −4.71 ± 0.49 pA in the saturated concentration of ≈0.4 mM $[diazoxide]_{cis}$ to −3.89 ± 0.43 pA in the saturated concentration of ≈0.4 mM $[diazoxide]_{cis}$ + 50 μM $[glibenclamide]_{cis}$.

The effects of $[diazoxide]_{cis}$ on ATP-induced inhibition of the SCl channel was also examined (Fig. 7). It was found that $[diazoxide]_{cis}$ at a saturating concentration of ≈0.4 mM (Fig. 7*C*), failed to activate or remove the ATP-induced inhibition of the SCl channel (Fig. 7*B*) or to have any effect on the reversibility of ATP-induced channel inhibition (Fig. 7*D*). Both I_{max} and I' significantly ($P < 0.01$) reduced from −5.07 ± 0.27 and −2.89 ± 0.60 pA in control solution to −2.41 ± 0.74 and −0.96 ± 0.40 pA in the presence of ≈0.4 mM $[diazoxide]_{cis}$ + 2 mM $[ATP]_{cis}$. However, there was no significant difference ($P \geqslant 0.05$) in I_{max} and I' values −2.48 ± 0.59 and

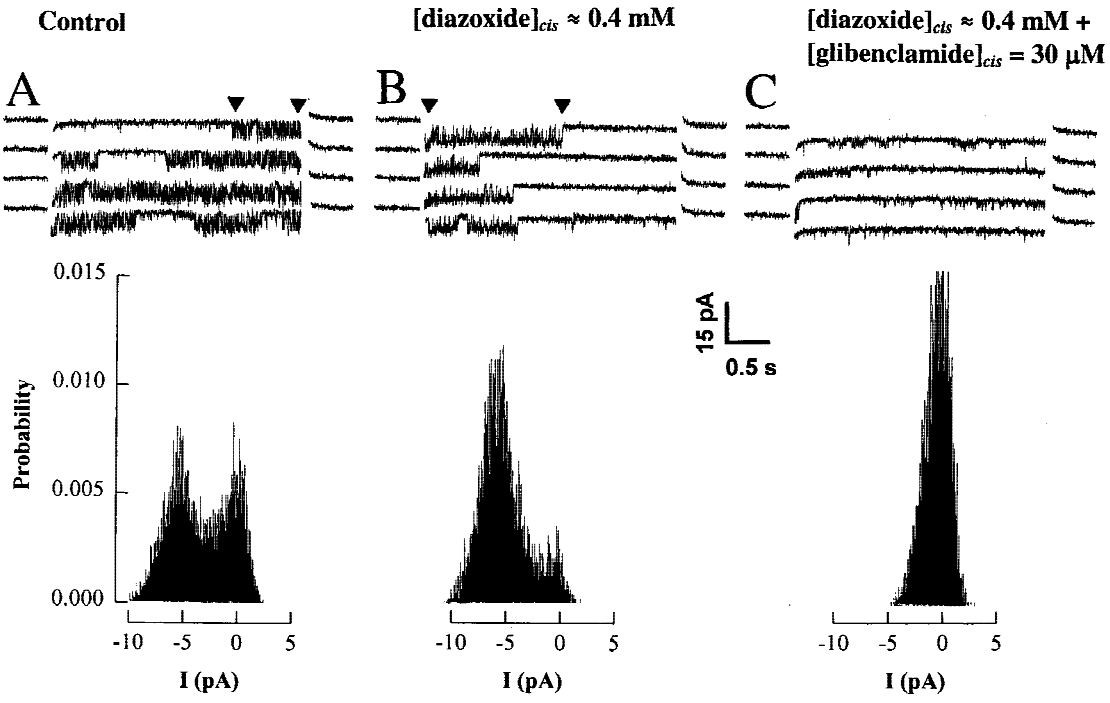

Fig. 6. Enhancement of SCl channel open state by $[diazoxide]_{cis}$ and its inhibition by $[glibenclamide]_{cis}$. Shown are four representative single-channel current traces at −40 mV and all-points histograms constructed from channel activity within bursts for (*A*) control, (*B*) saturation concentration of ≈0.4 mM $[diazoxide]_{cis}$, (*C*) saturation concentration of ≈0.4 mM $[diazoxide]_{cis}$ + 30 μM $[glibenclamide]_{cis}$. The two solid triangles above the current traces in (*A*) and (*B*) point to the start and the end of ''bursts'' of activity, respectively.

−0.79 ± 0.19 pA at 2 mM $[ATP]_{cis}$ and −2.60 ± 0.74 and −0.96 ± 0.40 pA in the presence of ≈0.4 mM $[diazoxide]_{cis}$ + 2 mM $[ATP]_{cis}$, respectively.

Effects of Minoxidil

The addition of 150 μM minoxidil, which belongs to the pyrimidine class of ATP-sensitive K^+ channel openers (Edwards & Weston, 1993), to the cytoplasmic side of the channel was examined (Fig. 8). The current traces in Fig. 8 and the corresponding all-points histograms show that 5 sec after the addition of minoxidil the probability of the channel being in the open state increased (Fig. 8*B*). However, this is followed by a reduction in the amplitude of the single channel current after 110 sec while the channel remained under the same conditions (Fig. 8*C*).

Analysis of current families consisting of 9 to 13 episodes reveals that the initial effect of minoxidil at 5 sec is characterized by a significant increase in I' ($P < 0.01$) from −1.70 ± 0.39 to −2.75 ± 0.44 pA while I_{max} was not affected significantly ($P > 0.05$) being −4.17 ± 0.66 and −4.08 ± 0.18 pA, respectively. In contrast, the effect of minoxidil at 110 sec is characterized by a significant decrease ($P < 0.01$) in I_{max} from −4.17 ± 0.66 to −3.07 ± 0.28 pA. The minoxidil-induced enhancement in I' is reduced to a level not significantly ($P > 0.05$) different from I' measured in control solution being −1.70 ± 0.39 and −1.58 ± 0.35 pA, respectively.

In the presence of ATP or glibenclamide on the cytoplasmic side of the channel, where the channel is inhibited, minoxidil failed to transiently activate or remove channel inhibition induced either by 2 mM ATP or 50 μM glibenclamide in the *cis* chamber ($n = 4$ and 2, respectively).

Effects of Cromakalim

Cromakalim (a potent smooth muscle relaxant) which belongs to the benzopyran class of ATP-sensitive K^+ channel openers (Edwards & Weston, 1993), at 80 μM, modified the SCl channel kinetics in the absence of ATP (Fig. 9). The inhibition is characterized by an increase in channel transitions to the closed state and a reduction in mean open time of the channel. The all-points histograms reveal that the amplitude of the maximal current was not affected. Channel analysis of 14 to 16 episodes confirms that cromakalim significantly ($P < 0.01$) reduced I' from −1.44 ± 0.34 to −0.76 ± 0.28 pA while I_{max} was not affected significantly ($P > 0.05$) being −4.27 ± 0.61 and −4.09 ± 0.09 pA, respectively. Also, the values of P_o and T_o were significantly ($P > 0.01$) reduced from 0.59 ± 0.16 and 18.42 ± 2.58 msec in control to 0.29 ±

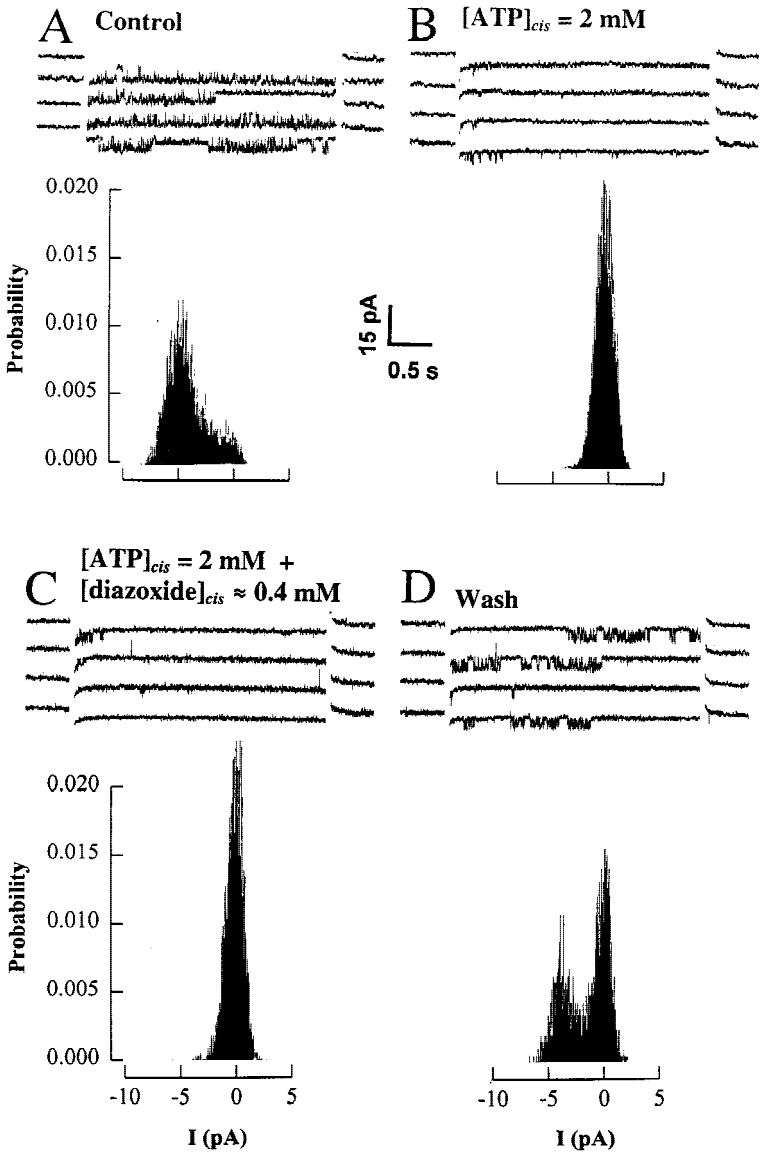

Fig. 7. Effects of $[diazoxide]_{cis}$ on SCl channel activity in the presence of $[ATP]_{cis}$. Shown are four representative single-channel current traces at −40 mV and all-points histograms constructed from ~50-sec long records (*A*) control, (*B*) 2 mM $[ATP]_{cis}$, (*C*) 2 mM $[ATP]_{cis}$ + ≈0.4 mM $[diazoxide]_{cis}$ and (*D*) wash, recovery after perfusion with ATP- and diazoxide-free solution.

0.07 and 9.18 ± 1.26 msec in *cis* solutions containing 80 μM cromakalim.

The presence of cromakalim on the cytoplasmic side of the channel did not prevent ATP from blocking the channel. Reduction in current amplitude is seen after the addition of 2 mM ATP to the cytoplasmic side of the channel in the presence of cromakalim (Fig. 9*C*). In the presence of $[cromakalim]_{cis}$ and 2 mM $[ATP]_{cis}$ both I_{max} and I' were significantly ($P < 0.01$) reduced to −1.41 ± 0.13 and −0.8 ± 0.05 pA, respectively.

Effects of Cl⁻ Channel Openers on the SCl Channel

The effects of diazepam and GABA on the SCl channel were also investigated to examine any possible similarities with the GABA receptor Cl⁻ channel whose structure and activation mechanism are better understood than those of the SCl channel. It was found that neither diazepam at 100 μM nor GABA at 150 μM had any significant effect on the SCl channel conductance and kinetics ($n = 3$ and 5, respectively).

Discussion

Mechanism of Glibenclamide-Induced SCl Channel Inhibition

Glibenclamide-modification of the kinetics and conductance of rabbit skeletal SR ATP-sensitive SCl channels is characterized by: (i) an initial increase in the transitions

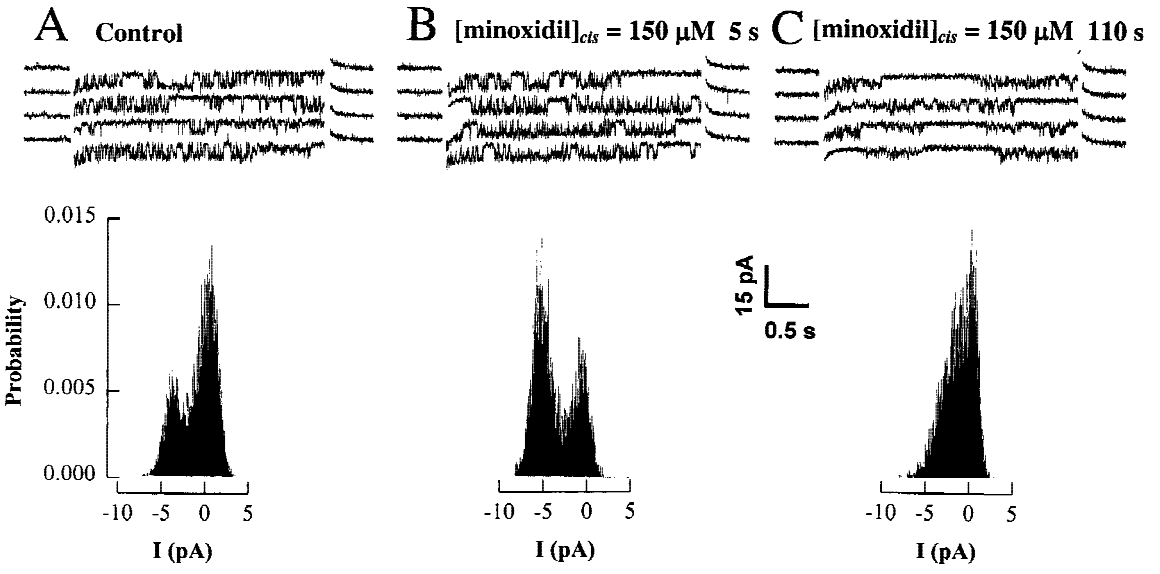

Fig. 8. The effects of 150 μM $[minoxidil]_{cis}$ on SCl channel activity. Four representative single-channel current traces recorded at −40 mV and all-points histograms (*A*) control, (*B*) 5 sec after the addition of 150 μM $[minoxidil]_{cis}$, (*C*) 110 sec after the addition of 150 μM $[minoxidil]_{cis}$.

to the maximal and submaximal open states within bursts, (ii) gradual increase in I' and I_{max}, followed by a progressive reduction in the number of events to high conductance levels and (iii) the appearance of incomplete transitions. During the burst the mean open time decreased and the frequency of current transitions to the closed state increased (Figs. 1–4).

These effects of glibenclamide are typical of a ''flicker block'' type (*see* Hille, 1992). In such a mechanism it is thought that glibenclamide induces its effects by interacting with the SCl channel gating mechanism, e.g., rapid binding and unbinding to the channel proteins, rather than by simple slow block of the conductive pathway. The structure of the SCl channel has not been examined yet, therefore, on a molecular level it is not known how this effect of glibenclamide is brought about.

In addition to differences in the conductance and kinetic properties, the pharmacological profile of the ATP-sensitive Cl^- channels is different from the ATP-activated Cl^- channels in cardiac (Tominaga et al., 1995) and epithelial cells (Sheppard & Welsh, 1992). For example, time- and voltage-dependent SCl channel activation and inactivation are seen in the absence of ATP. The SCl channel does not require ATP phosphorylation for burst activation and hydrolysis for burst termination as has been proposed for the CFTR Cl^- channel (*see* Carson, Travis & Welsh, 1995) and the cAMP-activated Cl^- conductance in cardiac muscle (Tominaga et al., 1995). The effects of glibenclamide on the SCl channel are not mediated via phosphorylation or ATP hydrolysis. Unlike the effects of glibenclamide on cAMP-activated Cl^- conductance (Sheppard & Welsh, 1992; Tominaga et al., 1995) and swelling-activated Cl^- conductance (Holevinsky et al., 1994) the glibenclamide-induced changes in current amplitude and fluctuations of the SCl channel of skeletal muscles were reversible immediately after wash with glibenclamide-free *cis* solution (Fig. 1*D*).

Glibenclamide is more potent from the cytoplasmic side than from the luminal side of the SCl channel. This is further confirmed by the finding that the presence of glibenclamide on the luminal side of the channel does not prevent ATP inhibition of the channel from the cytoplasmic side.

The micromolar glibenclamide inhibitory concentrations (Ki = ~ 30 μM) are much higher than those glibenclamide concentrations reported for ATP-activated Cl^- channels, 500 nM, in smooth muscle (Holevinsky et al., 1994), but comparable to those used for the inhibition of swelling-activated chloride conductance, 100 μM, (Meyer & Korbmacher, 1994) and cAMP-activated Cl^- channels, 20–38 μM, (Sheppard & Welsh, 1992; Tominaga et al., 1995). Similarly, these concentrations are close to those reported for single ATP-sensitive K^+ channels in smooth muscle, 20 μM, (Standen et al., 1989). They are also close to the concentrations of the inhibitor tolbutamide used to inhibit ATP-sensitive K^+ channels in skeletal, 60 μM, (Woll, Lönnendonker & Neumcke, 1989) and cardiac muscles, 380 μM, (Sturgess et al., 1988).

Interaction of ATP-Sensitive K^+ Channel Openers with SCl Channels

In this study it has been shown for the first time that diazoxide increases both the probability of the channel

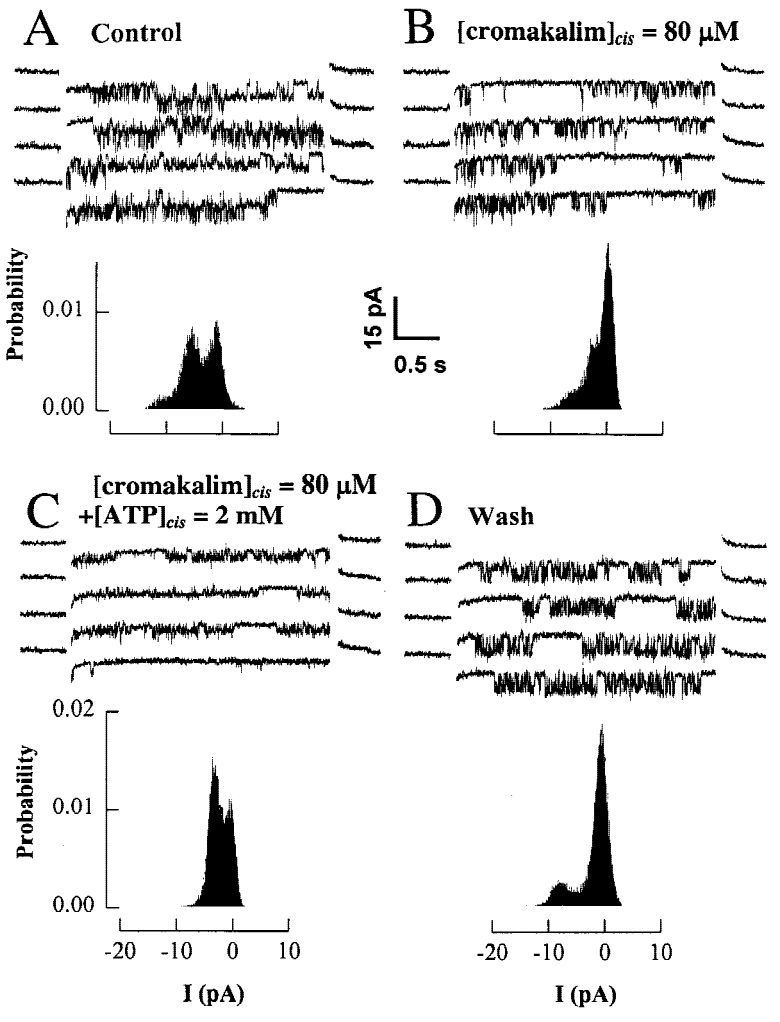

Fig. 9. The inhibitory effects of $[cromakalim]_{cis}$ on SCl channels activity. Four representative single-channel current traces recorded at −40 mV and all-points histograms (*A*) control, (*B*) 80 μM $[cromakalim]_{cis}$, (*C*) 80 μM $[cromakalim]_{cis}$ + 2 mM $[ATP]_{cis}$ and (*D*) wash with glibenclamide-free solution. This bilayer contains two overlapping SCl channels seen in (*A*) and (*B*) of which only one appears to recover after wash. (*D*).

being in the open state and the mean open time and hence the mean Ca^{2+} counter current flowing through the ATP-sensitive SCl channel in the SR of skeletal muscle. Unlike diazoxide, minoxidil transiently increases the probability of the channel being open but then it inhibits the maximal current whereas cromakalim modifies the channel kinetics. The molecular mechanism responsible for the differences in the action of these compounds is not known. These findings point to the fact that ATP-sensitive K^+ channel openers lack a common structure that is linked to their action on ATP-sensitive channels.

The fact that diazoxide interaction with the SCl channel protein causes modification of SCl channel kinetics without altering the single-channel current amplitude suggests that these effects may be due to an interaction with a binding site on the channel protein or coprotein outside the conductive pathway. Whether glibenclamide and ATP inhibit the diazoxide-enhanced SCl channel by interacting with the diazoxide binding site or with other distinct binding sites is also not known.

The minoxidil-induced initial increase in I' that was followed by inhibition of I_{max} may suggest that this compound becomes a more potent blocker of the channel conductance after modifying the channel kinetics in the open state. This suggestion is further supported by the finding that, in the presence of ATP or glibenclamide, minoxidil failed to transiently activate or remove channel inhibition.

Therapeutic Implications of ATP-Sensitive K^+ Channel Openers in Skeletal Muscle

The changes in conductance and kinetics of ion channels, e.g., Cl^- and Ca^{2+} channels, which underlie the electrical properties of the SR in skeletal muscle are coupled to the metabolism of the muscle fiber. The SCl channel is coupled to the muscle metabolism via cytosolic factors, e.g., Ca^{2+} (Kourie et al., 1996), ATP (Kourie, 1997*b*) and IP_3 (Kourie et al., 1997) that are also essential for the excitation-contraction mechanism. Hence, synthetic molecules that mimic the action of these coupling fac-

tors, i.e., by opening and closing Cl^- channels, are important in therapeutic approaches to Cl^- channelopathies that may underly abnormal muscle function.

The therapeutic effects of ATP-sensitive K^+ channel openers in smooth and cardiac muscles are well established (Edwards & Weston, 1993; Lawson, 1996). On the other hand, the effects of these agents on skeletal muscle pathologies are not well known. This is because the involvement of ATP-sensitive channels in pathologic conditions of skeletal muscle has not been well established. However, such involvement could be indicated from the following evidence:

(i) Lowering the intracellular pH, e.g., under hypoxia and pathologic fatigue, increases the activity of ATP-sensitive K^+ channels in skeletal muscle (Davies, 1990).

(ii) Ischemia-induced damage in rat skeletal muscle has been shown to be prevented by cromakalim (Hatton et al., 1991). However, it is not known whether this effect is mediated via ATP-sensitive K^+ and/or Cl^- channels. The fact that the ATP-sensitive SCl channel is inhibited under low oxygen pressure and reducing agent and activated by normoxic conditions and oxidizing agents indicates a possible role for this channel under hypoxic conditions (Kourie, 1997*a*).

(iii) The cromakalim, pinacidil and RP-49356-induced hyperpolarization, mediated via increase in P_o of the ATP-sensitive K^+ channels, in human skeletal muscle fibers from patients with myotonic dystrophy or hypokalaemic periodic paralysis is larger than those from normal volunteers (Spuler, Lehmann-Horn & Grafe, 1989; Quasthoff et al., 1990).

It is concluded that $[glibenclamide]_{cis}$ inhibits both ATP-activated Cl^- channels, present in epithelial, smooth and cardiac muscles, and ATP-sensitive Cl^- channels in SR skeletal muscle. ATP-sensitive K^+ channel openers (levcromakalim, minoxidil, diazoxide) inhibit ATP-activated Cl^- channels (Sheppard & Welsh, 1992; Holevinsky, 1992; Tominaga et al., 1995). In the SR cromakalim also exerts inhibitory effects on the ATP-sensitive SCl channel whereas diazoxide increases the probability of this channel being open. Diazoxide-induced muscle relaxation could be at least partly mediated via its effects on the Ca^{2+}-counter current through ATP-sensitive SCl channels. This suggestion does not seem unreasonable when taken together with the findings that diazoxide has no effects on ATP-sensitive K^+ channels in skeletal muscle (Weik & Neumcke, 1989, 1990).

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