J. Membrane Biol. 157, 39–51 (1997)

The Journal of
Membrane
Biology

ATP-sensitive Voltage- and Calcium-dependent Chloride Channels in Sarcoplasmic Reticulum Vesicles from Rabbit Skeletal Muscle

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Received: 4 September 1996/Revised: 6 December 1996

Abstract. Chloride channels in the sarcoplasmic reticulum (SR) are thought to play an essential role in excitation-contraction (E-C) coupling by balancing charge movement during calcium release and uptake. In this study the nucleotide-sensitivity of Cl^- channels in the SR from rabbit skeletal muscle was investigated using the lipid bilayer technique. Two distinct ATP-sensitive Cl^- channels that differ in their conductance and kinetic properties and in the mechanism of ATP-induced channel inhibition were observed. The first, a nonfrequent 150 pS channel was inhibited by *trans* (luminal) ATP, and the second, a common 75 pS small chloride (SCl) channel was inhibited by *cis* (cytoplasmic) ATP. In the case of the SCl channel the ATP-induced reversible decline in the values of current (maximal current amplitude, I_{max} and integral current, I') and kinetic parameters (frequency of opening F_O, probability of the channel being open P_O, mean open T_O and closed T_c times) show a nonspecific block of the voltage- and Ca^{2+}-dependent SCl channel. ATP was a more potent blocker from the cytoplasmic side than from the luminal side of the channel. The SCl channel block was not due to Ca^{2+} chelation by ATP, nor to phosphorylation of the channel protein. The inhibitory action of ATP was mimicked by the nonhydrolyzable analogue adenylylimidodiphosphate (AMP-PNP) in the absence of Mg^{2+}. The inhibitory potency of the adenine nucleotides was charge dependent in the following order $ATP^{4-} > ADP^{3-} >>> AMP^{2-}$. The data suggest that ATP-induced effects are mediated via an open channel block mechanism. Modulation of the SCl channel by $[ATP]_{cis}$ and $[Ca^{2+}]_{cis}$ indicates that (i) this channel senses the bioenergetic state of the muscle fiber and (ii) it is linked to the ATP-dependent cycling of the Ca^{2+} between the SR and the sarcoplasm.

Key words: ATP-sensitive — Chloride channel — Sarcoplasmic reticulum — Skeletal muscle — Counter-ion channel

Introduction

ATP-sensitive (inhibited) Cl^- channels (for definition *see* Edwards & Weston, 1993) are less common than their counterpart the ATP-sensitive K^+ channels, whereas ATP-activated Cl^- channels are recorded frequently (e.g., Anderson et al., 1991; Kawano et al., 1992; Nagel et al., 1992; Zhang & Jacob, 1994). Furthermore, ATP-activated Cl^- channels in epithelial cells (Sheppard & Welsh, 1992) and in smooth muscle fibers (Holevinsky et al., 1994) are inhibited by openers of the ATP-sensitive K^+ channel. Several anion channels have recently been reported to have a significant permeability to ATP. For example, the multidrug resistance (mdr) protein (Abraham et al., 1993; Valverde et al., 1992) and the cystic fibrosis transmembrane regulator (CFTR) (Reisin et al., 1994; Schwiebert et al., 1995) have been described as dual ATP and Cl^- channels, however, this has been disputed (Reddy et al., 1994; Wang et al., 1994) and remains controversial. So far, Cl^--selective channels that have been reported to be ATP-sensitive are found in human platelet surface membrane (Manning & Williams, 1989) and in nuclear membrane isolated from rat liver (Tabares, Mazzanti & Clapham, 1991) and sheep cardiac ventricular cells (Rousseau et al., 1996). An ATP-sensitive nonselective ion channel that allows the permeation of Cl^- has also been found in neonatal rat cultured central neurones (Ashford et al., 1988). In addition, a

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regulatory protein of chloride conductance, that has been obtained by expression cloning with cDNA from Madin-Darby canine kidney epithelial cells in *Xenopus oocytes,* induces an outwardly rectifying Cl^- current, defined as pI_{Cln}, and inhibited by extracellular nucleotides (Krapivinsky et al., 1994; Paulmichl et al., 1992). The properties of the ATP-inhibited Cl^- permeant channels are summarized in the Table. These properties include an inhibitory ATP concentration between 0.5 and 5 mM and a "flicker block" type of inhibition.

In recent studies, SR vesicles from skeletal muscle reconstituted into bilayers revealed the gating modes of a "big" Cl^- channel conductance (BCl) and a smaller Cl^- channel conductance (SCl) (Kourie et al., 1996*b*). Understanding the regulation of these channels is essential to our understanding of the function of the SR Cl^- channels as pathways for Ca^{2+} counter current. It is important to further our understanding of the regulation of the SCl channel by nucleotides which are essential for muscle function. They are known to reflect the bioenergetic state of the muscle fiber, play an important role in the mechanism of muscle contraction and relaxation and undergo changes during normal and/or adverse conditions, e.g., acidosis, muscle fatigue and hypoxia (*see* Rüegg, 1988). The aim of this investigation is to study the modulatory effects of nucleotides, which regulate various transport pathways including ion channels and active primary pumps, on the gating of the SR Cl channels. Specifically: (i) to characterize the ATP-sensitivity of the SCl channel, (ii) to examine whether this sensitivity is dependent on protein phosphorylation and (iii) to determine the specificity and efficacy of ATP, GTP, ADP, AMP and the nonhydrolyzable derivative of ATP (AMP-PNP). The data provide evidence for the presence of nucleotide-sensitive Cl^- channels in the SR of skeletal muscle.

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). Muscle was dissected from New Zealand rabbits and either used fresh or stored at −70°C. Cubes of muscle were homogenized in a Waring blender in homogenizing buffer (mM: imidazole, 20; sucrose, 300, adjusted to pH 7.1 with HCl). The homogenate was centrifuged (11,000× *g*, 15 min) and the pellet resuspended, rehomogenized (4 × 15 sec) and recentrifuged as above. The supernatant was filtered through cotton gauze and centrifuged at 110,000× *g* for 60 min. The crude SR vesicle pellet was layered onto a discontinuous sucrose gradient containing 28, 32, 34, 38 and 45% sucrose (in 20 mM imidazole, pH 7.1 adjusted with HCl), and centrifuged for 16 hr at 20,000 rpm using a Beckman SW28 rotor. Vesicles were collected from the following density interfaces: Band 1 (B1), 28–32%; Band 2 (B2), 32–34%; Band 3 (B3), 34–38%; Band 4 (B4), 38–45%. Vesicles were diluted 3-fold in 20 mM imidazole (pH, 7.1), pelleted at 125,000× *g*, resuspended in homogenizing buffer (at 10 to 20 mg/ml protein), frozen and stored in liquid N_2. All procedures were performed at 4°C and all buffers contained the protease inhibitors: leupeptin, 1 μM; pepstatin A, 1 μM; benzamidine, 1 mM and PMSF, 0.7 mM.

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). μl aliquots of nucleotides (Sigma) stock solutions, i.e., *cis* solution contained nucleotides, buffered to pH 7.4, were used to increment $[\text{nucleotides}]_{cis}$.

Lipid Bilayers and Vesicle Fusion

Bilayers were formed across a 150 μm hole in the wall of a 1 ml delrin^T cup using a mixture of palmitoyl-oleoyl-phosphatidylethanolamine, palmitoyl-oleoyl-phosphatidylserine and palmitoyl-oleoyl-phosphatidylcholine (5:3:2, by volume) (Ahern, Junankar & Dulhunty 1994; Kourie, 1996, 1997), obtained in chloroform from Avanti Polar Lipids (Alabaster, Alabama). The lipid mixture was dried under a stream of N_2 and redissolved in *n*-decane at a final concentration of 50 mg/ml. SR vesicles were added to the *cis* chamber to a final protein concentration of 1–10 μg/ml. 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 et al., 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* Figs. 2 and 3) 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 Corp.). 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*). The SCl channel activity and current levels shown in this and other studies (Kourie, 1996, 1997; Kourie, Foster & Dulhunty, 1997; Kourie et al., 1996*ab*) were representative of current records obtained from different preparations (n = 18) of SR vesicles which incorporated into more than 250 different bilayers, each containing single active channel.

Data Analysis

Data were obtained only from bilayers having a specific capacitance >0.42 μF/cm^2 which contained one active SCl channel. The criteria for defining ion currents as belonging to a "single channel" have been described elsewhere (Colquhoun & Hawkes, 1983). Episodes of 50 to 200 sec of single-channel activity were analyzed for overall characteristics using CHANNEL 2 (developed by P.W. Gage & M. Smith, JCSMR) to obtain the open probability, P_O, i.e., the fraction of time that the channel was open. The analysis was repeated three times

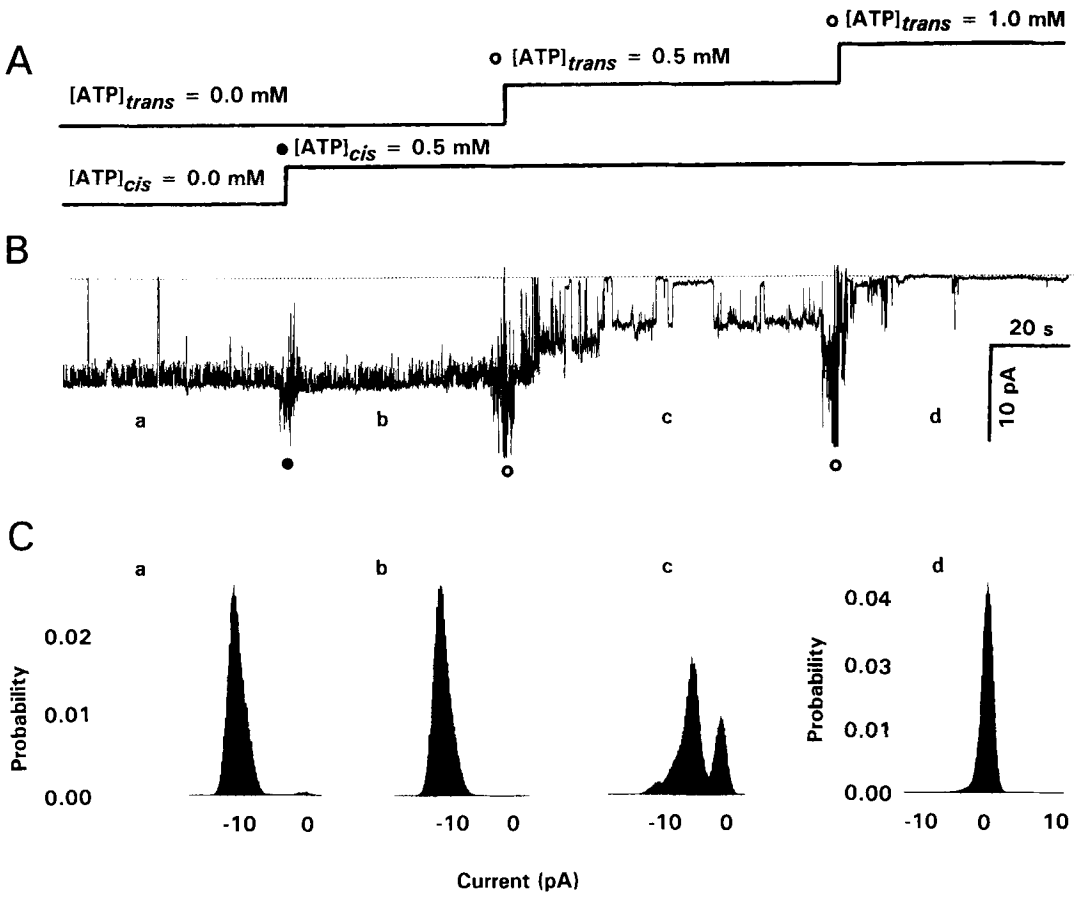

Fig. 1. Effects of increasing $[ATP]_{cis}$ and $[ATP]_{trans}$ on a large Cl^- permeant anion channel. (*A*) The solid straight lines show the time course for ATP additions to the *cis* (filled circle) and *trans* (*open circles*) chambers, respectively. (*B*) Continuous current trace illustrating the time course of $[ATP]_{cis}$ and $[ATP]_{trans}$ effects on the channel activity. The optimal bilayer which has a specific capacitance $C_b > 0.42$ $\mu F/cm^2$, is held at −20 mV (with respect to *trans* chamber) and in asymmetrical choline-Cl (250 mM/50 mM; *cis/trans*). The circles indicate artifacts (mechanical noise) associated with additions and stirring of 0.5 mM ATP into the *cis* (filled) or *trans* (open) solutions. The dotted line indicates zero current level. Downward deflections denote activation of the inward Cl^- current. The current trace is filtered at f_c = 0.2 kHz for display only. (*C*) The all-points histograms (*a–d*) are constructed from the corresponding current segments marked *a–d* which were filtered originally at 1 kHz and digitized at 2 kHz.

where the threshold was set at 9, 18 and 27% of the maximal current (I_{max}), to include current transitions to substates less than 50% of the maximum conductance. Such a prudent analysis of substates was necessary since transitions to these substates contribute significantly to channel activity. This was particularly essential in the presence of nucleotides where transitions to substates were very 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 kinetic parameters of the SCl channel before and after the channel had been subjected to any treatment.

ABBREVIATIONS:

ADP	Adenosine-5′-diphosphate (sodium salt from bacterial source)
AMP	Adenosine-5′-monophosphate (from yeast)
AMP-PNP	adenylylimidodiphosphate (lithium salt)
ATP	Adenosine-5′-triphosphate (magnesium salt of disodium salt)
GTP	Guanosine 5′-triphosphate (sodium salt, Type III)
I'	average mean current
I_{max}	maximum current
SCl channel	Small Cl^- channel
ER	endoplasmic reticulum
SR	sarcoplasmic reticulum

Results

ATP-Sensitivity of a Large Cl^- Permeant Anion Channel

Figure 1 shows the effects of ATP added to the *cis* and *trans* chambers on the activity of a single Cl^- permeant channel recorded at −20 mV in Mg^{2+}-free solutions (250 mM/50 mM Cl^-; *cis/trans*). The channel is voltage independent with P_O of ~ 1 (*not shown*). The maximal con-

ductance is 150 pS and there are two subconductance states of 107 and 71 pS. This channel was insensitive to the presence of ATP on the cytoplasmic side of the channel ($n = 4$) as shown in the segment marked *b* on the current trace in Fig. 1 where the conductance and kinetic properties of the channel are unaffected by 0.5 mM $[ATP]_{cis}$. The channel was sensitive to ATP added to the luminal side of the bilayer (corresponding to the lumen of the SR) where the subconductance states are uncoupled (*see* segment *c* of the current trace). At luminal [ATP] of 0.5 the dominant maximal channel conductance gave way to distinct subconductance states of 107, 71 and 10 pS with P_O of 0.32 and the current transitions, which were mainly between the 150 and 107 pS states, gave way to slow transitions between 71, 10.6 and 0 pS (closed) states with P_O of 0.15. At luminal [ATP] of 1.0 mM these slow transitions to the 71 pS became shorter and transitions to the 10.6 pS state and the closed state became longer. The frequency of recording this channel was low and hence this investigation focuses only on the effects of nucleotides on the well characterized SCl channel.

ATP-Sensitivity of the SCl Channel

Unlike the activity of the ATP-sensitive K^+ channel (e.g., Ashcroft & Kakei, 1989) which experiences rundown with time, the SCl channel activity is stable in the absence of ATP at cytoplasmic side of the channel facing the *cis* solution. However, the SCl channel inactivates at negative voltages and a depolarizing prepulse can remove this inactivation. A voltage protocol (Fig. 2*A*) was used to examine the voltage-dependence of the channel in ATP-free control solution (Fig. 2*B*) and during exposure to 0.5 mM $[ATP]_{cis}$ (Fig. 2*C*). Single-channel activity was characterized by bursts of openings. All channels demonstrated the usual voltage-dependent activity with a maximum conductance of ~75 pS, and openings to several subconductance states, as has been detailed previously (Kourie et al., 1996*b*). The channel activity was affected rapidly by $[ATP]_{cis}$ at all voltages. To study the nature of the ATP-induced channel block at different voltages the dependency of I' and P_O on the bilayer potential was examined (Fig. 3). In Fig. 3*A* it is apparent that in the presence of ATP, I' values were reduced to a similar level at voltages between 0 and −70 mV. In addition, at voltages between +30 and +60 mV there was a semiparallel reduction in I' and a shift in the reversal potential from +35 to +42 mV. At −40 and −70 mV the values of P_O for the SCl channel shown in Fig. 2 were reduced from 0.76 and 0.29 at control to similar values of 0.09 and 0.06 at 0.5 mM $[ATP]_{cis}$, respectively. However, bursts of channel activity and channel inactivation were still apparent e.g., the last current trace at −70 mV in Fig. 2*C*. ATP also induced ~ +10 mV shift in the voltage-dependency of P_O (Fig. 3*B*). The effects of

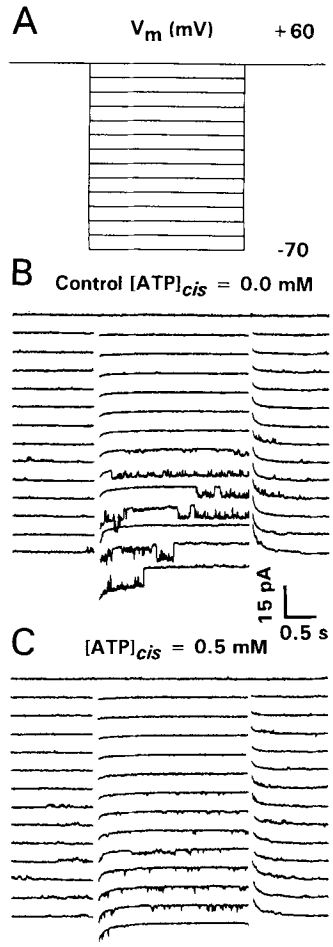

Fig. 2. Effects of $[ATP]_{cis}$ on the voltage-dependence of SCl channel activity. (*A*) Voltage protocol. (*B*) Control current traces recorded in asymmetrical choline-Cl (250 mM/50 mM; *cis/trans*) and (*C*) current traces recorded from the same channel after the addition of 0.5 mM ATP to the *cis* 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 an optimal bilayer having a specific capacitance, C_b, 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 has been removed and the traces have been offset by 9 pA for a better display.

$[ATP]_{cis}$ on conductance and kinetic properties of the SCl channel were rapidly reversible ($n = 7$) after perfusion with ATP-free solution in a manner similar to that shown e.g., in Figs. 4*B*, and 7*D*.

Concentration-Dependency of ATP-Inhibition of the SCl Channel

The [ATP] dependency of the SCl channel was examined using the voltage protocol shown in Fig. 4*A*. The current traces shown in Fig. 4*B* reveal that the ATP-sensitivity of the SCl channel is characterized by: (i) an initial increase in the transitions to the maximal and submaximal open states within bursts (indicated by filled circles), (ii)

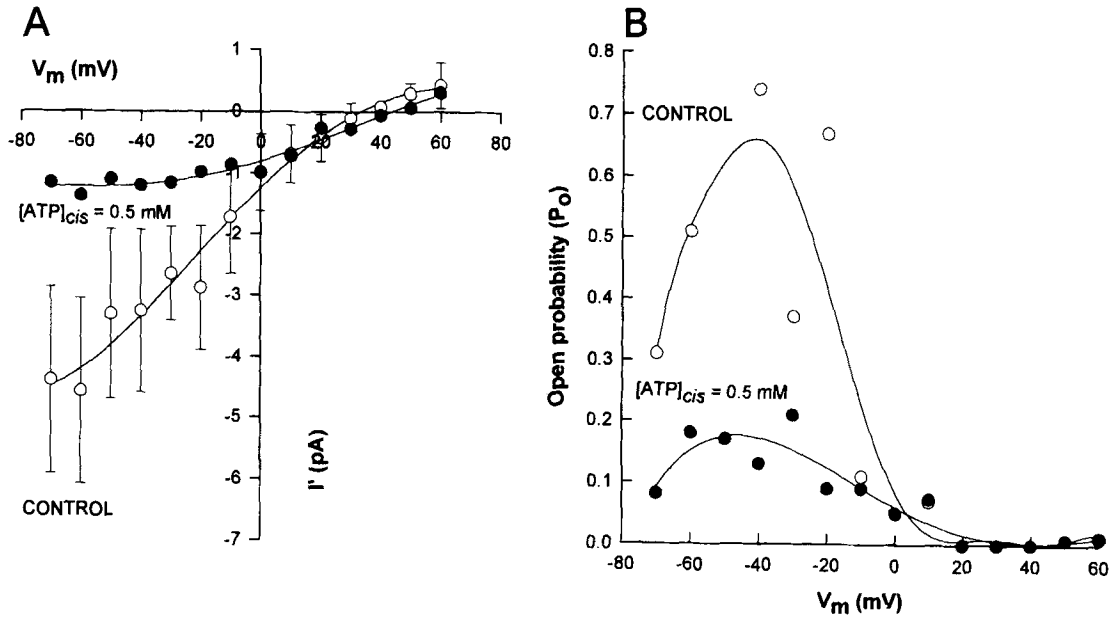

Fig. 3. Effects of 0.5 mM $[ATP]_{cis}$ on the voltage-dependence of conductance and kinetic parameters of an SCl channel. (*A*) Mean current, I', the integral of the current with respect to time. Open symbols and vertical bars denote mean of I' and standard deviations for four runs on the channel in the absence of ATP. Closed symbols denote I' of the same channel for a single run in the presence of ATP. The solid lines are drawn to a fourth order polynomial fit. (*B*) Open probability (P_O) of the same channel under same conditions as in *A*. The solid lines are drawn to the fourth and eighth order polynomial fit in presence and absence of ATP. The optima bilayer is held at −40 mV in asymmetrical choline-Cl (250 mM/50 mM; *cis/trans*). ATP

gradual decrease in I_{max}, followed by: (iii) a progressive reduction in the number of events to high conductance levels and the appearance of many incomplete transitions at $[ATP]_{cis}$ between 0.3 and 0.5 mM and (iv) the inhibition is fully reversible after washing with ATP-free control solution. The all-point histograms shown in Fig. 4*C–F* constructed from longer segments (16 episodes) of recording at −40 mV (*see* legends) confirm that I_{max} (the distance between the two peaks) is reduced significantly by increasing $[ATP]_{cis}$ (*see* Fig. 4*D* and *E*). However, the effects on I_{max} were reversible (Fig. 4*F*; see also Fig. 6*A*).

Although bursts of channel activity appear to be conserved in the presence of ATP (Fig. 4*B*), the records show a progressive change in channel activity corresponding to increments in $[ATP]_{cis}$. A detailed analysis of typical single channel activity at −40 mV (*see* also Kourie et al., 1996*ab*) revealed ATP-induced modification of channel gating as deduced from the kinetic parameters (Fig. 5). It is apparent that the current level set to detect channel opening qualitatively influenced the measured probability of the SCl channel being open (P_O) (Fig. 5*A*), the frequency of events (F_O) (Fig. 5*B*) and the mean closed time (T_c) (Fig. 5*D*) but not the mean open time (T_O; i.e., the total time that the channel was open to all conductance levels, divided by the length of the record) (Fig. 5*C*). Exponential decreases in P_O and T_O in response to increases in $[ATP]_{cis}$ are apparent in Fig. 5*A* and Fig. 5*C*, respectively. T_c increased gradually and also F_O but then decreased between 0.3 and 0.5 mM $[ATP]_{cis}$. The physiologically important mean current, I' i.e., the integral of the current with respect to time, divided by the total time, like I_{max}, was also reversibly reduced in the presence of $[ATP]_{cis}$ (Fig. 6*A*).

Figure 6 shows the dose-response curves for the inhibitory effects of ATP on I_{max} (Fig. 6*B*), I' (Fig. 6*C*) and P_O (Fig. 6*D*). All three parameters I_{max}, I' and P_O expressed as fractions of that in the ATP-free control solution. The data are fitted to a second order polynomial equation. It is apparent that the concentration of the $[ATP]_{cis}$ for the half inhibitory constant, K_i, is dependent on the type of the parameter ratio used as criterion. For example, K_i values were 0.43 and 0.17 mM for the parameter ratios $I_{max(ATP)}/I_{max}$ and $I'_{(ATP)}/I'$, respectively. K_i value is also dependent on current threshold set for data analysis (*see* data analysis in Materials and Methods). The K_i values for the parameter ratio $P_{O(ATP)}/P_O$ obtained at a threshold of 0.5 and 1.5 pA were 0.36 and 0.24 mM, respectively.

The Effects of Nucleotides are not due to Ca^{2+} Chelation

To ascertain that the inhibitory effects of ATP and other nucleotides on the Ca^{2+}-activated SCl channel are not due to Ca^{2+} chelation by these nucleotides, the $[Ca^{2+}]_{cis}$ or $[Ca^{2+}]_{trans}$ were raised to 6.0 mM in the presence of 0.5

Table. Properties of currently known ATP-sensitive anion channels

Membrane type	Single channel conductance (pS)	Method and [Cl] *cis/trans* or bath/ pipette (mM)	Current-voltage (*I-V*)	[ATP] (mM)	Membrane side *cis/trans* or extracellular/ intracellular	Mode of action	[AMP-PNP]	Tissue type	Reference
Nuclear	30	Lipid bilayer 250/50 CsCl	Linear inward current between −100 and +30 mV	2	*Cis* (cytoplasmic)	Flicker block at −60 mV	?	Sheep cardiac ventricular cells	(Rousseau et al., 1996)
cDNA encoding protein expressed in *Xenopus oocytes*	?	Voltage clamp whole cell 104/? Cl E_{Cl} −40 to −30 mV	Outward rectifying current at −100 to +40 mV	0–1	Extracellular	Reduced whole-cell current amplitude	?	MDCK epithelial cell line	(Paulmichl et al., 1992)
Nuclear	58	Patch clamp inside-out 144/144 Cl	Outward rectifying current at −40 to +60 mV	0.5–5	Extracellular and intracellular	Flicker block at +60 mV	?	Rat liver	(Tabares et al., 1991)
Surface	160 at −40 mV 70 at +10 mV No subconductances	Lipid bilayer 450/450 KCl	Inward rectifying current at −80 to +70 mV	1–5	Added to *cis* and *trans*	Flicker block only at −10 to −70 mV	?	Human platelet	(Manning & Williams, 1989)
Surface	57 at 0 to +50 mV 18.6 at 0 to −50 mV	Patch clamp inside-out 140/140 Cl	Outward rectifying current at -70 to + 70 mV	2	Cytoplasmic	Flicker block only at +50 mV	Not effective	Neonatal rat cultured central neurones	(Ashford et al., 1988)
SR	75 Five subconductances	Lipid bilayer 250/50 choline-Cl	Linear inward current between −70 and +60 mV	0–1	*Cis* (cytoplasmic)	Flicker block and then reduction in current amplitude at −70 to +60 mV	Effective	Skeletal muscle	This study
SR	150 Two subconductances	Lipid bilayer 250/50 choline-Cl	Linear inward current between −60 and +60 mV	0–1	*Trans* (luminal)	Uncoupling subconductances at −20 mV	?	Skeletal muscle	This study

mM [ATP] (n = 5). This was sufficient to saturate the buffering capability of the nucleotides. Figure 7 shows the results of such an experiment in which control channel activity at −40 mV (Fig. 7*A*), inhibited by 0.5 mM $[ATP]_{cis}$ (Fig. 7*B*), was not significantly reactivated by further addition of 5.0 mM Ca^{2+} (the control solution initially contained 1.0 mM Ca^{2+}) in the continued presence of ATP (Fig. 7*C*). For example, analysis of channel activity (16 episodes; where the amplitude of current threshold was set at 1.0 pA) in the presence of 0.5 mM $[ATP]_{cis}$ the values of P_O, F_O, T_O, and T_c were 0.17, 102 events/sec, 0.98 msec and 4.9 msec, respectively. Similarly, the values of these parameters were 0.19, 97 events/sec, 0.95 msec and 5.2 msec, respectively, in the

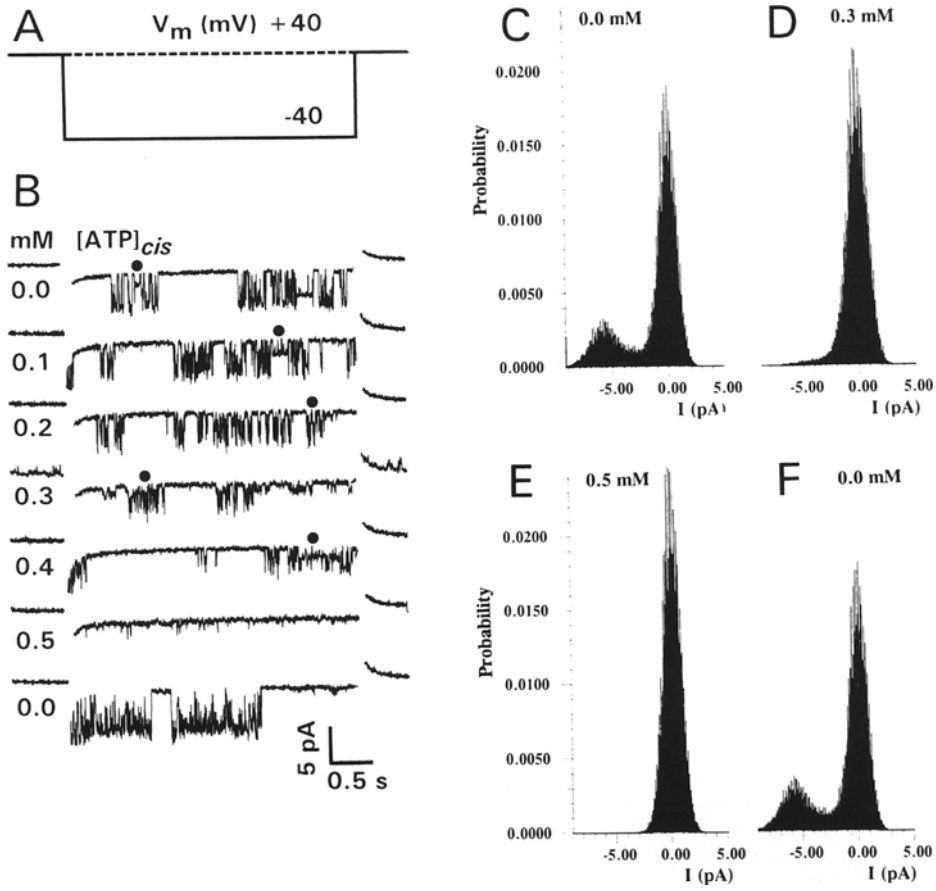

Fig. 4. The dose response of the ATP-sensitive SCl channel. (*A*) Voltage protocol used to obtain current traces in this and following figures and (*B*) current traces recorded at different $[ATP]_{cis}$ shown on the left of the current traces. Each current trace is a representative of 16 traces (episodes) obtained sequentially in response to the voltage episode shown in (*A*) applied 16 times across the same bilayer which contained a single active SCl channel. Representative all-points histograms for the SCl channel shown in (*B*) illustrating the effects of ATP on the probability of various current levels recorded at −40 mV. (*C*) control, (*D*) 0.3 mM $[ATP]_{cis}$, (*E*) 0.5 mM $[ATP]_{cis}$ and (*F*) wash with *cis* control solution. The duration of the each dissected data segment (16 episodes) in *A–D* was between 50 and 60 sec. The histograms are constructed from data filtered at 1 kHz and sampled at 2 kHz. The optimal bilayer is held at −40 mV in asymmetrical choline-Cl (250 mM/50 mM; *cis/trans*).

presence of 0.5 mM $[ATP]_{cis}$ + further addition of 5.0 mM $[Ca^{2+}]_{cis}$. However, channel activity recovered fully after washing with ATP-free control solution (Fig. 7*D*).

Inhibitory Effects of Nucleotides (Cytoplasmic Side Vs. Luminal Side of the SCl Channel

The effect of the luminal (*trans*) additions of ATP on the SCl channel was also examined. ATP was less effective from the luminal side of the channel by more than three fold. For example, compare current trace at 1.5 mM $[ATP]_{trans}$ in Fig. 7*F* with that of only 0.5 mM $[ATP]_{cis}$ in Fig. 4*B*. The ratios $P_{O(ATP)}/P_O$ (obtained at a threshold of 1.0 pA), $I_{max(ATP)}/I_{max}$ and $I'_{(ATP)}/I'$ were 0.16, 0.35 and 0.06 at 0.5 mM $[ATP]_{cis}$ and 0.82, 0.61 and 0.55 at 1.5 mM $[ATP]_{trans}$, respectively. The effectiveness of ATP in inhibiting the SCl channel from the cytoplasmic side over that from the luminal side was also demonstrated in the same channel ($n = 3$). The channels were not reactivated by further addition of 5.0 or 10.0 mM Ca^{2+} (the *trans* control solution initially contained 1.0 mM Ca^{2+}) in the continued presence of 1.5 mM ATP ($n = 2$). However, channel activity recovered fully after wash with ATP-free *trans* control solution (Fig. 7*F* bottom trace) in a manner similar to that observed after wash with ATP-free *cis* control solution (Fig. 7*D*).

The SCl Channel Inhibition by AMP-PNP in the Absence of Mg^{2+}

The addition of 1–2 mM Mg^{2+} to the *cis* chamber (close to physiological concentrations at rest) had no effect on the Ca^{2+}-activated SCl channel at 1 mM $[Ca^{2+}]_{cis}$ nor on the inactivated channel at 10^{-7} M $[Ca^{2+}]_{cis}$ ($n = 3$). The

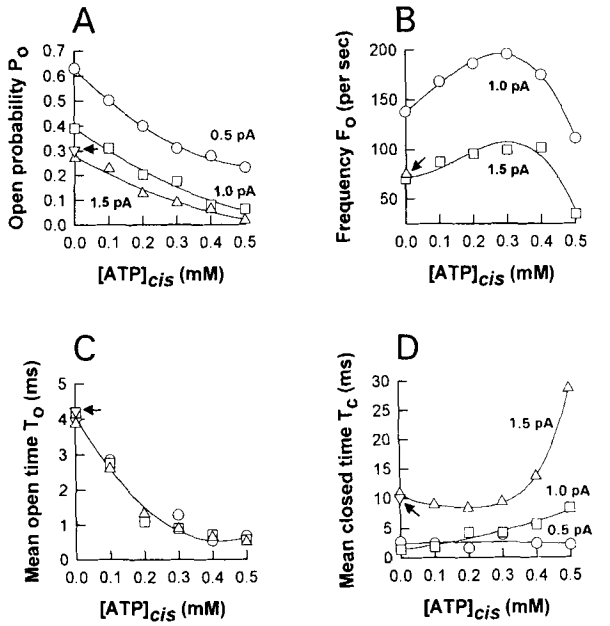

Fig. 5. ATP-dependency of the SCl channel kinetic parameters. (*A*) Open probability (P_O) (*B*) frequency F_O, (*C*) mean open time T_O and (*D*) mean closed time T_c. Single-channel currents are recorded after an SR vesicle of skeletal muscle incorporated in an optimal bilayer held at −40 mV and in asymmetrical choline-Cl (250 mM/50 mM; *cis/trans*). The data were obtained from CHANNEL 2 analysis using 0.5 pA (○), 1.0 pA (□) and 1.5 pA (△) threshold i.e., the current amplitude from the base line where the channel is closed and current amplitude is 0 pA. At all three thresholds, the effects of ATP on the kinetic parameters were reversible and an example in each panel *A–D* is given as an arrow pointing to a single additional symbol. The analysis is for data filtered at 1 kHz and samples at 2 kHz. The solid lines are drawn to a second order polynomial fit in *A,C* and *D* (at 1.5 pA threshold in *D* the fit is to a fourth order polynomial) and to a third order in *B*.

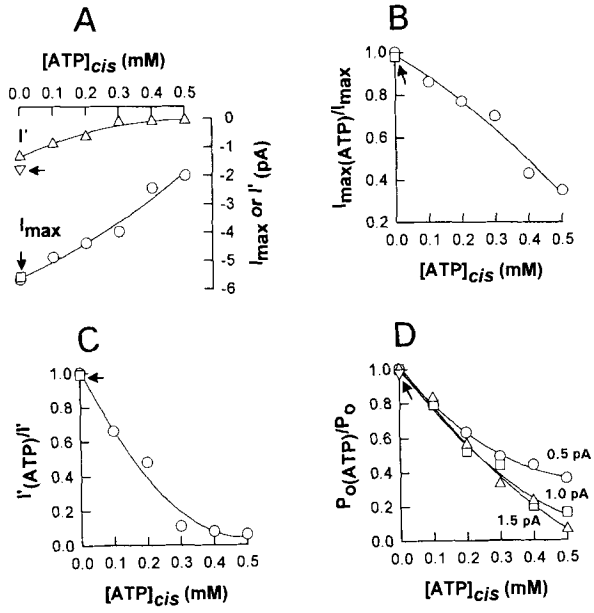

Fig. 6. Dose-dependent effects of ATP on SCl channel parameters. (*A*) I_{max} (○) and I' (△). The (□) and (▽) denote recovery of I_{max} and I' respectively, after wash with *cis* control solution (*B*) $I_{max(ATP)}/I_{max}$ (*C*) $I'(ATP)/I'$ (*D*) $P_{O(ATP}/P_O$ obtained at a threshold of 0.5 pA (○), 1.0 pA (□) and 1.5 pA (△), respectively. The solid lines are drawn to a second order polynomial fit *A–D*. The effects of ATP on current and kinetic parameters were reversible and an example in each panel *A–D* is given as an arrow pointing to a single additional symbol.

SCl channel was sensitive to the presence of the non-hydrolyzable analogue of ATP, adenylylimidodiphosphate (AMP-PNP), in the *cis* chamber (n = 3) (see Fig. 8*A* and *B*). At 0.5 mM [AMP-PNP]$_{Cis}$, the ratios $P_{O(AMP\text{-}PNP)}/P_O$, (obtained at a threshold of 1.0 pA) $I_{max(AMP\text{-}PNP)}/I_{max}$ and $I'_{(AMP\text{-}PNP)}/I'$ were 0.11, 0.29 and 0.05, respectively. These values are similar to those obtained in the presence of 0.5 mM [ATP]$_{Cis}$. An additional similarity was that at 0.5 mM, the [AMP-PNP]$_{cis}$-induced inhibition of the SCl channel activity was fully reversible after wash with *cis* control solution (n = 3).

Specificity and Potency of other Adenine Nucleotides

The nucleotide-inhibition of the SCl channel was not specific for [ATP]$_{cis}$ since 0.5 mM [ADP]$_{cis}$ also reversibly inhibited the channel (n = 5) (e.g., Fig. 8*C–F*), though to a lesser extent than 0.5 mM [ATP]$_{cis}$, [AMP-PNP]$_{cis}$ or [GTP]$_{cis}$. For example, the ratios $P_{O(nucleotide)}/P_O$, $I_{max(nucleotide)}/I_{max}$ and $I'_{(nucleotide)}/I'$ were 0.48, 0.56 and 0.43 at 0.5 mM [ADP]$_{cis}$ and 0.16, 0.35 and 0.06 at 0.5 mM [ATP]$_{cis}$, respectively. The relative potency of different adenine nucleotides was also examined by comparing the effects of nucleotides on the same SCl channel (e.g., Fig. 9). The effect of up to 10.8 mM [AMP]$_{cis}$ on the SCl channel was negligible. The order of inhibition ATP > ADP > > >AMP was the same as that reported for nucleotide sensitive K^+ channel in cardiac myocytes (Kakei, Noma & Shibasaki, 1985), frog skeletal muscle (Spruce, Standen & Stanfield, 1987), and pancreatic β-cells (Davies, Standen & Stanfield, 1991). The inhibitory effects of 0.5 mM [ATP]$_{cis}$ were also observed in the presence of 0.5 mM [ADP]$_{cis}$ (n = 3).

The effect of [GTP]$_{cis}$ was compared with that of [ATP]$_{cis}$ on the same channel (n = 2). Figure 10 shows sequential families of current traces recorded during an experiment in which the effects of 0.5 mM [GTP]$_{cis}$ or [ATP]$_{cis}$ on the SCl channel was examined. It is apparent from the representative current episodes obtained at the same time intervals after the addition of the nucleotides that: (i) the effect of GTP was slow (Fig. 10*B*) and that of ATP was immediate (Fig. 10*D*) and (ii) the effects of GTP on the channel activity, like those of ATP were reversible after exposure to nucleotide-free control solutions (Fig. 10*C*).

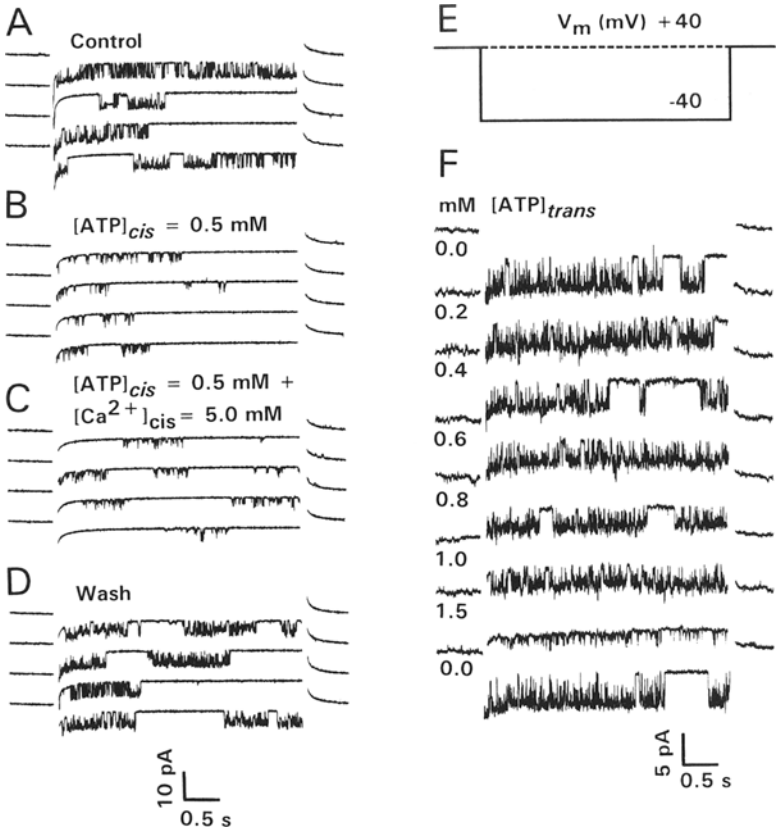

Fig. 7. The inhibitory effects of *cis* ATP on the SCl channel activity are not due to Ca^{2+} chelation by ATP. (*A*) Control, representative current traces of single SCl channel activity and (*B*) channel activity ~15 sec after the addition of 0.5 mM ATP to the *cis* solution. (*C*) Current traces of the same channel as in *A* and *B* immediately after addition of 5.0 mM $[Ca^{2+}]_{cis}$ in the presence of ATP. (*D*) Wash, recovery of channel activity after perfusion with ATP-free control solution. The ATP-dose response for another SCl channel. (*E*) Voltage protocol used to obtain current traces and (*F*) current traces recorded at different $[ATP]_{trans}$ shown on the left of the current traces.

Discussion

The data obtained in this study reveal that in addition to ATP-sensitive K^+ channels that are present in the sarcolemma of skeletal muscle (Spruce, Standen & Stanfield, 1985; Spruce et al., 1987; Weik & Neumcke, 1989), the presence of two distinct ATP-sensitive Cl^- channels in the SR. These channels differ in their conductance and kinetic properties and in the mechanism of ATP-induced channel inhibition (Table). The 150 pS channel has a high open probability and the ATP-induced block from the luminal side uncouples the subconductances of the channel (Fig. 1). It has been postulated that the voltage dependent anion channel (VDAC) provides a transport pathway for polyanions e.g., $citrate^{3-}$ and adenine nucleotides (ATP^{3-} and ADP^{3-} (Colombini, 1989) which are needed for the phosphorylation of the SR luminal proteins such as sarcalumenin, calsequestrin and histidin-rich binding protein (*see* Shoshan-Barmatz et al., 1996). Whether the $[ATP]_{cis}$-insensitive multiconductance anion channel could transport polyanions into the SR lumen needs to be examined. The luminal ATP-induced complete block of the channel activity via several subconductance states points to a tight modulatory mechanism that regulates any Ca^{2+}-leak from the SR through this channel. The inhibitory effects of the nucleotides on this Ca^{2+}-activated SCl channel were not mediated via Ca^{2+} chelation (due to ATP affinity for Ca^{2+}). The inhibition was observed even after the Ca^{2+}-buffering capability of ATP was saturated by a high $[Ca^{2+}]_{cis}$ (Fig. 7*C*). The nucleotide-induced block of the SCl channel from the cytoplasmic side is related to the associated charge, and other polyanions block, concentration dependent (Fig. 4*B*) with an increase in the flicker of both maximal and submaximal conductance states (Fig. 4*B*) and voltage dependent changes in I' and P_O (Fig. 3). ATP also induced reduction in mean open time (T_O), initial increase in the mean closed time (T_c) with increasing ATP (Fig. 5) and shifted the voltage dependency of P_O. These findings point to ATP-induced effects that are mediated via an open channel block mechanism described in the following kinetic scheme:

$$\text{Closed channel} \underset{\text{ATP}}{\rightleftharpoons} \text{Open channel} \underset{\text{ATP}}{\rightleftharpoons} \text{Blocked channel}$$

The results in Figs. 4*B* and Fig. 7*F* concerning SCl channel activity in response to *cis* and *trans* [ATP] indicate that the polyanion binding site (*see below*), involved in channel inhibition, was easier to access from

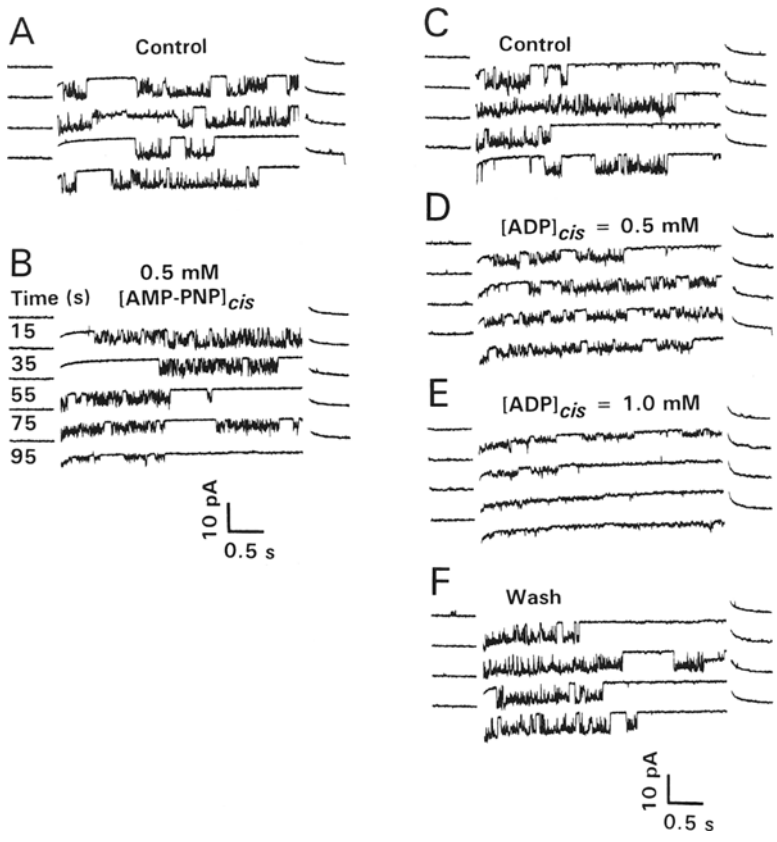

Fig. 8. Effects of the nonhydrolyzable nucleotide, AMP-PNP, on SCl channel activity. (*A*) Control, four representative current traces and (*B*) selected current traces of the same channel recorded sequentially ~10 sec after the addition of 0.5 mM AMP-PNP to the *cis* chamber. The times at which current traces are selected to demonstrate the time-course of $[AMP\text{-}PNP]_{cis}$ effects on the channel activity are indicated on the left of the current traces. Effects of $[ADP]_{cis}$ on another SCl channel activity recorded at (*C*) control, (*D*) 0.5 mM $[ADP]_{cis}$, (*E*) 1.0 mM $[ADP]_{cis}$ and (*F*) Wash, recovery after perfusion with ADP-free solution.

the cytoplasmic side than from the luminal side of the channel. This finding is similar to that reported for the ATP-inhibited Cl^- permeant channels in the surface membrane of human platelets (Manning & Williams, 1989) and ATP-sensitive K^+ channels in β cells and in skeletal and cardiac muscles (*see* Terzic, Jahangir & Kurachi, 1995). In contrast, the outwardly rectifying Cl^- current, pI_{Cln}, is nucleotide-sensitive from the extracellular side of the membrane (Paulmichl et al., 1992; Priebe, Friedrich & Benndrof, 1996). The ATP inhibited Cl^- channel in the nuclear membrane from rat liver is inhibited from either side of the channel (Tabares et al., 1991).

Phosphorylation is not Involved in Gating the SCl Channel

The activity of the SCl channel could be maintained, for as long as the bilayer is stable, in the absence of any added ATP or Mg^{2+} contaminant. The SCl channel activity was inhibited by various nucleotides (Figs. 2, 9 and 10) and AMP-PNP (Fig. 8*B*). These findings suggest that phosphorylation is not required for SCl channel inhibition and are in agreement with the properties of the ATP-sensitive K^+ channel in pancreatic β-cell (Cook & Hales, 1984) and cardiac myocytes (Terzic et al., 1995). In contrast, protein phosphorylation is required for the activation of other Cl^- channels (e.g., Anderson et al., 1991; Anderson & Welsh, 1992; Kawano et al., 1992; Reisin et al., 1994; Zhang & Jacob, 1994). The inactivation of the reconstituted 71 pS (250 mM/50 mM *cis/trans*) Cl^- channel from cardiac SR could be prevented by millimolar concentrations of Mg-ATP or reactivated by the catalytic subunit of protein kinase A (PKA) (Kawano et al., 1992). The ATP-sensitive SCl channel also differs from this Mg-ATP sustained cardiac Cl^- channel (Kawano et al., 1992) in being both Ca^{2+}- and voltage-dependent. Furthermore, the SCl channel retains its sensitivity to $[ATP]_{cis}$ after recovery from inhibition by $[AMP\text{-}PNP]_{cis}$. Hence, this channel is also unlike the nonselective ATP-sensitive ion channel which loses its sensitivity to ATP after treatment with AMP-PNP (Ashford et al., 1988).

The Mechanism of ATP-Induced Modification in the SCl Channel

In pancreatic β cells the influence of ATP on K^+ channel activity is the result of ATP interaction with two pharmacologically distinct sites present on or near the channel proteins: (i) an inhibition site and (ii) availability site (Ashcroft & Kakei, 1989). The first does not require

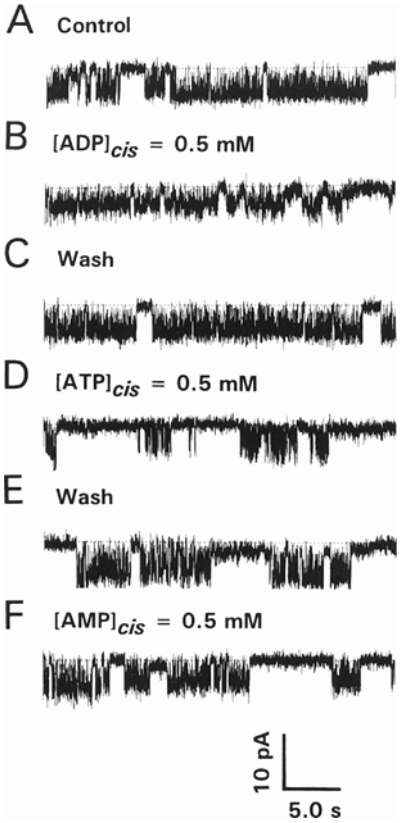

Fig. 9. Single SCl channel activity recorded sequentially at (*A*) control (*B*) 0.5 mM $[ADP]_{cis}$, (*C*) wash (*D*) 0.5 mM $[ATP]_{cis}$ (*E*) wash, and (*F*) 0.5 mM $[AMP]_{cis}$.

phosphorylation whereas the second requires phosphorylation to prevent the rundown of channel activity. The SCl channel does not appear to require the second site since it does not experience rundown. The SCl channel undergoes voltage-dependent inactivation which could be removed by depolarizing pre-pulse. ATP does not appear to interact with the inactivation of the SCl channel. It neither hastens the inactivation nor prevents channel activation. Like the ATP-activated Cl^- channel (Anderson & Welsh, 1992), the ATP-sensitive SCl channel is not highly specific to nucleotide triphosphates. However, unlike the ATP-activated CFTR Cl^- channel, ATP-sensitive SCl channel activation does not require ATP hydrolysis or Mg^{2+} as a cofactor. It is of interest to note that ATP increases P_O of the CFTR Cl^- channel by increasing the frequency F_O of channel activation and shortening the duration of the closed state. The CFTR Cl^- channel is mainly in the closed state in the first place where the channel activation is characterized by single transitions between the closed and the open states (*see* Fig. 1 in Anderson & Welsh, 1992). Similar observation has been reported for the ATP-activated outwardly rectifying Cl^- channel (*see* Fig. 4*A* in Schwiebert et al., 1995). On the other hand, ATP reduced the P_O of the SCl channel by decreasing the mean open time T_O and by increasing the mean closed time T_c and the frequency F_O of channel activity (at least at [ATP] between 0 and 0.3 mM) within the burst i.e., a channel which is initially in the open state (*see* Fig. 4). It has also been found that ATP-induced flickering in channel activity contributes, together with the decline in the open time, to the decrease in P_O of a 30 pS Cl^- channel in nuclei membranes from mammalian cardiac muscle (*see* Fig. 6 in Rousseau et al., 1996). The findings concerning the ATP-induced increase in F_O could indicate a common mechanism of action on both ATP-activated and ATP-inactivated Cl^- channel proteins.

The efficacy of adenine nucleotides in inhibiting the SCl channel is in the following order $ATP^{4-} > ADP^{3-} > > > AMP^{2-}$. This suggests that nucleotide-induction channel inhibition is dependent on adenine nucleotide charge as has been previously suggested for the ATP-sensitive K^+ channel (Ashford & Kakei, 1989). These findings are also in agreement with the finding of a nonspecific inhibition by polyanions, including inositol polyphosphates, of the SCl channel gating mechanism (Kourie et al., 1997). However, the potency of a certain structural configuration that is highly effective in channel inhibition could not be ruled out. The findings in Fig. 10 concerning the role of onset of inhibition by ATP and GTP indicate that, like the ATP-sensitive K^+ channels in skeletal muscle (Spruce et al., 1985), the action of adenine nucleotide could also involve base specificity to the binding site.

Physiological Significance of ATP-Inhibited SCl Channels in the SR

In vivo, the probability for the SCl channels to be open, as for all ATP-sensitive ion channels (*see* Terzic et al., 1995), is low at physiological concentrations of ATP. However, ATP-induced changes in the SCl channel properties may occur as a result of cyclic changes in: ATP hydrolysis (at least in the channel vicinity) or intracellular compartmentation, ATP sensitivity of the SCl channel and other channel modulators such as intracellular H^+ and extracellular adenosine, particularly under metabolic stress e.g., extremely fatigued muscles. By analogy with functional coupling between the ATP-sensitive K^+ channels and the ATP consuming Na^+-K^+ pump in cardiac (Priebe et al., 1996) and in proximal convoluted tubule (Hurst et al., 1993), the physiological significance of ATP-sensitive SCl channels could be viewed in terms of their possible coupling with the ATP consuming electrogenic Ca^{2+}-pump in the SR membrane. Evidence that supports such a functional coupling to Ca^{2+} uptake is found in another internal membrane. For example, a voltage-dependent Cl^- channel acts as a charge carrier to facilitate electrogenic Ca^{2+} uptake in the

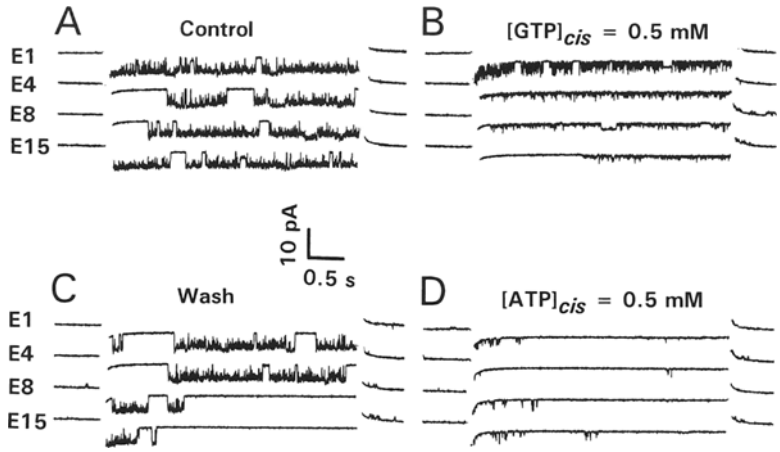

Fig. 10. The inhibitory effects of $[ATP]_{cis}$ on SCl channel activity are mimicked by other nucleotide triphosphates. (*A*) control, four representative single-channel current traces (*B*) 0.5 mM $[GTP]_{cis}$, (*C*) wash and (*D*) 0.5 mM $[ATP]_{cis}$. After the addition of GTP or ATP to the *cis* chamber, the episodes E1, E4, E8 and E15 are taken at times 0, 15, 35 and 70 sec, respectively.

endoplasmic reticulum (ER) (Kemmer et al., 1987; Schmid et al., 1988). Under normal physiological conditions the ATP-inhibition of the SCl channel, as a counter charge pathway for the SR electrogenic Ca^{2+}-pump, would put a "brake" on this pump and prevent a decline in cellular ATP. Such an energy conserving role has also been proposed for the K^+ channel in cardiac muscle (Noma, 1983). The modulation of the SCl channel by $[ATP]_{cis}$ (this study) and $[Ca^{2+}]_{cis}$ (Kourie et al., 1996*b*) strongly suggests that this channel senses the bioenergetic state of the fiber. Therefore, it may be part of ATP-dependent mechanism for recycling of the Ca^{2+} between the SR and the sarcoplasm, e.g., during thermogenesis in aerobic fast-twitch fiber.

It is concluded that in addition to the known direct role of ATP on Ca^{2+}-release channels (Ma & Zhao, 1994; Smith, Coronado & Meissner, 1985) and on the electrogenic Ca^{2+}-pump, ATP-induced modifications in the conductance and kinetic properties of the SCl channels also contribute indirectly to the regulation of $[Ca^{2+}]_{cytoplasm}$. Therefore, adverse physiological and pathological conditions which alter $[ATP]_{cytoplasm}$ can modify the Ca^{2+} counter-current through the SCl channel and consequently may contribute to changes in the $[Ca^{2+}]_{cytoplasm}$ underlying muscle function.

I thank Professor K. Kirk, Drs. A.F. Dulhunty and P.S Foster for critical reading of the manuscript, S. Pace and J. Stivala for isolating and characterizing SR vesicles, L. Bartlett, R. McIlroy and J. Wilkes for technical assistance, Dr. G. Ahern who independently observed the ATP-sensitivity of the SCl channel. Dr. G. Ahren made his initial observation while studying the ryanodine Ca^{2+}-release channel in the presence of ATP and CsCl 250 mM/50 mM (*cis/trans*). I gratefully dedicate this work to Dr. A.F. Dulhunty leader of the Muscle Research Group for introducing me to the research field of muscle physiology. I also thank the reviewers for their helpful suggestions.

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