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Hydrogen Peroxide Inhibits Chloride Channels of the Sarcoplasmic Reticulum of Skeletal Muscle

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Abstract. Data obtained with the lipid bilayer technique indicate that *cis* (cytoplasmic) concentration of 4.4–22 mM hydrogen peroxide (H_2O_2), is a water-soluble oxidant. $[H_2O_2]_{cis}$ ($n = 26$) reversibly inhibits the multisubconductance SCl channel of the sarcoplasmic reticulum vesicles from rabbit skeletal muscle. At −40 mV, the mean values of the current amplitude (I) and the probability of the SCl channel being open (P_o) were reduced significantly ($n = 8$) from −6.14 ± 0.42 pA and 0.69 ± 0.06 (for all conductance levels) in control 0.0 mM $[H_2O_2]_{cis}$ to −1.10 ± 0.51 pA and 0.13 ± 0.04 (for the intermediate subconductance states) in 8.8 mM $[H_2O_2]_{cis}$, respectively. The $[H_2O_2]_{cis}$-induced decrease in P_o is mainly due to a decrease in the mean open time T_o. The mechanism of $[H_2O_2]_{cis}$ effects on the multiconductance SCl channel is characterized by a mode shift in the channel state from the main conductance state to the low subconductance states. The estimated concentration of the $[H_2O_2]_{cis}$ for the half inhibitory constant, K_i, was 11.78 mM, higher than the estimated 8.0 and 8.1 mM for the parameters P_o and T_o, respectively, indicating that the conductance of the SCl channel is less sensitive than the gating kinetics of the channel. After a lag period of between 30 to 60 sec, the lipophilic SH-oxidizing agent 4,4′-dithiodipyridine (4,4′-DTDP) added to the *cis* side at 1.0 mM removed the inhibitory effects of 8.8 mM $[H_2O_2]_{cis}$. The 4,4′-DTDP-enhanced SCl channel activity was blocked after the addition of 0.5 mM ATP to the *cis* side of the channel. The addition of 1.0 mM 4,4′-DTDP to the *cis* or *trans* solutions facing an SCl channel already subjected to 0.5 mM $[ATP]_{cis}$ or $[ATP]_{trans}$ failed to activate the ATP-inhibited SCl channel. These findings suggest that 4,4′-DTDP is not preventing the binding of ATP to its binding site on the channel protein. The interaction of H_2O_2 with the SCl channel proteins is consistent with a thiol-disulfide redox state model for regulating ion transport, where SH groups can directly modify the function of the channel and/or the availability of regulatory sites on the channel proteins. The H_2O_2 effects on the Ca^{2+} countercurrent through the SCl channel are also consistent with H_2O_2-modification of the mechanisms involved in the Ca^{2+} regulation, which underlies excitation-contraction coupling in skeletal muscle.

Key words: Reactive oxygen species — Sulfhydryl group (SH)-oxidizing and SH-reducing agents — ATP-sensitive channels — Bilayer technique — Calcium countercurrent

Introduction

It is well established that reactive oxygen species (ROS) such as superoxide radical anion (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH) contribute to the malfunction of smooth and cardiac muscles under hypoxia-reperfusion conditions. In fatigued skeletal muscle, and also in cardiac and smooth muscles experiencing ischemic-reperfusion conditions, an early result of the ROS-induced modifications in the transmembrane signal is the change in the homeostasis of the cytoplasmic calcium $[Ca^{2+}]_{cyto}$ (for review *see* [20] and references within). In muscles, this change in $[Ca^{2+}]_{cyto}$ can result from direct effects of ROS on Ca^{2+} transport pathways in the sarcolemma and in the sarcoplasmic reticulum (SR) and/or indirectly on the Ca^{2+} countercurrent, through Cl^- channels. It is known that chloride is important for Ca^{2+}-release from the SR (for review *see* [3]).

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Because the SR is an internal membrane, the lipid bilayer technique is the only way to directly study the regulation and function of ion channels in this otherwise inaccessible membrane. Despite some limitations of the technique, e.g., in mimicking the cytoplasmic environment of channels, it has been proven powerful in understanding the fundamental regulatory mechanisms of Cl^-, K^+ and the ryanodine Ca^{2+}-release channels. In this context, the use of the lipid-bilayer technique to study the ATP-sensitive SCl channel [2, 18] provides the best available means of examining the physiological mechanistic aspects of channel function and regulation. This continues in the direction of work already performed in our laboratories, and in others, on Cl^- channels, which has already yielded important information about the regulation and function of Cl^- channels. The regulation and function of chloride channels in muscle function has been reviewed in detail [3, 19]. They play an important role in providing countercurrent during Ca^{2+} release via the Ca^{2+}-release channel, and uptake via the electrogenic Ca^{2+}-pump (Ca^{2+}-Mg^{2+}-ATPase). Some chloride channels are also involved in the transport of high-energy compounds such as ATP and other polyanionic metabolites (J.I. Kourie, *unpublished data*).

A survey of the literature reveals that to date there is no direct evidence for ROS-induced modifications in the activity of single Cl^- channels [20]. Pharmacological probing with anion channel blockers, e.g., 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), points to the possible involvement of anion conductances in the mechanism of ROS-induced injury [13]. However, DIDS is not a specific anion channel blocker. For example, it has also been shown that DIDS affects the activity of the ryanodine Ca^{2+}-release channel [35]. ROS interaction with Cl^--transport mechanisms could be indirectly indicated by the finding that the sulfhydryl group (SH)-oxidizing and SH-reducing modify the activity of one of the SR small Cl^- channels (SCl channel) [17], the human skeletal muscle Cl^- current (hClC-1) expressed in *Xenopus* oocytes and in human embryonic cells [23], the surface membrane Cl^- channel of bovine trachea [28] and the voltage-dependent anion-selective channel (VDAC) in the mitochondrial outer membrane [36].

The aims of this study are: (i) to examine the direct interaction of the water-soluble H_2O_2 (as a ROS investigative probe) with a single SCl channel protein, (ii) to compare the H_2O_2-induced effects on the SCl channel activity with those effects induced by the lipophilic and specific SH-oxidizing agent 4,4′-dithiodipyridine (4,4′-DTDP) [17] and (iii) to probe the effects of H_2O_2 and 4,4′-DTDP on the SCl channel in the presence of ATP. The findings reported in this study provide the first direct evidence for H_2O_2-induced modifications in the electrical properties of a Cl^- channel.

Materials and Methods

Preparation of SR Vesicles

Terminal cisternae or longitudinal SR vesicles from rabbit skeletal muscle [31] were incorporated into lipid bilayers [26] as described previously [17, 21].

Solutions

Solutions contained choline-Cl (250 mM *cis*/50 mM *trans*) plus 1 mM $CaCl_2$ and 10 mM HEPES (pH_{cis} 7.2–7.4, adjusted with Tris). The [H_2O_2] of the *cis* and *trans* solution was adjusted by adding aliquots of 10% H_2O_2 (in water) stock solutions. The maximum dilution of the *cis* or *trans* solutions at the highest [H_2O_2] used in this study was less than 5%, e.g., the addition of 10–50 μl of 10% H_2O_2 is added to a chamber of 1,000 μl.

Lipid Bilayer and Vesicle Fusion

The artificial lipid bilayer technique is widely used to record ion channels from internal membranes which otherwise are inaccessible by patch-clamp electrodes. Lipid bilayers were formed across a 150 μm hole in the wall of a 1 ml delrin™ cup by using a mixture of palmitoyl-oleoyl-phosphatidylethanolamine, palmitoyl-oleoyl-phosphatidylserine and palmitoyl-oleoyl-phosphatidylcholine (5:3:2, by volume) [2, 16–18], which was 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 is defined as *cis*, and the other side as *trans*. The cytoplasmic side of the vesicle is thought to face the *cis* chamber [26]. This was verified by using common ligands, which are known to bind to the cytoplasmic domain of the ryanodine Ca^{2+} release channel protein [1]. The reversal potential was determined by using minimum polynomial fits of the current-voltage curves. The reversal potential was then corrected for the liquid junction potential by using the JPCalc software [4]. The experiments were conducted at 20–25°C.

Recording Single Channel Activity

The pClamp program (Axon Instruments, Foster City, CA) was used for voltage command and acquisition of Cl^- current families 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 bilayer was held at −40 mV in asymmetrical choline-Cl (250/50 mM; *cis/trans*).

Data Analysis

Kinetic analysis was conducted only for optimal bilayers that contained a single active channel [8]. Data were recorded at 5 kHz, filtered at 1 kHz (4-pole Bessel, −3dB) and digitized *via* a TL-1 DMA interface (Axon Instruments) at 2 kHz. An in-house analysis program, CHANNEL 2 (developed by P.W. Gage and M. Smith, at The John Curtin School of Medical Research), was used to measure the following parameters of channel activity: mean open time, T_o (i.e., the total time that the channel was not closed and including openings to all conductance levels, divided by the number of events); T_c (i.e., the total time that the channel was closed divided by the number of events), frequency of opening to all conductance levels F_o and the open probability, P_o (i.e., the sum of all open times as a fraction of the total time). Since measurement of open times and P_o included openings to submaximal conductance levels, the analysis was repeated three times where the threshold for channel opening or closing was set at 9, 18 and 27%, rather than at 50%, of the maximum current amplitude to allow the inclusion of current transitions to subconductance states that are less than 50% of the maximum conductance. Ideally, P_o of each individual subconductance state should be determined. However, it was not possible to determine the P_o dependence of the different individual subconductance states of the SCl channel as has been done for the double barrel Cl^- channel of the *Torpedo electroplax* [25]. Under various ionic conditions the three substates of the double barrel Cl channel of the *Torpedo electroplax* are always equally spaced in conductance. One of these states is the closed state, 0 pS, and this leaves the channel with only two equally spaced conductive substates, 9.4 and 18.5 pS. In addition, the frequencies of these substates follow a binomial distribution so if the frequency of one of these substates is measured, the other two could be accurately predicated by a binomial distribution. Furthermore, the open time distribution has only a single time constant. In contrast, the SCl channel, even under control conditions, is much more complex because: (i) it has a larger number of substates (six subconductance states), not equally spaced; (ii) the channel has three open-time constants and three closed-time constants; (iii) reducing- and oxidizing agents have unequal effects on these substates; and (iv) the channel undergoes voltage-dependent inactivation that can only be removed by a depolarizing voltage step.

Current amplitude, *I*, was obtained by measuring the difference (in pA) between the peak at 0 pA (representing the closed state) and the extreme peak on the left (representing the open state for the majority of distinct events) in the all-points histogram generated by using CHANNEL 2. The value of the current amplitude was also obtained by measuring the difference (in pA) between two lines, one set on the maximum baseline noise of the closed level, where the current amplitude is considered to be 0 pA, and the other set on the noise of the majority of distinct events, longer than 0.5 msec, which were in the open state. Both methods were used and the results were generally in agreement.

Statistics

Unless stated, each SCl channel was used as its own control and the comparison was made between kinetic parameters of the channel both before and after changing the $[H_2O_2]_{cis}$, $[4,4'\text{-DTDP}]_{cis}$, and/or $[ATP]_{cis}$. Data are reported as means ± SEM of channels and the difference in means was analyzed by Student's *t*-test. Data were considered statistically significant when probability (*P*) values were <0.05.

Results

Effects of H_2O_2 on the SCl Channel

H_2O_2 can readily be converted to ·OH *via* the Fenton reaction, which induces lipid peroxidation in the presence of iron or copper [14]. Although it is not known whether the incorporated SR vesicles in the artificial bilayer retain any iron or copper that may catalyze ·OH production, it is important to rule out the possibility of indirect effects of H_2O_2 on the channel activity *via* lipid peroxidation. Experiments conducted on bilayers before the addition of the SR vesicles revealed that the biophysical properties of the phospholipids forming the bilayer ($n = 3$) were not affected by $[H_2O_2]_{cis}$ between 4.4 and 22 mM. The bilayers maintained a specific bilayer capacitance value of ~0.42 $\mu F/cm^2$ and a cord conductance value for the leak of ~12.5 pS in the presence of 4.4, 17.6 and 22 mM $[H_2O_2]_{cis}$. Under the experimental conditions reported here, these values are characteristic of optimal bilayers [16].

Figure 1*A* shows typical SCl channel activity recorded from an optimal bilayer (250 mM *cis*/50 mM *trans* Cl^-) where the cytosolic side of the channel protein is considered to be facing the *cis* solution to which H_2O_2 was added. At −40 mV, the current amplitude of the SCl channel was reversibly reduced, not gradually, but rather in a steplike manner after the addition of 8.8 mM $[H_2O_2]_{cis}$ ($n = 8$) (e.g., Fig. 1*B–G*). The effects of 8.8 mM $[H_2O_2]_{cis}$ are reversible after a wash period of 78 ± 17 sec ($n = 3$) and they can be repeated, after a wash, on the same channel. The effects of $[H_2O_2]_{cis}$ on this multiconductance SCl channel are characterized by a transition from the main conductance state to the low subconductance levels (current traces Fig. 1*B–F*) and recovery after wash to the main conductance state (Fig. 1*G*). This transition can also be indicated by the shift of probability peaks towards the intermediary peaks (histograms Fig. 1*B–F*). Brief prepulses to positive potentials that remove the inactivation of SCl channels [22] failed to reverse the $[H_2O_2]_{cis}$-induced channel transitions to subconductance states (*data not shown*). Current amplitude, *I* (the distance between the closed and open states for the majority of distinct events) and P_o were affected significantly ($P < 0.05$ or $P < 0.01$, $n = 26$) by 8.8–22 mM $[H_2O_2]_{cis}$. At −40 mV, the mean values of *I* and P_o were reduced significantly ($n = 8$) from −6.14 ± 0.42 pA and 0.69 ± 0.06 (for all conductance levels) in control 0.0 mM $[H_2O_2]_{cis}$ to −1.10 ± 0.51 pA and 0.13 ± 0.04 (for the intermediate subconductance states) in 8.8 mM $[H_2O_2]_{cis}$, respectively. *I* and P_o values recovered to −5.88 ± 0.23 pA and 0.69 ± 0.05, respectively, after a wash with the H_2O_2-free control *cis* solution.

Voltage-Dependency of H_2O_2 Effects

To examine the voltage dependency of $[H_2O_2]_{cis}$-induced effects on the SCl channel activity (Fig. 2), a voltage protocol was used to obtain single channel currents. From an initial holding potential of +60 mV the bilayer

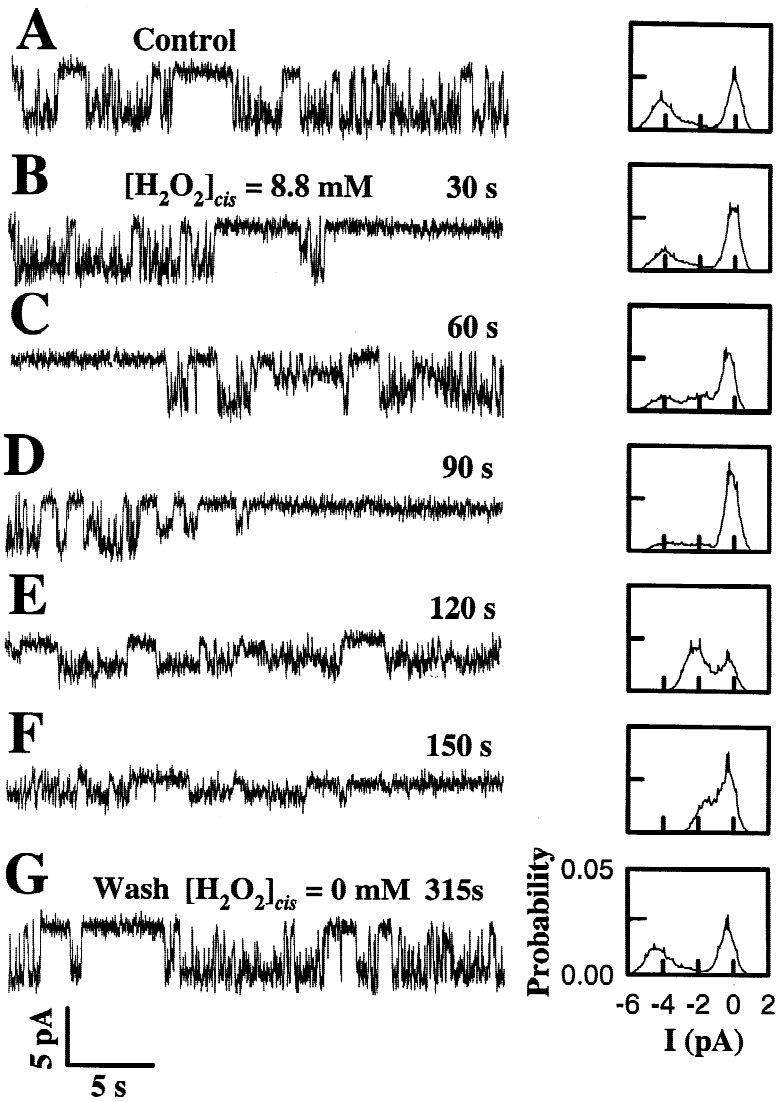

Fig. 1. Effects of 8.8 mM $[H_2O_2]_{cis}$ on the SCl channel activity recorded at −40 mV. Shown are single-channel current records (left) and corresponding all-points histograms (right) measured for: (*A*) control treatment, (*B–F*) continuous 8.8 mM $[H_2O_2]_{cis}$ treatment at different times after its addition to the *cis* chamber and (*G*) wash treatment with H_2O_2-free *cis* solution. For clarity the data are reduced by a factor of 10. The electrical noise between (*A*) and (*B*) and between (*F*) and (*G*) due either to a mechanical stirring of H_2O_2 in the *cis* chamber or to a perfusion of the *cis* chamber with control solution has been removed. Channel opening is downward. The all-points histograms were constructed from longer data segments of 84.29 sec (*A*), 30 sec segments (*B–F*) and 95.14 sec (*G*). The bin width was 0.05 pA.

potential was stepped to voltages ranging from +60 to −70 mV, in steps of −10 mV (Fig. 2*A*). Following the first few milliseconds of the clamp, during which a capacitative transient current occurred, a single channel current was activated, following hyperpolarizing voltage steps (Fig. 2*B*). It is apparent that the effects of $[H_2O_2]_{cis}$ on the SCl channel activity (Fig. 2*C*) were reversible (Fig. 2*D*) and that neither the voltage-dependency of the channel nor the transitions to maximal and submaximal levels had remained modified after a return to perfusion with a control solution for all voltages between −70 and +60 mV. Similarly, H_2O_2, which readily crosses lipid membranes (*see* [20]), inhibited the SCl channel activity at all voltages when it was added to the *trans* side of the channel protein (Fig. 3).

The current-voltage (*I–V*) relationship for the current amplitude, *I*, shows that it is reduced significantly (Fig. 4*A*). For example, at −40 mV, *I* was reduced from −5.64 pA in control to −1.81 pA in 8.8 M $[H_2O_2]_{cis}$. However, the value of *I* recovered to −5.52 pA after washing with an H_2O_2-free control solution. When two and three exponentials were fitted to the data the shift in the reversal potential was 5.58 ± 1.5 mV ($n = 3$) with a maximal shift of 8.7 mV and a minimal shift of 3.8 mV. This small shift in the reversal potential indicates no significant changes in the nature of the $[H_2O_2]_{cis}$-reduced current, which remains a Cl^- current. The effect of $[H_2O_2]_{cis}$ on the voltage-dependent open probability of the SCl channel was also examined at voltages between −70 and +60 mV (Fig. 4*B*). It is apparent that the presence of 8.8 mM $[H_2O_2]_{cis}$ modified the typically bell-shaped voltage dependency of P_o (Fig. 4*B*). The peak of the bell-shaped voltage dependency of P_o shifted towards more negative values, from −40 mV in control *cis* solu-

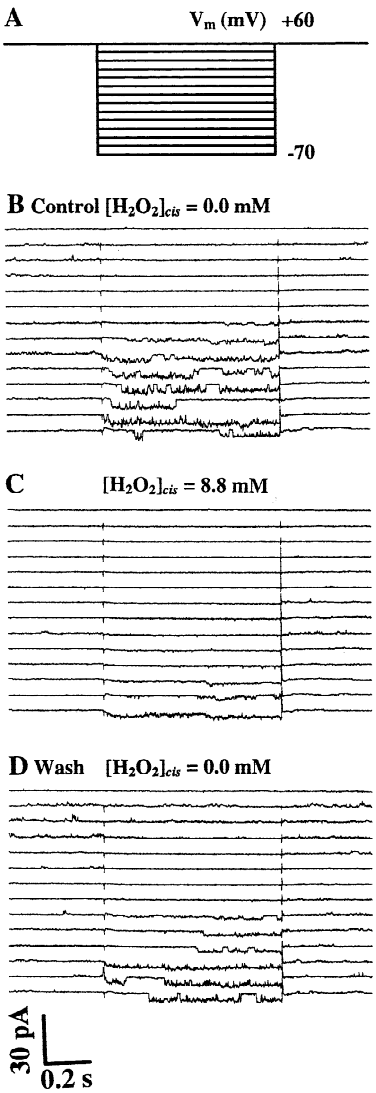

Fig. 2. Effects of $[H_2O_2]_{cis}$ on the voltage-dependence of SCl channel activity. (*A*) The bilayer was clamped from a holding potential of +60 mV to a range of voltages between −70 mV (bottom trace) and +60 mV (top trace) in steps of −10 mV. (*B*) Control current traces recorded in asymmetrical choline-Cl (250 mM/50 mM; *cis/trans*). (*C*) Current traces recorded ~60 sec after the addition of 8.8 mM $[H_2O_2]_{cis}$ to the *cis* chamber. (*D*) Wash, recovery of the channel activity after perfusion with H_2O_2-free *cis* 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 capacitative and leakage currents were removed by using the pClamp software (Axon Instruments) which allows the subtraction of a family of current traces containing no channel activity from another containing SCl channel activity. The current traces are filtered at f_c = 0.2 kHz, and the traces are offset by 9.0 pA for a better display.

tion to at least −70 mV in 8.8 mM $[H_2O_2]_{cis}$. The voltage-dependency of the conductance, as indicated from the *I–V* relationships (Fig. 4*A*), recovers after a wash with an H_2O_2-free control *cis* solution.

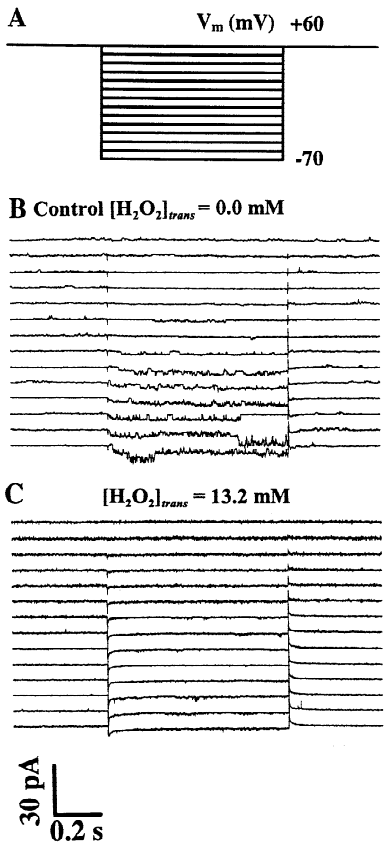

Fig. 3. Effects of $[H_2O_2]_{trans}$ on the voltage-dependence of SCl channel activity. (*A*) Voltage protocol. (*B*) Control current traces recorded in asymmetrical choline-Cl (250 mM/50 mM; *cis/trans*). (*C*) Current traces recorded ~60 sec after the addition of 13.2 mM H_2O_2 to the *trans* chamber. The rest as in Fig. 2.

Concentration-Dependency of H_2O_2 Effects on Conductance and Kinetic Properties

The $[H_2O_2]_{cis}$ dependency of the conductance and kinetic properties of the SCl channel at −40 mV was also examined. The current amplitude declines in steps to around −2.90 and −2.86 pA at 8.8 and 13.2 mM $[H_2O_2]_{cis}$, respectively (Fig. 5). Both values are close to the current amplitude of the intermediate subconductance levels (−2.96 and −2.86 pA) of the SCl channel. Whereas the decline of the current to −1.86 and −0.66 pA at 17.6 and 22 mM $[H_2O_2]_{cis}$, respectively, represents a transition in channel activity to the first two subconductance levels (−0.71 and −1.62 pA) from the closed state. The estimated concentration of the $[H_2O_2]_{cis}$ for the half inhibitory constant, K_i, was 11.78 mM, higher than those estimated for the parameters P_o and T_o (*see below*). This may indicate that the conductance of the SCl channel is less sensitive than the gating kinetics of the channel.

As previously reported [18], the changes in the ki-

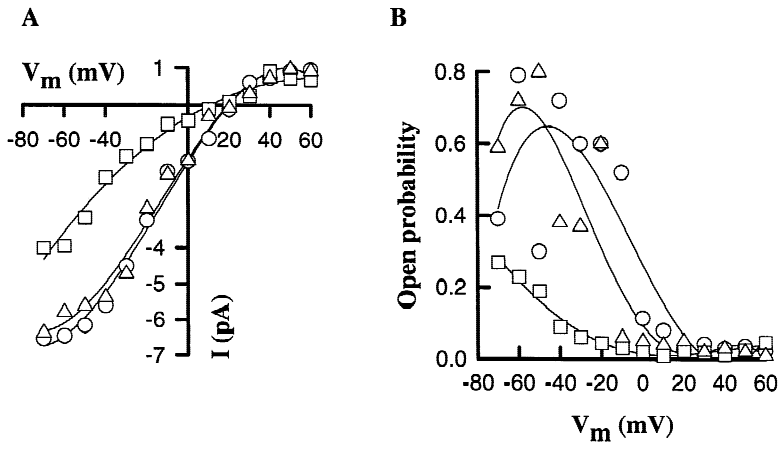

Fig. 4. Effects of $[H_2O_2]_{cis}$ on the voltage-dependence of SCl channel current parameters. (*A*) Current amplitude, *I*, and (*B*) open probability, P_o. (○) Control, (□) 8.8 mM, and (△) wash. The solid lines are drawn to a second-order exponential fit (*A*) and a third order polynomial fit (*B*).

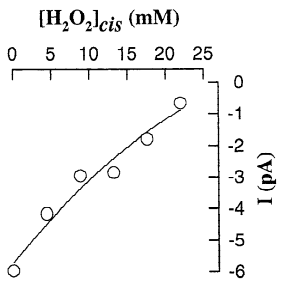

Fig. 5. Current amplitude of different H_2O_2 concentrations-induced steps in single SCl channel activity recorded after an SR vesicle of skeletal muscle incorporated into an optimal bilayer held at −40 mV and in asymmetrical choline-Cl (250 mM/50 mM; *cis/trans*). The amplitude values of the measured current steps of −0.66, −1.81, −2.85, −2.96 pA are similar or close to the current amplitude values of the immediate and intermediate subconductance states of the SCl channel at −0.71, −1.62, −2.68, −2.96 and −5.43 pA, respectively. The solid line is drawn to a second-order exponential fit.

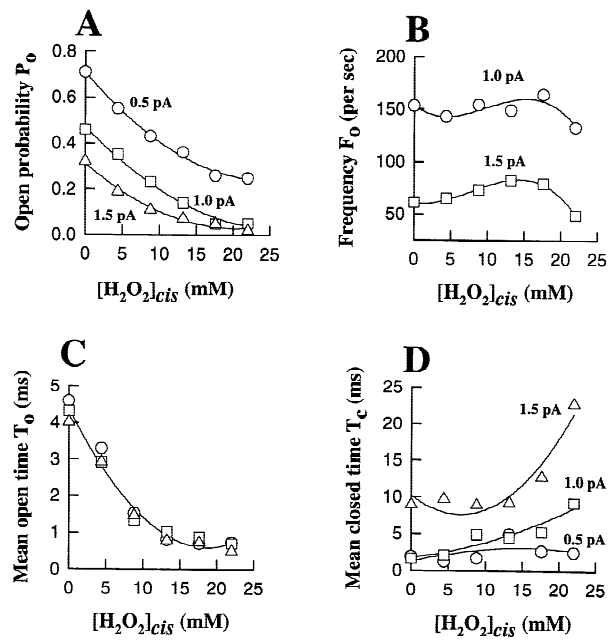

Fig. 6. Concentration-dependency of H_2O_2 effects on the SCl channel kinetic parameters. (*A*) Open probability, P_o, (*B*) frequency, F_o, (*C*) mean open time, T_o, and (*D*) mean closed time, T_c. Single-channel currents are recorded after an SR vesicle of skeletal muscle incorporated in an optimal bilayer held at −40 mV and in asymmetrical choline-Cl (250 mM/50 mM; *cis/trans*). The solid lines are drawn to second exponential fits or third order polynomial fits. 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.

netic parameters P_o, F_o and T_c, but not in T_o, obtained for SCl channel activity at −40 mV are dependent on the current level that has been set to detect channel openings. Typical changes in the kinetic parameters of the SCl channel in response to channel inhibitors include: decreases in P_o and T_o and an increase in T_c (for the larger conducting states), respectively (Fig. 6). Similarly, increasing the $[H_2O_2]_{cis}$ reduces the P_o of the SCl channel (Fig. 6*A*) mainly because of a decrease in the mean open time T_o (Fig. 6*C*). The changes in F_o of the SCl channel (Fig. 6*B*) are characterized by an initial increase at $[H_2O_2]_{cis}$ of less than 17.6 mM, followed by a decrease at $[H_2O_2]_{cis}$ of more than 17.6 mM. The initial increase in F_o at $[H_2O_2]_{cis}$ between 0 and 17.6 mM confirms that the decline in P_o of the SCl channel is caused by a decrease in T_o. At $[H_2O_2]_{cis}$ higher than 17.6 mM, the decline in P_o of the SCl channel is due not only to a decrease in T_o but also to a decrease in F_o and a slight increase in T_c for the larger conducting states. The estimated concentrations of $[H_2O_2]_{cis}$ for the half-inhibitory constant, K_i, were 8.0 and 8.1 mM, for the parameters P_o and T_o, respectively.

Differences in the Effects of 4,4′-DTDP and H_2O_2

The effects of H_2O_2 on the SCl channel activity were in contrast to the reported effects of the 4,4′-DTDP [17].

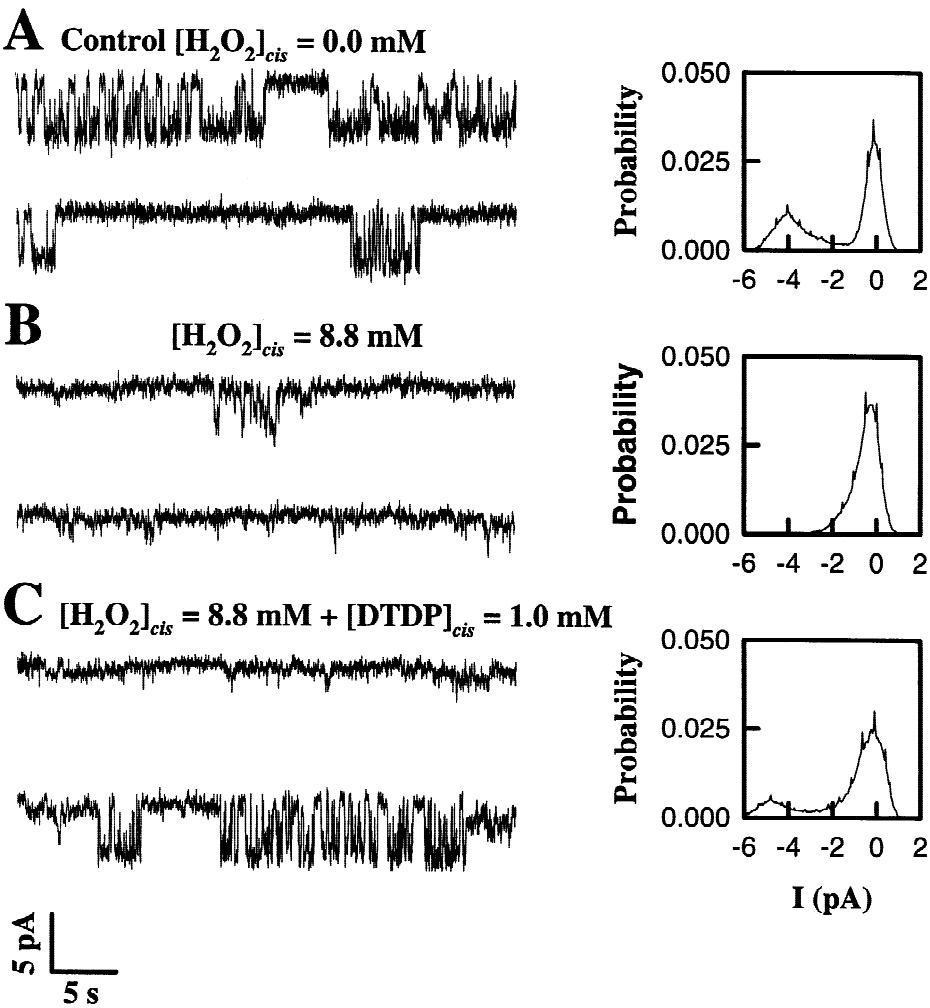

Fig. 7. Effects of $[4,4'\text{-DTDP}]_{cis}$ on the SCl channel activity recorded at −40 mV in the presence of $[H_2O_2]_{cis}$. Shown are representative single-channel current traces (each two are continuous) and corresponding all-points histograms measured for (*A*) control, (*B*) 8.8 mM $[H_2O_2]_{cis}$ and (*C*) 8.8 $[H_2O_2]_{cis}$ + 1.0 mM $[4,4'\text{-DTDP}]_{cis}$. The all-points histograms were constructed from longer data segments in *A, B* and *C* 83.12, 106.14, and 80.89 sec, respectively.

To ascertain the nature of these differences, the effects of these oxidizing agents were examined on the same SCl channel (Fig. 7*A–C*). After a lag period of between 30 to 60 sec, 1.0 mM $[4,4'\text{-DTDP}]_{cis}$ removed the inhibitory effects of 8.8 mM $[H_2O_2]_{cis}$ (Fig. 7*C*). The recovered SCl channel activity is characterized by the presence of the main conductance and the intermediate subconductance states as seen in control (Fig. 7*A*). The values obtained for I and P_o for three SCl channels were: −6.01 ± 0.59 pA and 0.67 ± 0.04 in control 0.0 mM $[H_2O_2]_{cis}$, reduced significantly ($P < 0.01$, $n = 3$) to −1.23 ± 0.58 pA and 0.13 ± 0.03 in 8.8 mM $[H_2O_2]_{cis}$ (Fig. 7*B*); and restored to −5.84 ± 0.33 pA and 0.66 ± 0.07, in 1.0 mM $[4,4'\text{-DTDP}]_{cis}$ + 8.8 mM $[H_2O_2]_{cis}$, respectively. In contrast, the reducing agent GSH, and also DTT, which inhibits the SCl channel activity [17] failed to remove $[H_2O_2]_{cis}$-induced channel inhibition (Fig. 8*C*). The values obtained for I and P_o for three SCl channels were: −5.78 ± 0.72 pA and 0.64 ± 0.05 in control 0.0 mM $[H_2O_2]_{cis}$, reduced significantly ($P < 0.01$, $n = 4$) to −1.45 ± 0.38 pA and 0.09 ± 0.03 in 8.8 mM $[H_2O_2]_{cis}$; and remained at −1.34 ± 0.47 pA and 0.08 ± 0.02, in 1.0 mM $[GSH]_{cis}$ + 8.8 mM $[H_2O_2]_{cis}$, respectively.

Effects of H_2O_2 and 4,4′-DTDP in the Presence of ATP

It was shown that reducing and oxidizing agents, respectively, reduce and increase the probability of the ATP-sensitive SCl channel being open, *via* redox-sensitive SH groups that are involved in gating the channel [17]. It has also been reported that an SH group is located in the vicinity of the ATP-binding site of the ATP-sensitive K^+

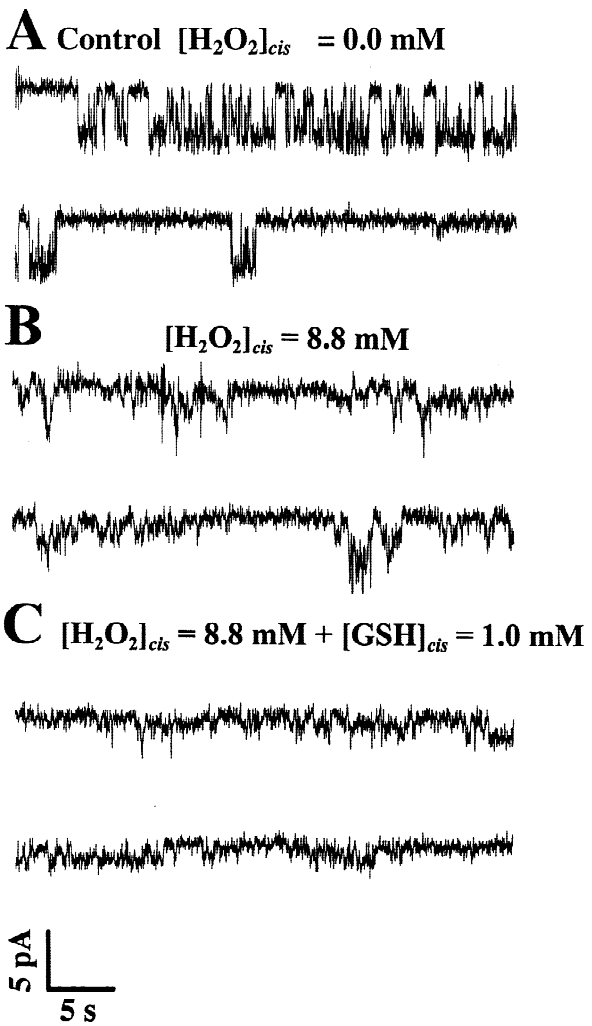

Fig. 8. Effects of $[GSH]_{cis}$ on the SCl channel activity recorded at −40 mV in the presence of $[H_2O_2]_{cis}$. (*A*) Control, (*B*) 8.8 mM $[H_2O_2]_{cis}$ and (*C*) 8.8 $[H_2O_2]_{cis}$ + 1.0 mM $[GSH]_{cis}$.

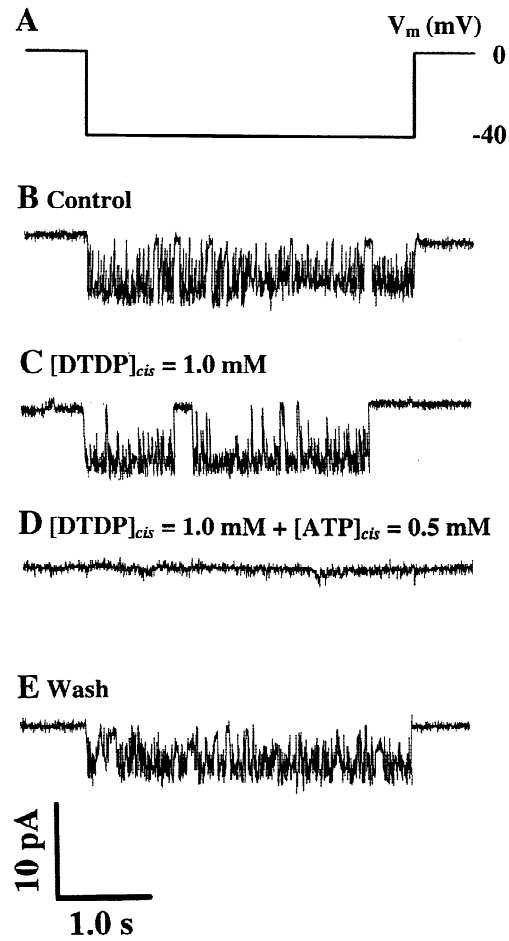

Fig. 9. ATP block of the activating action of the SH-oxidizing agent 4,4′-DTDP on the SCl channel activity. Channel activity recorded in response to a single voltage step protocol from 0 to -40 mV (*A*). (*B*) Control current activity of a single SCl channel, (*C*) the activity of the same SCl channel 15 sec after the addition of 1 mM $[4,4'\text{-DTDP}]_{cis}$, (*D*) in the presence of 1 mM $[4,4'\text{-DTDP}]_{cis}$ and 0.5 mM $[ATP]_{cis}$ and (*E*) wash.

channels in skeletal muscle [34]. Hence, the location of the ATP-binding site relative to the cysteine residues containing the SH-group of the SCl channel was probed. However, because both $[ATP]_{cis}$ and $[H_2O_2]_{cis}$ induce SCl channel inhibition, the addition of 2.0 mM ATP to the *cis* solution had no effects on the 8.8 mM $[H_2O_2]_{cis}$-induced channel inhibition and *vice versa* (*data not shown*). This finding is different from the findings below for the sequence effects of $[ATP]_{cis}$ and $[DTDP]_{cis}$ on the SCl channel (Figs. 9 and 10).

In agreement with previous finding [17], the *cis* addition of 1.0 mM SH-oxidizing agent 4,4′-DTDP typically increases the probability of the SCl channel being in the open state (Fig. 9*C*). The 4,4′-DTDP-enhanced SCl channel activity was blocked after the addition of 0.5 mM ATP to the *cis* side of the channel (Fig. 9*D*). The values of I and P_o increased from −5.87 ± 0.17 pA and 0.69 ± 0.03 in control to −6.12 ± 0.27 pA and 0.81 ± 0.03 in 1.0 mM $[4,4'\text{-DTDP}]_{cis}$ and significantly decreased to −0.68 ± 0.18 pA and 0.10 ± 0.3, in 1.0 mM $[4,4'\text{-DTDP}]_{cis}$ + 0.5 mM $[ATP]_{cis}$. The effects of 4,4′-DTDP and ATP were rapidly reversible, after a mean period of 5.8 sec ($n = 2$) (*see also* Fig. 9*E*) where the values of I and P_o recovered to −5.81 ± 0.71 pA and 0.68 ± 0.02, respectively.

Furthermore, it was found that the addition of 1.0 mM SH-oxidizing agent 4,4′-DTDP to the *cis* solution facing an SCl channel already subjected to 0.5 mM $[ATP]_{cis}$ (Fig. 10*C*), failed to activate the SCl channel (Fig. 10*D*). However, the ATP-inhibited SCl channel recovered rapidly 6.7 ± 1.4 sec ($n = 3$) after perfusion with the ATP- and 4,4′-DTDP- free control *cis* solutions (Fig. 10*E*). The values of I and P_o decreased significantly ($P < 0.01$, $n = 4$) from −5.84 ± 0.28 pA and 0.69

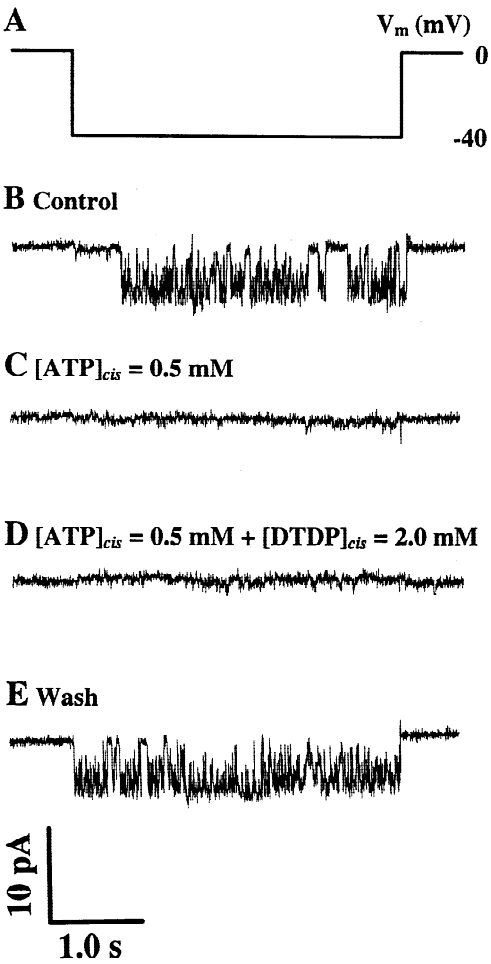

Fig. 10. 4,4′-DTDP effects on ATP-inhibited SCl channel activity. Channel activity recorded at in response to a voltage step from 0 to −40 mV (*A*). (*B*) control current activity of a single SCl channel, (*C*) the activity of the same SCl channel 15 sec after the addition of 0.5 mM $[ATP]_{cis}$, (*D*) in the presence of 0.5 mM $[ATP]_{cis}$ and 2.0 mM $[4,4'\text{-}DTDP]_{cis}$ and (*E*) Wash.

± 0.02 in control to −0.86 ± 0.14 pA and 0.08 ± 0.02 in 0.5 mM $[ATP]_{cis}$, and remained low at −1.06 ± 0.14 pA and 0.10 ± 0.01, in 0.5 mM $[ATP]_{cis}$ + 2.0 mM $[4,4'\text{-}DTDP]_{cis}$. The I and P_o values respectively recovered to −5.65 ± 0.23 pA and 0.67 ± 0.03 after washing with an ATP- and 4,4′-DTDP-free *cis* solution.

The lipophilic and SH-oxidizing agent 4,4′-DTDP [7] at 1.0 mM in the *trans* solution increased the probability of the SCl channel being in the open state. This increase in the open probability of the channel was blocked after the addition of 0.5 mM ATP to the *trans* side of the channel in a manner typical of that shown in Fig. 9 for the addition of 1.0 mM $[4,4'\text{-}DTDP]_{cis}$. The mean value of P_o increased from 0.65 in control to 0.88 in 1.0 mM $[4,4'\text{-}DTDP]_{trans}$ and significantly decreased to 0.12, in 1.0 mM $[4,4'\text{-}DTDP]_{trans}$ + 0.5 mM $[ATP]_{trans}$. These effects of 4,4′-DTDP and ATP on the P_o of the SCl channel were rapidly reversible. After ~45 sec of wash with the control *cis* solution P_o recovered to 0.71. The addition of 1.0 mM GSH to the *cis* or/and *trans* sides of the channel also did not remove the ATP-induced inhibition of the SCl channel ($n = 6$).

Discussion

The SCl channel protein(s) has not been isolated, sequenced, cloned nor has its structure been determined. In this study the role of the reactive cysteine residues in the function of the SCl channel was indirectly assessed by examining the changes in the channel activity induced by SH-oxidizing and -reducing agents. Such pharmacological probes have also been used to examine other SR channel proteins [9, 10]. The major findings of this study provide novel information on the regulation of ATP-sensitive SCl channels of the skeletal SR by H_2O_2. Specifically, we have found: (i) that H_2O_2 modified SCl channel functions, as indicated by conductance and kinetic parameters of the channel activity; (ii) that the effects were not mediated via changes in the bilayer but directly on the SCl channel proteins; (iii) that H_2O_2-induced effects differ from those of 4,4′-DTDP; (iv) that modulation of the SCl channel by H_2O_2 and 4,4′-DTDP depends on the presence of ATP, which interferes with the binding of these substances to the channel protein.

Modification of SCl Channel Conductance and Gating Kinetics

The conductance and kinetics of the SCl channels ($n = 26$) exposed to $[H_2O_2]_{cis}$ are dominated by the conductance and kinetics of the submaximal states. The data in Fig. 1 show that steplike transitions to subconductance states, which are initially seen along with higher conductance states under control conditions, become dominant in the presence of 8.8 mM $[H_2O_2]$ on the cytoplasmic side of the SCl channel. The kinetic parameters of the SCl channel show that the P_o and the frequency of transitions, F_o, from the closed and partially open subconductance states to the main open conductance state, and the mean open time, T_o, at the maximal conductance state, 65–75 pS, decreased (Figs. 6*A–C*). The open probability, P_o, of SCl channels exposed to $[H_2O_2]$ higher than 8.8 mM is mainly that of current transitions between the closed conductance state and the unequally spaced submaximal conductance states. Since H_2O_2 readily crosses biological membranes (*see* [20]), the $[H_2O_2]_{trans}$-induced inhibition of the SCl channel could be due to binding to an inhibitory site(s) on the *trans* and/or on the *cis* side(s) of the channel protein.

Concentrations and Mechanisms of H_2O_2 Action on Ion Channels in Muscles

The response of the SCl channel to either H_2O_2 or 4,4′-DTDP was not mediated via changes in the biophysical

properties of the phospholipids forming the bilayer. Rather, the effects occurred directly on the SR SCl channel proteins (*see also* [5]). The inhibition of the SCl channel activity exposed to mM concentrations of H_2O_2 in this study was in contrast to the H_2O_2-enhanced channel activation reported for RYR Ca^{2+}-release channels [5, 27]. Oba et al. [27] have shown that in skeletal muscle, 1.5–5 mM [H_2O_2] induced Ca^{2+}-release from the SR by increasing the P_o of RYR Ca^{2+}-release channels. Similarly, in cardiac SR from sheep, after a lag period of 1–3 min. H_2O_2 (3–5 mM) modified the gating of the RYR Ca^{2+}-release channel causing an increase in the P_o, without affecting the conductance or channel modulation with ATP, caffeine, Mg^{2+} or RY [5]. Previously, Holmberg et al. [15] reported that ROS (in the form of superoxide anion radical (O_2^-) and singlet oxygen ($^1O_2$) increase the P_o of the cardiac RYR Ca^{2+}-release channels. However, this ROS-induced increase in P_o was followed by irreversible loss of channel function and the appearance of subconductance states. In the SR of rabbit skeletal muscle, $[H_2O_2]_{cis}$ at less than 1 mM increased the P_o of the RYR Ca^{2+}-release channel whereas 10 mM $[H_2O_2]_{cis}$ decreased the P_o [12]. The ATP-sensitive K^+ channel in skeletal muscle is irreversibly inhibited, via oxidation of SH groups, by 50 mM [H_2O_2] [34]. The mM concentrations of H_2O_2 used in the in vitro studies of muscle ion channels recorded in patch-clamp and artificial lipid bilayer membrane techniques are higher than those (μM) generated during skeletal muscle fatigue and in cardiac and smooth muscles under the hypoxia-reperfusion process (*see* [20]). Therefore, the relative contribution of H_2O_2 and the in vivo concentration threshold necessary to induce changes in the muscle ion channels underlying contraction remain to be quantified.

The interaction of H_2O_2 with the SCl channel proteins is consistent with a thiol-disulfide redox state model for regulating ion transport [17]. Changes in the redox state of the SH groups on the ion channel proteins result in physical changes that can directly modify the function and/or the availability of regulatory sites on the channel proteins. Vega-Saenz de Miera and Rudy [32] have proposed that H_2O_2 modifies the redox state of the K^+ channel protein by oxidizing the cysteine residues which are involved in the "ball" and "chain" mechanism gating the channel. The oxidizing agent of H_2O_2 on ion channel proteins in muscles, and also in other tissues, is confirmed by the findings that H_2O_2-induced changes in ion channel proteins can be reversed with SH-reducing agents, e.g., dithiothreitol (DTT). Such H_2O_2-induced DTT-reversible effects on the biophysical properties of ion transport proteins include: (i) increase in P_o of RYR in both cardiac and skeletal muscle [12, 27] and (ii) decline in the activity of the Ca^{2+}-activated K^+ channels in bovine aortic endothelial cells [6]. It is assumed that SH-reducing agents act on H_2O_2-induced disulfide bonds, e.g., by dissociating the H_2O_2-induced disulfide linked RYR protein complex [12].

Differences in the Effects of H_2O_2 and 4,4′-DTDP

Oxidizing agents have different properties. Hydrophilic agents can affect the channel by interacting with the channel proteins on the side to which they were added and/or by passing through the channel to the modulating site. Lipophilic agents partition the lipid membrane and thus can modify the channel activity by interacting with sites present on both sides of the channel, regardless of the side to which they are added. Additionally, a lipophilic agent may reach a binding-site by passing through the channel.

The differences in the effects of the oxidizing agents 4,4′-DTDP (activation) and H_2O_2 (inhibition) on the SCl channel reported in this study suggest that channel activation is dependent on the oxidation of certain sites on the SCl channel protein. There is also evidence of oxidizing agents inhibiting other ion channels in muscles that support this suggestion [33]. The precise molecular mechanisms of action of these agents are not known. If these agents were competitively oxidizing the same site the consequences of their binding to this site on the channel activity would be expected to be the same. It is unlikely that the variation in the effects of H_2O_2 and 4,4′-DTDP on the SCl channel is due to differences in their binding affinity. The differences in the specificity and reversibility effects of H_2O_2, and other oxidizing agents, on ion channels, e.g., RCK4 channel [30], KShI-IDC and KShIID.1 channels [32] and on the SCl channel (this study) may be due to: (i) a difference in the location of the SH groups on these channel proteins and the relative significance of these SH groups in the channel function; (ii) binding to other sites on the channel protein that may not involve SH-groups; (iii) oxidizing agents differ in their action, e.g., oxidizing agents may form different disulfide bridges. Oxidation of K^+(Ca^{2+}) channels by H_2O_2 forms disulfide bonds that differ from those induced by SH-oxidation with 5,5′-dithio-bis(2-nitrobenzoic acid) (5,5′ DTNB) and thimerosal (*see* Cai and Sauvé [6]). Also oxidized glutathione, GSSG, which is unlike other oxidizing agents (4,4′-DTDP, H_2O_2, thimerosal, and DTNB), is not effective in releasing Ca^{2+} [9, 10]. (iv) The activation state of the channel protein, e.g., the effects of the oxidizing agent 4,4′-DTDP on the ryanodine receptor Ca^{2+}-release channel is dependent on the channel being activated by Ca^{2+} or caffeine [10]. (v) Biphasic effects of some oxidizing agents, e.g., 4,4′-DTDP-induced activation and then inactivation of the ryanodine receptor Ca^{2+}-release channel [11]. The ability of 4,4′-DTDP to activate the H_2O_2-inhibited SCl channel may indicate that 4,4′-DTDP reverses the effect of H_2O_2 by binding to a site located deeper in the conductance pathway away from the H_2O_2 binding site.

Effects of H_2O_2 and 4,4′-DTDP in the Presence of ATP

The fact that both ATP and H_2O_2 inhibit the SCl channel makes it difficult to determine the H_2O_2 binding site, which contains the SH groups, relative to the ATP binding site on the channel protein. However, the results in Fig. 10 point to the inability of the SH-oxidizing agent 4,4′-DTDP to activate the SCl channel in the presence of 0.5 mM $[ATP]_{cis}$. Therefore, the binding of the ATP to the channel protein blocks the channel regardless of the presence of 4,4′-DTDP. ATP-induced inhibition of another ATP-sensitive muscle channel is also observed regardless of the presence of the irreversible inhibitor N-ethylmaleimide (NEM) [34]. The ATP-induced inhibition of 4,4′-DTDP-enhanced SCl channel opening (Fig. 9) suggests that 4,4′-DTDP is not preventing and/or dislodging ATP from its binding site on the channel protein.

Physiological Significance of H_2O_2-Inhibited SCl Channel

The H_2O_2-induced potentiation of twitch tension in cardiac and skeletal muscles [27, 29] which is decreased by catalase [29], is not mediated *via* end effects on the myofilaments [24, 27]. This indicates that the H_2O_2 induces modifications in ion transport mechanisms, which regulate Ca^{2+} homeostasis. In the SR of cardiac and skeletal muscles, both SCl channels (this study and [17]) and Ca^{2+}-release channels [5] are modulated by reducing and oxidizing agents, which include H_2O_2. These studies are in agreement with (i) a linkage between the metabolism of the muscle fiber and the electrical properties of the SR membrane via H_2O_2-production and (ii) a functional linkage between the SCl and Ca^{2+}-release channels. It is not unreasonable to suggest that these SR channels could be components of an affected molecular mechanism underlying ROS role in Ca^{2+}-loading under hypoxic-reperfusion conditions. Other observations in agreement with this suggestion include: (i) the fact that low $[H_2O_2]$ induced modification of these multisubconductance Ca^{2+}-activated SCl channels in the presence of $[Ca^{2+}]_{cis}$, implies that H_2O_2 remains active when cytosolic $[Ca^{2+}]$ of $>10^{-4}$ M is achieved during muscle contraction and (ii) the hypoxia-induced transitions to subconductance states [17] would be further enhanced by an increase in $[H_2O_2]_{cyt}$. The mechanism of H_2O_2-induced mode shift in the conductance of the SCl channel is physiologically significant since the subconductance states of this channel are known to differ from main conductance states in their regulation and this is in agreement with the possibility of multiregulatory mechanisms involved in vivo.

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