Involvement of thiols in the induction of inward current induced by silver in frog skeletal muscle membrane

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Abstract. Exposure of voltage-clamped frog skeletal muscle fibres to silver caused a maintained inward current which could be carried by Ca^{2+}, Mg^{2+} or Na^+. Inorganic Ca^{2+} channel blockers and dithiothreitol (SH reducing agent) diminished this current, but a Na^+ channel blocker did not. Thus, silver activates the Ca^{2+} channel by acting on SH groups in a Ca^{2+} channel protein.
Key words. Silver ion; calcium channel; inward current; SH group; contraction; skeletal muscle deterioration.

Industrial or medical exposure to silver increases blood concentrations to levels which can have toxic effects, such as induction of sarcomas[1], anemia[2] and enlargement of the heart[3]. Previous studies have shown that the silver ion has three different sites of action in skeletal muscle[4–10]: Ca^{2+} channels in surface and transverse (T) tubular membranes, the Ca^{2+} releasing channel and Ca^{2+}-ATPase in the sarcoplasmic reticulum (SR), and actomyosin ATPase. When silver was extracellularly applied to isolated muscle fibres, phasic contraction was produced by binding of silver to the α_1 subunit of the Ca^{2+} channel on the T-tubule, even in solutions containing no Ca^{2+}[11]. In solutions with external Ca^{2+}, fibres developed phasic contraction followed by tonic tension[12]. Thus, silver ions may activate Ca^{2+} channels on the sarcolemma and cause the muscle fibre to deteriorate by allowing excess Ca^{2+} to enter the cell. In this study, we examined this possibility by measuring membrane current in voltage-clamped fibres and involvement of thiols in the induction of the inward current after application of Ag^+ to muscle fibres.

Materials and methods

After frogs were decapitated, single muscle fibres (60–140 μm in diameter) were dissected from the musculus tibialis anterior of *Rana japonica* and from the musculus flexor brevis digiti V of *Rana catesbiana* in Ringer solution (in mM: 115 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 3 Na phosphate buffer, pH 7.0). Inward current in the voltage-clamped fibre was measured using a double mannitol gap method[10,13]. The test (central) and mannitol compartments were 200 and 250 μm in width, respectively. Both end compartments were filled with Ringer solution. Each compartment was separated by a 100-μm Vaseline layer. The Vaseline boundary layers ensured that no muscle movement could develop after silver (Ag^+) application. For the gap, 245 mM mannitol solution was prepared with ultrapure water (Barnstead, Boston, MA) immediately before each experiment. Membrane potential was held at −90 mV, except for the experiment to determine the current-voltage (I–V) relationship. Membrane current was amplified by a voltage-clamp amplifier (CEZ-1100, Nihon Koden) and fed to a memory oscilloscope (VC-10, Nihon Koden) for recording on a paper oscillograph (PMP-3004, Nihon Koden).

For Ag^+ application, Ringer solution in the central compartment was replaced by chloride-free nitrate MOPS solution (in mM: 115 $NaNO_3$, 2.5 KNO_3, 3 $Mg(NO_3)_2$ or 1.8 mM $Ca(NO_3)_2$, 10 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.0). After equilibration for 5 min in MOPS solution containing 1.8 mM $Ca(NO_3)_2$, the fibre was exposed to a test solution containing 10 μM Ag^+. Following development of an inward current, 1 μM tetrodotoxin and 1 mM nickel ion or 1 mM cadmium ion were applied to check whether the inward current is inhibited by Na^+ channel and Ca^{2+} channel blockers. Further characteristics of Ag^+-induced current were examined in fibres soaked in solutions containing a single species of cation (1.8 mM Ca^{2+}, 3 mM Mg^{2+} or 115 mM Na^+) as current carrier. In Ca^{2+} or Mg^{2+} solution, Na^+ was replaced by N-methyl-d-glucamine or choline, and NO_3^- was used as anion.

To determine the effect of sulfhydryl groups on Ag^+-induced inward current, dithiothreitol (DTT) at 1 mM was applied to the fibre which had developed an inward current after treatment with 10 μM Ag^+.

All experiments were done at 20–22 °C. Results are given as means ±SEM.

Results and discussion

Exposure of voltage-clamped fibres to 10 μM Ag^+ elicited an inward current (9 ± 1 μA, n = 7) with a short lag time. The current slowly reached a maximum and was

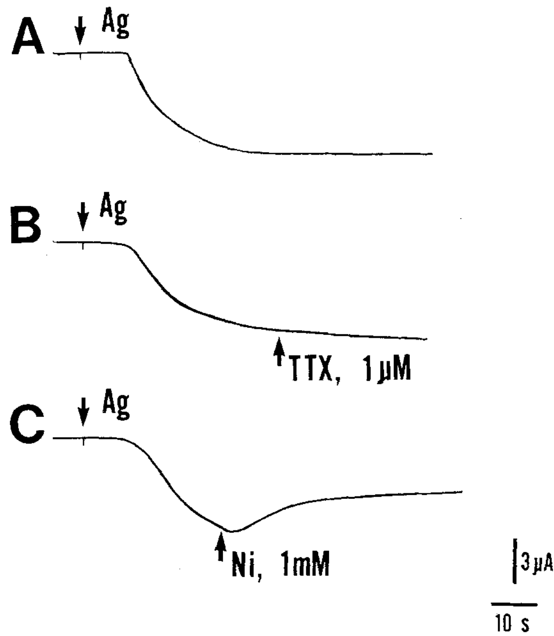

Figure 1. Silver-induced inward current (*A*) and effects of Na^+- or Ca^{2+}-channel blocker (*B* and *C*). Ag^+ at 10 μM was applied to each fibre at time indicated by arrows. Tetrodotoxin (TTX, 1 μM) and nickel (Ni^{2+}, 1 mM) were given after the inward current had reached a steady level. Calibration: 3 μA and 10 s.

maintained at that level (fig. 1a). This current was not blocked by 1 μM tetrodotoxin applied after the current had reached a plateau (fig. 1b). However, 1 mM Ni^{2+}, an inorganic Ca^{2+} channel blocker, reduced the inward current (fig. 1c). Thus, the inward current is likely carried through a Ca^{2+} channel, but not through a Na^+ channel. In this regard, our previous findings show that the inward current evoked by 10 μM Ag^+ was partially inhibited by application of 1 mM Cd^{2+} during the development of the inward current, but was almost completely inhibited when 1 mM Cd^{2+} and Ag^+ were applied simultaneously[10].

In skeletal muscle, Ca^{2+} channels are mainly located in the T-tubular membrane and are of the slowly activating, dihydropyridine-sensitive type[14,15], belonging to the L-type of Nowycky et al.[16]. Previously, we showed that activation of the Ca^{2+} channel voltage sensor by perchlorate and Bay K 8644 potentiates Ag^+-induced contraction, whereas nifedipine and felodipine, dihydropyridine derivatives, inhibited the contraction. Ag^+ was found to bind to the partially purified Ca^{2+} channel from T-tubules of rabbit skeletal muscle[11]. In fibres treated with 10 μM nifedipine, the inward current induced by 10 μM Ag^+ was markedly reduced[10]. Thus, Ag^+ applied extracellularly probably binds to the sarcolemmal Ca^{2+} channel (DHP receptor) of fibres.

We have reported that Ag^+ extracellularly applied to single fibers elicited a phasic contraction, followed by a tonic tension in the presence of physiological concentrations of external Ca^{2+} [5,10,11,12]. The phasic contraction was blocked by detubulation and by treatment of fibers with DHP derivatives (nifedipine and felodipine), but not by external Ca^{2+} deprivation or Cd^{2+} treatment. Activators of the voltage sensor in the T-tubules, Bay K 8644 and perchlorate, potentiated the phasic tension. On the other hand, the tonic contraction depended on external Ca^{2+} concentrations and was blocked by Cd^{2+}, but still occurred after detubulation. Ca^{2+} current induced by Ag^+ was completely inhibited by 1 mM Cd^{2+} and partially inhibited by 10 μM nifedipine. Therefore, we concluded that the phasic contraction was activated via the voltage sensor and the tonic via the inward Ca^{2+} current. Ca^{2+} entering the cell through this channel may lead to maintained contraction with resultant deterioration of skeletal muscle fibres.

When DTT at 1 mM was added to fibres immediately after a small inward current was induced by addition of 10 μM Ag^+, the current reverted rapidly and almost completely to the level before Ag^+ application (fig. 2). However, when applied after a delay, DTT was much less effective in reducing the Ag^+-induced inward current. We previously showed that fibres did not respond to the second exposure of Ag^+ if DTT was applied at the relaxation stage of the first Ag^+-induced contraction[8]. These results suggest that Ag^+ has much higher affinity for SH groups on the Ca^{2+} channel than for SH groups on DTT, and that Ag^+ once bound on the Ca^{2+} channel protein is not easily removed by DTT. Alternatively, the Ca^{2+} channel may have two binding sites with different affinities for Ag^+, i.e. a weak and a strong binding site. Ag^+ bound on the binding site with strong affinity may not be removed by DTT. Chemical analysis of the binding sites of Ag^+ on the Ca^{2+} channel was not performed in the present study. In both cases, this characteristic action of Ag^+ might lead to continuous influx of extracellular

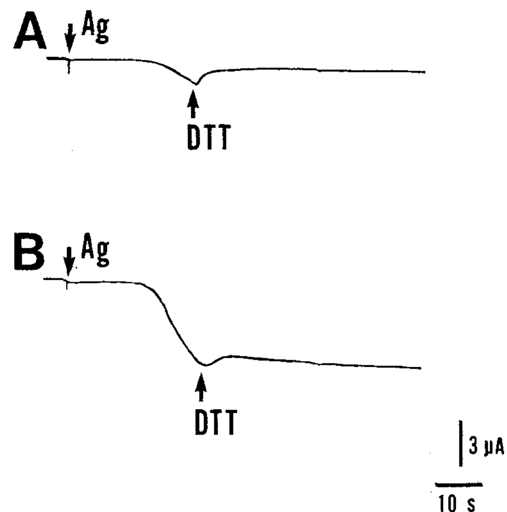

Figure 2. Effect of dithiothreitol (DTT) on silver-induced inward current. DTT at 1 mM was applied immediately after the inward current had occurred (*A*) or just before the maximum current had developed (*B*). See figure 1 legend for other notations.

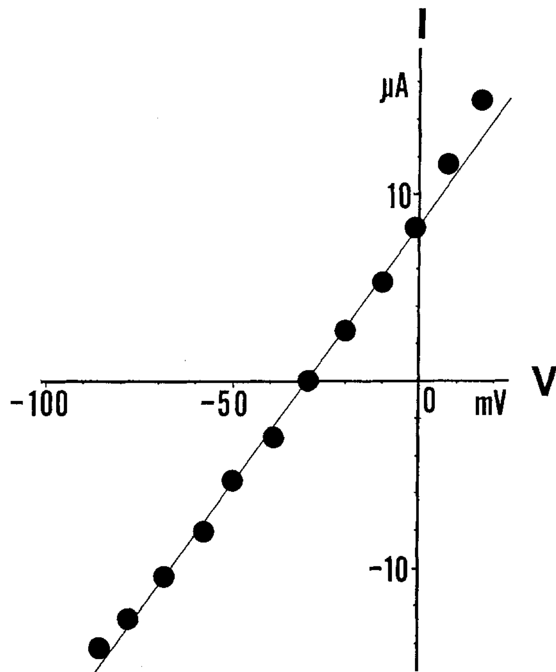

Figure 3. I–V relationship of silver (10 μM)-induced current in the steady state level. A straight line is obtained over a voltage range between −80 and 0 mV.

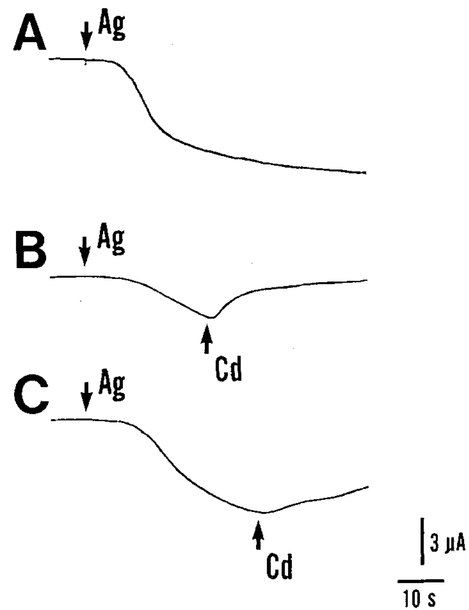

Figure 4. Divalent and monovalent cations as carrier for Ag^+-induced current and blockage of inward current by 1 mM Cd^{2+} (Cd). Ca^{2+} at 1.8 mM (*A*), Mg^{2+} at 3 mM (*B*) and Na^+ at 115 mM (*C*) as sole cation could carry the inward current through the channel. Na^+ was replaced by N-methyl-d-glucamine (*A*) or choline (*B*). Cd^{2+} was applied to each fibre at time indicated with arrows. See Figure 1 for calibration.

Ca^{2+} and in turn to muscle deterioration due to supercontraction. Because oxidation of free SH groups by H_2O_2 or dithionitrobenzene (DTNB) prevented Ag^+ contraction whereas reduction of oxidized SH by DTT restored it[11], we concluded that Ag^+ activates the Ca^{2+} channel by modifying SH groups on this channel.

The I–V relation after 10 μM Ag^+ exposure was linear over a voltage range between −80 mV and 0 mV (fig. 3). Reversal potential was -37 ± 6 mV (n = 6), indicating that the Ag^+-induced inward current is produced by an inflow of more than one kind of ion through the muscle membrane. Thus, we explored which ion could carry the inward current through the channel by using solutions containing 1.8 mM Ca^{2+}, 3 mM Mg^{2+} or 115 mM Na^+ as the sole permeable cation. As shown in figure 4, all three ions could carry the Ag^+-induced inward current. The magnitude of the inward current carried by 115 mM Na^+ was similar to that carried by 1.8 mM Ca^{2+}. In solutions containing either Mg^{2+} or Na^+, the inward current was partially blocked by 1 mM Cd^{2+}. In this regard, the L-type Ca^{2+} channel is reported to be able to carry various cations in the absence of extracellular Ca^{2+}[17,18]. No inward current was observed after application of Ag^+ to fibres in choline-Ringer solution (Na^+ was replaced by choline and divalent cations were omitted)[10], indicating that Ag^+ and choline are not carriers for inward current. The present study, therefore, shows that extracellular Ag^+ activates sarcolemmal Ca^{2+} channel by binding to SH groups and that cations such as Ca^{2+}, Mg^{2+} and Na^+ flow through this activated channel in intact fibres.

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