brief communication

Modulation of the Ca^{2+} channel voltage sensor and excitation–contraction coupling by silver

Toshiharu Oba,* Mamoru Yamaguchi,* Songtao Wang,‡ and J. David Johnson‡

*Department of Veterinary Anatomy and Cellular Biology, College of Veterinary Medicine, and ‡Department of Medical Biochemistry, College of Medicine, The Ohio State University, Columbus, Ohio 43210 USA

ABSTRACT Ag^+ (0.5–10 μM) is known to produce a transient contraction of intact frog skeletal muscle fibers followed by complete inhibition of excitation–contraction (E–C) coupling. We have carried out physiological and biochemical experiments to investigate the basis of this effect.

Dihydropyridine (DHP) Ca^{2+} channel blockers, which inhibit the voltage sensor of the Ca^{2+} channel, completely inhibit Ag^+ contractions. Removal of extracellular Ca^{2+}, or blockade of Ca^{2+} entry with cadmium, does not inhibit Ag^+ contractions. Activation of the Ca^{2+} channel's voltage sensor with the Ca^{2+} channel agonists Bay K 8644 or with perchlorate, potentiates the Ag^+-induced contraction.

Ag^+ binds to the partially purified rabbit skeletal muscle Ca^{2+} channel and inhibits DHP binding (IC_{50} = 1.1 μM) and sulfhydryl (SH) reactivity (IC_{50} = 0.11 μM) over the concentration range where it inhibits E–C coupling. Oxidation of free SH groups by H_2O_2 or their reaction with DTNB prevents Ag^+ contractions, while DTT reduction of oxidized SH groups restores Ag^+ contractions.

These results suggest that Ag^+ binds to critical SH groups on the DHP receptor Ca^{2+} channel, resulting in modification of the channel's voltage sensor and the failure of E–C coupling.

INTRODUCTION

During excitation of skeletal muscle, depolarization of the sarcolemmal membrane is propagated to the transverse (T) tubular system. The T-tubular Ca^{2+} channels (DHP receptors) contain a voltage sensor, which "senses" this depolarization, produces charge movement (1), and presumably triggers the release of Ca^{2+} from the sarcoplasmic reticulum (SR) to cause contraction (1–5). Although several mechanisms for E–C coupling have been proposed (6), recent studies of muscular dysgenic mice suggest that the T-tubule Ca^{2+} channel facilitates E–C coupling in skeletal muscle (see references 2, 4).

In muscular dysgenesis, a defect in the α_1 subunit of the Ca^{2+} channel is associated with a marked decrease in DHP binding, Ca^{2+} influx, charge movement, and a loss of E–C coupling (2, 4). Thus, the DHP receptor serves both as a Ca^{2+} channel and as a voltage sensor that is essential for E–C coupling in skeletal muscle. Rios and Brum have shown that DHPs inhibit the Ca^{2+} channel's voltage sensor, resulting in a decrease of charge movement and E–C coupling (3).

Bay K 8644 and perchlorate shift the voltage dependence of Ca^{2+} channel activation (and charge movement) to more negative potentials and act as Ca^{2+} channel agonists (see references 12–15). Inorganic Ca^{2+} channel blockers including La^{3+} and Cd^{2+} block Ca^{2+} influx without affecting the voltage sensor (see reference 9).

Previously, Oba and Hotta have shown that Ag^+ induces a transient contraction of intact skeletal muscle fibers followed by inhibition of E–C coupling (7). These Ag^+ effects occur without membrane depolarization and require an intact T-tubule membrane system (7, 8). In this paper we examine the effect of DHP calcium channel agonists and antagonists on Ag^+ contractions and determine the effects of Ag^+ on DHP binding and SH reactivity of the partially purified Ca^{2+} channel.

MATERIALS AND METHODS

Single fibers were isolated from anterior tibialis of *Rana temporaria* or toe muscle of *Rana catesbeiana* in ice-cold Ringer solution (mM; 115 NaCl, 2.5 KCl, 2.15 Na_2HPO_4, 0.85 NaH_2PO_4, and 1.8 $CaCl_2$, pH 7.0) and mounted in a 3.0 ml, 20°C chamber, with a slight stretch over slack length (2.3–2.4 μm sarcomere length). Tetanus (0.2 ms duration at 100 Hz for about 1 s) and twitch tensions were measured isometrically with a tension transducer (model 400A; Cambridge Technology, Cambridge, MA) with sensitivity of 2 V/g (±0.1 g) and a resolution of 0.3 ms with instrumentation previously described (see reference 18). All Ag^+ contractions were in a Ca^{2+} free nitrate Ringer (mM: 115 $NaNO_3$, 2.5 KNO_3, 3 $Mg(NO_3)_2$, and 10 3-(*N*-morpholino) propanesulfonic acid (MOPS), pH 7.0) to prevent precipitation of AgCl and irreversible prolonged contraction (see reference 8). Tetanus tension was only slightly greater (1.03 times ± 0.05 SD, n = 10) in Ca^{2+} free nitrate Ringer than in Ringer solution, while twitch tension was significantly potentiated (1.41 times ± 0.18 SD, n = 10).

Skeletal muscle Ca^{2+} channel was purified to 85% homogeneity (as determined by silver staining of SDS gels) by digitonin solubilization of rabbit skeletal muscle microsomes, ultracentrifugation, and wheat germ agglutinin chromatography on a WGA-Affi-Gel 10 column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ), per the method described in (19). Binding of [^{3}H]-PN200-110 ([^{3}H]isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-methoxycarbonylpyridine-3-carboxylate) was conducted at 30°C, per the

Address correspondence to Dr. J. David Johnson, Department of Medical Biochemistry, College of Medicine, Room 333 Hamilton Hall, The Ohio State University, 1645 Neil Avenue, Columbus, OH 43210.

Dr. Toshiharu Oba's present address is Department of Physiology, Nagoya City University Medical School, Mizuho-Ku, Nagoya 467, Japan.

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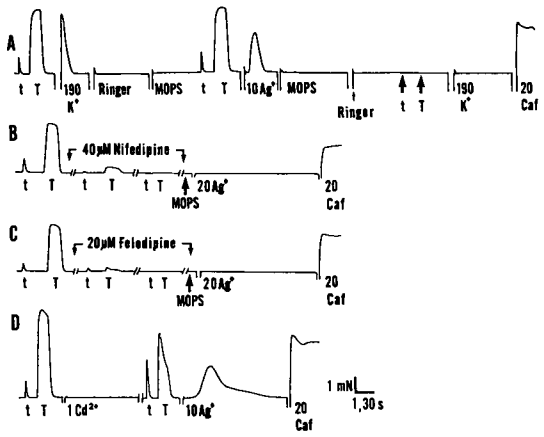

FIGURE 1 Effects of DHPs and Cd^{2+} on Ag^+ contractions. (*A*). After twitch (*t*), tetanus (*T*), and 190 mM KCl (K^+) contractions the fiber was washed with Ringer and with Ca^{2+} free nitrate Ringer. A *t* and *T* in Ca^{2+} free nitrate Ringer buffer were followed by a typical Ag^+ (10 μM) transient contraction (Ag^+ tension was 0.61 × *T*). After a wash with Ca^{2+} free nitrate Ringer and Ringer, no *t*, *T*, nor K^+ contractions were observed, but caffeine (20 mM) produced contracture (0.8 × *T*). (*B*) and (*C*). Nifedipine (40 μM) or felodipine (20 μM) were applied to the fibers after *t* and *T* in Ringer, and *t* and *T* were elicited once every 1 or 2 min for the first 10 min, and once every 5 or 10 min for the next 30 min. The *t* (but not *T*) tension, was increased 132% over controls 1 min after DHP application (not shown). *T* and *t* tension were reduced 90–97% by both DHPs after 15 min (first *t* and *T* shown + drug), and were inhibited completely after 30 min (second *t* and *T* shown + drug). After a further 30 min +DHP, the fiber was washed (three times) with Ca^{2+} free nitrate Ringer buffer and no *t* or *T* tensions were observed (not shown). Subsequent addition of 20 μM Ag produced no tension, but 20 mM caffeine produced contractures. (*D*). After *t* and *T* in Ringer the fiber was washed with Ca^{2+} free nitrate Ringer buffer containing 1 mM Cd^{2+}, which decreased *T* tension. Ag^+ (10 μM) produced contraction in the presence of Cd^{2+} although, the magnitude of the Ag^+ contraction was reduced by 25% and the rate of its relaxation was reduced by nearly 40%. Cd^{2+} did not affect caffeine contractions. Calibration: 1 mN, 1 s for *t* and *T*, and 30 s for Ag^+-, K^+- and caffeine-contractions (// = break in time).

method of (19), except the buffer had no KCl. It consisted of 50 mM MOPS, pH = 7.0, 0.1% digitonin, 1 mg/ml bovine serum albumin, 100 μM $CaCl_2$, and 0.5 nM [^{3}H]-PN200-110 (Amersham Corp., Arlington Heights, IL), 4 μg/ml of Ca^{2+} channel, and the indicated-added [$AgNO_3$]. Nonspecific binding was determined in the presence of 2 μM cold felodipine.

SH reactivity of the calcium channel was monitored at 22°C using the fluorescent maleimide, 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid, (MIANS; Molecular Probes, Inc., Eugene, OR), which undergoes a large fluorescence increase when it reacts with free SH groups on proteins (20).

RESULTS

Treatment of a frog skeletal muscle fiber with 10 μM Ag^+ induced a transient contraction (Fig. 1 *A*) as previously reported by Oba and Hotta (7, 8). Even after a subsequent wash with Ca^{2+} free nitrate Ringer and with Ringer (to remove Ag^+), there was a complete inhibition of twitch, tetanus, and K^+-induced tension. Caffeine-induced contractions still occurred after Ag^+ treatment, suggesting that SR-Ca^{2+} release and the contractile apparatus were functional. Twitch and tetanus tension were rapidly (<1 s) inhibited after an Ag^+ contraction (data not shown).

These Ag^+ contractions occur without membrane depolarization and in the presence of tetrodotoxin (8). They are abolished when the fiber is detubulated (by glycerine Ringer), suggesting a direct effect of Ag^+ on E–C coupling at the level of the T-tubular system (7, 8). Since the T-tubular Ca^{2+} channels are essential to E–C coupling, we examined the possibility that Ag^+ interacts with the Ca^{2+} channel to produce contraction and the subsequent failure of E–C coupling.

Treatment of fibers with DHPs (nifedipine or felodipine) initially potentiated twitch tension (not shown), then inhibited twitch and tetanus tension (Fig. 1, *B* and *C*), as reported by others (3, 9–11). After three washes with Ca^{2+} free nitrate Ringer, twitch and tetanus were still inhibited and 20 μM Ag^+ produced no contraction in either the felodipine- or the nifedipine-treated fiber. Subsequent treatment with caffeine induced release of SR-Ca^{2+} and produced tension. Thus, DHPs, which block the Ca^{2+} channel's voltage sensor and inhibit charge movement (3, 9, 11), completely suppress Ag^+-induced contractions.

In the presence of 1 mM cadmium (Cd^{2+}), which inhibits Ca^{2+} influx through the Ca^{2+} channel but not its voltage sensor (9), twitch and tetanus tension were still produced and Ag^+ still produced a transient contraction (Fig. 1 *D*). Thus, Cd^{2+} blockade of Ca^{2+} entry does not inhibit Ag^+ contractions. Moreover, normal Ag^+ contractions were observed in the absence of extracellular Ca^{2+} (Fig. 1 *A*) and in the presence of 2-mM EGTA (8). Thus, the entry of extracellular Ca^{2+} through the Ca^{2+} channel is not required for Ag^+ contractions.

These findings are consistent with the hypothesis that (1) DHP channel antagonists inhibit Ag^+-induced contractions by inhibiting the voltage sensor of the Ca^{2+} channel and not by blockade of Ca^{2+} entry and (2) that the failure of E–C coupling after Ag^+ treatment may be due to Ag^+ inhibition of the Ca^{2+} channel voltage sensor.

If Ag^+'s site of action is the voltage sensor of the Ca^{2+} channel, then perchlorate (ClO_4^-), which selectively shifts the voltage-dependence of charge movement to more negative potentials (12–14), might be expected to potentiate Ag^+ contractions. ClO_4^- shifted the mechanical threshold (as measured by K^+ contracture) to more negative potentials in a dose-dependent manner (8.3 mV shift at 0.3 mM, 14.3 mV at 1 mM, 19.8 mV at 10 mM, and 23.8 mV at 30 mM ClO_4^-), consistent with the results of Gomolla et al. (12). Fibers were treated with 0 mM, 10 mM, or 30 mM ClO_4^- for 3 min in Ca^{2+} free nitrate Ringer, followed by addition of 5 μM Ag^+ (Fig. 2, *A*, *B*, or *C*, respectively). With increasing concentrations of ClO_4^-, the relative values for the maximum rate of rise of the Ag^+ contraction were increased significantly

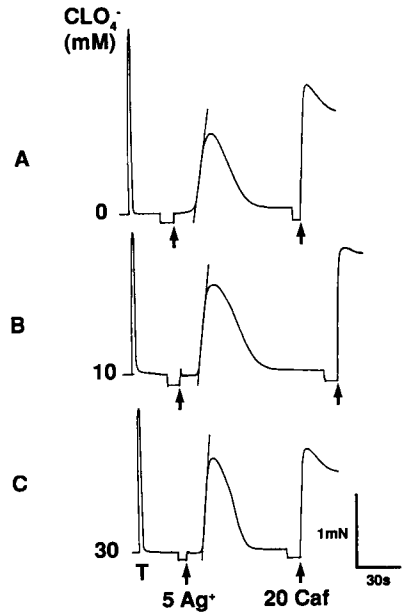

FIGURE 2 Effect of perchlorate on Ag^+ contractions. $NaClO_4$ was applied to single fibers at a final concentration of 0 mM, control (*A*), 10 mM (*B*), or 30 mM (*C*). Maximum tetanus tension (*T*) was checked in Ca^{2+} free nitrate Ringer buffer with each [$NaClO_4$] before addition of 5 μM Ag. The relative maximum rate of rise of Ag^+ tension (relative T_{max}) was determined as the maximum rate of rise of tension (T_{max}) divided by the maximum tension (*T*), because *T* depends upon fiber diameter. Twitch amplitude was potentiated dose-dependently immediately after exposure to $NaClO_4$, 1.37 ± 0.11, (SD, n = 5) at 0.3 mM $NaClO_4$, 2.26 + 0.23 at 1 mM, 3.30 ± 0.71 at 3 mM, 4.72 ± 1.50 at 10 mM, and 4.18 ± 1.70 at 30 mM. *T* tension was slightly decreased (0.96 ± 0.04 (SD, n = 4) and 0.94 ± 0.09 times control at 10-mM and 30-mM ClO_4, respectively). Caffeine (20-mM) contractions were not affected by $NaClO_4$.

from 0.11 ± 0.02 s^{-1} (±SD, n = 7) for controls, to 0.14 ± 0.02 s^{-1} ($P < 0.05$, n = 5) for 10 mM, and to 0.20 ± 0.04 s^{-1} ($P < 0.01$, n = 4) for 30 mM ClO_4^-. Other tension parameters (relative Ag^+ peak tension, maximum rate of fall, contractile duration) were not significantly affected. Thus, ClO_4^-, which selectively potentiates activation of the DHP receptor's voltage sensor, also potentiates Ag^+ contractions.

The DHP Ca^{2+} channel agonist, Bay K 8644 (see reference 15) produced a similar potentiation of Ag^+ contractions. Bay K 8644 (60 min treatment with 100 nM) potentiated 20 mM KCl contractions from P/T (amplitude of Ag^+ contraction/amplitude of tetanus tension) = 0.2 ± 0.07 (n = 8), to P/T = 0.30 ± 0.18 (n = 11), as previously reported by Frank (16). Bay K 8644 significantly ($P < 0.05$) increased the relative rate of rise of a 2 μM Ag^+ contraction from 0.11 ± 0.04 s^{-1} (n = 5) to 0.21 ± 0.07 s^{-1} (n = 4), and increased the magnitude (P/T) of the Ag^+ contraction from 0.36 ± 0.09 to 0.48 ± 0.13 (data not shown). The observations that perchlorate and Bay K 8644, which directly affect the voltage sensor of the Ca^{2+} channel (12–15), potentiate Ag^+ contractions suggest that Ag^+ might directly affect the voltage sensor component of the DHP receptor-Ca^{2+} channel or that the voltage sensor may directly affect the Ag^+ binding site in skeletal muscle.

Ag^+ inhibits DHP binding to the partially purified rabbit skeletal muscle Ca^{2+} channel with an IC_{50} of 1.1 μM (Fig. 3). Oba and Hotta (7) have previously shown that Ag^+ contractions in skeletal muscle fibers occur half-maximally near 1.5 μM Ag^+. Thus Ag^+ interacts with the purified Ca^{2+} channel to inhibit DHP binding, over the same concentration range where it induces Ag^+ contractions, and subsequently inhibits twitch and tetanus tension.

Ag^+ interacts with SH groups on proteins and Murphy et al. (17) have shown that modification of free SH groups on the Ca^{2+} channel inhibits DHP binding. We, therefore, examined the effectiveness of Ag^+ in inhibiting a fluorescent maleimide, 2-(4-maleimidoanilino) naphthalene-6-sulfonic acid (MIANS) reactivity with the Ca^{2+} channel. Ag^+ inhibited MIANS reactivity with the Ca^{2+} channel with an IC_{50} of 0.11 μM Ag^+ (Fig. 3). This suggests that Ag^+ reacts with free SH groups on the Ca^{2+} channel, blocking their reaction with maleimide, and subsequently inhibits DHP binding. Thus, over a similar concentration range where Ag^+ induces contractions and inhibits E–C coupling of skeletal muscle fibers, it binds to the Ca^{2+} channel and inhibits its SH reactivity and DHP binding. This suggest that Ag^+ could inhibit E–C coupling and DHP binding by interacting with critical SH groups on the Ca^{2+} channel.

Treatment of the muscle fiber with 0.1% H_2O_2 inhibited neither twitch nor tetanus tension but was effective in inhibiting subsequent Ag^+ contractions (Fig. 4 *A*).

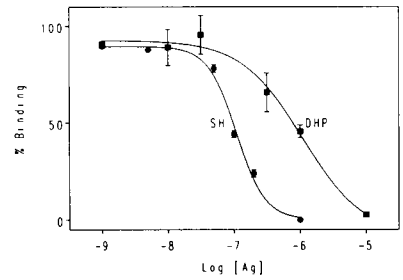

FIGURE 3 Ag^+ inhibition of sulfhydryl (SH) reactivity (●– – –●) and DHP binding (■– – –■) to partially purified Ca^{2+} channel. [^{3}H]-PN200-110 binding is shown as a function of increasing concentrations of $AgNO_3$. DHP binding was conducted as described in Materials and Methods. Each point is the average of six determinations, except for pAg = 9.0 and 5.0, where n = 3. Standard error is shown for each point unless it was less than 2%; 100% = 12.5 pmol PN200-110 bound/mg protein. Ag^+ inhibition of MIANS reactivity with calcium channel SH groups was conducted in 1-ml 50 mM MOPS, pH = 7.0, 0.1% digitonin, 2 mM EDTA, 10 μM MIANS with the indicated [$AgNO_3$]. Each reaction was started by the addition of 14 μg of Ca^{2+} channel and the initial rate of the fluorescence increase was followed on a spectrofluorometer (model LS-5; Perkin-Elmer Corp., Norwalk, CT) with excitation at 320 nm and emission at 440 nm. Each point is the average of three determinations.

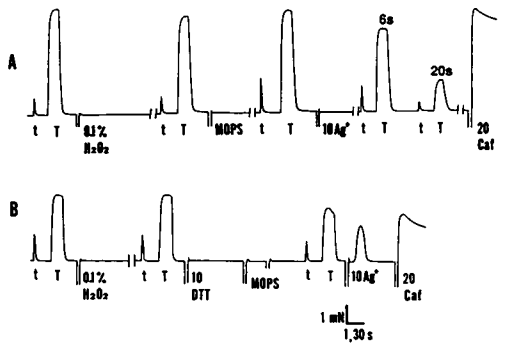

FIGURE 4 Effect of H_2O_2 and DTT on Ag contractions. (*A*). After a twitch (*t*) and tetanus (*T*), the fiber was treated with 0.1% H_2O_2 for 10 min in Ringer followed by a second *t* and *T*. After a rinse with Ca^{2+} free nitrate Ringer, and *t* and *T*, the fiber was exposed to 10 μM Ag^+. The *t* and *T* tensions are shown at 6 and 20 s after Ag^+ addition. Ag^+ fully inhibited *t* and *T* only after 5 mins. Caffeine (20 mM) produced full contraction. (*B*). After a *t* and *T* the fiber was washed with Ringer + 0.1% H_2O_2 for 10 min, followed by a second *t* and *T*. The fiber was then rinsed with Ringer containing 10 mM DTT for 2 min and then washed with Ca^{2+} free nitrate Ringer three times, followed by *t* and *T* tensions (with slightly decreased amplitudes). Ten μM Ag^+ produced a typical Ag^+ contraction. Calibration: 1 mN, 1 s for *t* and *T*, and 30 s for Ag^+- and caffeine contractions.

H_2O_2 treatment markedly slowed Ag^+ inhibition of twitch and tetanus: twitch and tetanus were still observed 6 and 20 s after Ag^+ treatment (Fig. 4 *A*) but were completely inhibited after 5 min of Ag^+ treatment. Thus, H_2O_2 treatment (presumably by oxidation of SH groups) prevents Ag^+ contractions and dramatically slows Ag^+ inhibition of twitch and tetanus tension. If the H_2O_2-treated fibers were treated with DTT (to reduce SH groups), the Ag^+ contraction was fully restored (Fig. 4 *B*). Further, the SH-selective reagent DTNB (5,5′-dithiobis(2-nitrobenzoic acid), 50 mM) produced a 50% reduction in twitch tension and completely inhibited Ag^+ (10 μM) contractions (data not shown). Consistent with this, the selective alkylation of SH groups by maleimides completely inhibits Ag^+ contractions in intact skeletal muscle fibers (18). These data suggest that free SH groups, presumably on the Ca^{2+} channel, are necessary to produce Ag^+ contractions and the subsequent Ag^+-induced inhibition of E–C coupling.

DISCUSSION

Our results indicate that Ag^+ binds to the partially purified, voltage sensitive Ca^{2+} channel of skeletal muscle T-tubules to inhibit DHP binding and block SH reactivity over a similar concentration range where Ag^+ elicits a transient contraction and inhibits E–C coupling. This suggests that the DHP receptor may be the primary site of action for Ag^+ in intact skeletal muscle. Consistent with this, DHP Ca^{2+} channel antagonists, which block the Ca^{2+} channel's voltage sensor (3, 9, 11), and ClO_4^- and Bay K 8644, which potentiate the Ca^{2+} channel voltage sensor (12–15), effectively inhibit and potentiate Ag^+ contractions, respectively. Thus, it is likely that Ag^+ produces a transient contraction, followed by complete failure of E–C coupling, by its interaction with the voltage sensor of the Ca^{2+} channel. Confirmation of this hypothesis awaits electrophysiological examination of the effect of Ag^+ on charge movement and Ca^{2+} channel current–voltage relationships in skeletal muscle.

Currently we do not know why Ag^+ initially induces a contraction followed by a failure of E–C coupling. It is possible that Ag^+ can interact with the SH groups, which inhibit E–C coupling only after a contraction has occurred. Such "use-dependent" phenomena are often seen with drug binding to Ca^{2+} channels. Our observation that oxidation of SH groups by H_2O_2 prevents Ag^+ contractions and Ag^+ inhibition of E–C coupling, in a manner that is reversed by DTT reduction of oxidized SH groups, suggests that free SH groups are necessary for Ag^+ action. Further, Ag^+ inhibits SH reactivity in the partially purified Ca^{2+} channel, suggesting that Ag^+ binding to critical SH groups on the Ca^{2+} channel may be responsible for its inhibition of E–C coupling in skeletal muscle fibers.

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