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Different effects of two gold compounds on muscle contraction, membrane potential and ryanodine receptor

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Abstract

Effects of gold sodium thiomalate and $NaAuCl_4$ on skeletal muscle function were studied using intact single fibres of frog skeletal muscle and fragmented sarcoplasmic reticulum prepared from frog and rabbit skeletal muscles. Gold sodium thiomalate at a concentration of 500 μM decreased tension amplitude by 27% and resting membrane potential by 5.3% after 30 and 22 min, respectively. The duration of tetanus tension was markedly shortened by 500 μM gold sodium thiomalate. When 10 μM $NaAuCl_4$ was applied to gold sodium thiomalate-pretreated fibres, the fibres lost the ability to contract upon electrical stimulation, similar to the effects of 10 μM $NaAuCl_4$ alone. In the presence of thiomalic acid, on the other hand, $NaAuCl_4$ did not completely block tetanus tension even at 50 μM. Thiomalic acid also inhibited $NaAuCl_4$-induced membrane depolarization. These findings suggest that thiomalate masks the effects of gold ion on muscle function. When sarcoplasmic reticulum vesicles were incorporated into lipid bilayers, exposure of the *cis* side of the Ca^{2+}-release channel to 100 μM gold sodium thiomalate rapidly increased the open probability of the channel 3.3-fold, from 0.032 in controls to 0.105, with an increase in number of open events and a decrease in mean closed time. The ability of $NaAuCl_4$ to activate the Ca^{2+}-release channel was much stronger than that of gold sodium thiomalate. Only 1 μM $NaAuCl_4$ was enough to activate the channel and this gold was effective from either side of the channel. These results suggest that gold sodium thiomalate could be used as an antirheumatic drug without considering severe side-effects on skeletal muscle. Coexistent thiomalate probably contributes to protection of muscle function from side-effects of gold ion.

Keywords: Gold sodium thiomalate; $NaAuCl_4$; Thiomalic acid; Muscle contraction; Membrane potential; Ca^{2+}-release channel/ryanodine receptor

1. Introduction

Gold salts such as gold sodium thiomalate (aurothiomalate) and auranofin have been used as remittive therapies in patients with rheumatoid arthritis. Long-term intramuscular treatment with gold seems to prevent premature death in patients with rheumatoid arthritis (Lehtinen and Isomaki, 1991), and leads to a long-lasting improvement in patient mortality (Munro et al., 1998). Japanese acupuncturists sometimes have used gold needles in patients with rheumatoid arthritis to relieve severe pain and to slow the progression of joint destruction. However, gold has been linked to side effects such as proteinurea and peripheral neuropathy (Koh and Boey, 1992; Klinkhoff and Teufel, 1997). The molecular mechanism(s) underlying the actions of gold remains obscure, but it probably acts via the immunological system and alteration of lysosomal enzyme activity (Chaffman et al., 1984). Previous investigation showed that in vitro exposure of skeletal muscles to Au^{3+} (as $NaAuCl_4$) spontaneously elicited a large irreversible contraction, probably mediated through modification of sulfhydryl groups in the L-type Ca^{2+} channel on the transverse tubular membrane (Nihonyanagi and Oba, 1993). Another action of Au^{3+} on skeletal muscle was to activate the ryanodine receptor, when the Ca^{2+}-release channel/ryanodine receptor was isolated from the sarcoplasmic reticulum of frog skeletal muscle and was incorporated into planar lipid bilayers (Nihonyanagi and Oba, 1996). If extracellular Au^{3+} can cross the plasma

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membrane and enter the cell, then Au^{3+} may contribute to muscle contraction through the release of Ca^{2+} from the sarcoplasmic reticulum. This possibility arises from the histological observation that Au^{3+}, released from implanted acupuncture needles can enter cells (Suzuki et al., 1993). To our knowledge, however, there is no information about the direct effects of gold sodium thiomalate on skeletal muscle function. It is very important to gather data for skeletal muscle, because gold sodium thiomalate is administered via the intramuscular route in the therapy for rheumatoid arthritis.

We compared the effects of gold sodium thiomalate on physiological attributes of skeletal muscle, resting and action membrane potentials, twitch and tetanus tensions, and the open probability of the ryanodine receptor/Ca^{2+}-release channel, with the effects of $NaAuCl_4$. The results show that gold sodium thiomalate has a less profound effect on skeletal muscle than does $NaAuCl_4$. Therefore, we suggest that gold sodium thiomalate could be used as an antirheumatic drug without concern for severe effects on skeletal muscles, unlike the serious toxic effects of Au^{3+}.

2. Materials and methods

2.1. Materials

Single fibres for isometric contraction experiments were dissected from the toe muscle (flexor brevis digiti IV or V) of bullfrogs (*Rana catesbeiana*) and placed in ice-cold Ringer solution (in mM: 115 NaCl, 2.5 KCl, 1.8 $CaCl_2$, and 3 sodium phosphate buffer, pH 7.0), as described previously (Oba et al., 1981). Sartorius muscles isolated from bullfrogs were used for measurements of resting and action membrane potentials. Experiments were started after incubation of the fibers for 15 min in cold Ringer solution.

Heavy sarcoplasmic reticulum vesicles were isolated to record the Ca^{2+}-release channel activity from leg muscle of the bullfrog (Koshita and Oba, 1989). In some experiments, we used heavy sarcoplasmic reticulum prepared from leg and back muscles of the rabbit, kindly provided by Dr. T. Murayama, Dept. of Pharmacology, Juntendo University. Heavy sarcoplasmic reticulums were suspended in a small amount of buffer solution containing 100 mM KCl, 20 mM Tris-malate (pH 6.8), 20 μM $CaCl_2$ and 0.3 M sucrose for single channel current recording experiments using planar lipid bilayers. The sarcoplasmic reticulum vesicles were quickly frozen in liquid N_2, and stored at −80°C until use. Protein concentration was determined by the biuret reaction, using bovine serum albumin as the standard.

Stock solutions for each chemical were prepared by dissolving $NaAuCl_4$ (5 mM solution, Nakarai Chem., Kyoto), ruthenium red (5 mM solution, Sigma, St. Louis, MO) and thiomalic acid (0.2 M solution, Wako, Osaka) in ultra-pure water (18.3 MΩ/cm, Barnstead, Boston, MA), caffeine (50 mM solution, Sigma) in Ringer solution, and ryanodine (1 mM solution, Wako) in 99.5% ethanol, respectively. Gold sodium thiomalate was purchased from Shionogi (Osaka) as ampoules (Shiosol, 10 mg/ml). L-α-phosphatidyl-ethanolamine, L-α-phosphatidyl-L-serine and L-α-phosphatidylcholine were from Avanti Polar-Lipids, (Alabaster, AL). Decane was purchased from Wako. Other chemicals were of analytical grade.

2.2. Muscle contraction experiments

After isometric twitch and tetanus tensions (100 Hz for 1 s, 0.2 ms duration) were measured at room temperature as described previously (Oba et al., 1981), the single fibre was exposed to 10 μM gold sodium thiomalate. The fibre was stimulated once every 5 min for 30 min to check the effect of gold sodium thiomalate on twitch and tetanus tensions. After 30 min, the gold sodium thiomalate concentration was increased to 100 μM and the same experimental protocol was followed. Then, tension experiments were repeated after an increase in the gold sodium thiomalate concentration to 500 μM. After that, 10 μM $NaAuCl_4$ was applied to gold sodium thiomalate-treated fibres to evaluate the additive effect of Au^{3+} on muscle contraction. Finally, caffeine, 5 mM, was added to the fibre to determine whether the sarcoplasmic reticulum and contractile proteins were still functional after gold treatment.

The effect of thiomalic acid on twitch and tetanus tension was used as a control response. The single fibre was exposed to 500 μM thiomalic acid after twitch and tetanus tensions in Ringer's solution were observed, similar to the gold sodium thiomalate experiment described above. The fibre was stimulated once every 5 min for 30 min in the presence of thiomalic acid and then the inhibitory effect of 10, 50 and 100 μM or sometimes 200 μM $NaAuCl_4$ on tetanus tension was checked.

2.3. Resting and action potential measurements

Membrane potentials were measured intracellularly by a conventional glass microelectrode technique. Resting membrane potentials were recorded as a function of incubation time from sartorius muscles which had been soaked in Ringer solution containing 10, 50, 100 or 500 μM gold sodium thiomalate and 10 or 100 μM $NaAuCl_4$. Action potentials were measured in the sartorius muscle in the presence of either 500 μM gold sodium thiomalate or 100 μM $NaAuCl_4$. As a control, we evaluated the effects of 500 μM thiomalic acid on resting and action potentials. In some experiments, the effects of 100 μM $NaAuCl_4$ were checked in fibres pretreated with 500 μM thiomalic acid for 30 min. All experiments were performed at room temperature (20 ± 1°C).

2.4. Single-channel recording experiment

Single channel recordings were carried out by incorporating heavy sarcoplasmic reticulum vesicles into planar lipid bilayers (Oba et al., 1998). Lipid bilayers consisting of a mixture of L-α-phosphatidylethanolamine, L-α-phosphatidyl-L-serine and L-α-phosphatidylcholine (5:3:2 w/w) in *n*-decane (40 mg/ml) were formed across a hole 300 μm in diameter in a polystyrene partition separating two chambers (*cis* and *trans*). The *cis* (1 ml)/*trans* (1.5 ml) solutions consisted of 250/50 mM $CsCH_3SO_3$ and 10 mM CsOH (pH 7.4 adjusted by HEPES). Sarcoplasmic reticulum vesicles (~2 μg/ml) were added to the *cis* chamber. After channel incorporation had been confirmed by the occurrence of flickering currents, further incorporation of vesicles was prevented by adding an aliquot of 2.2 M $CsCH_3SO_3$ (pH was adjusted to 7.4 by HEPES) to the *trans* compartment. The cytoplasmic surface of the ryanodine receptor faced the *cis* side, as previously shown, using application of ATP to the *cis* chamber (Oba et al., 1996).

Single channel currents were amplified by a patch clamp amplifier (Axopatch 1D, Axon Instrument, CA) and filtered at 1 kHz using an eight-pole low-path Bessel filter (Model 900, Frequency Devices, MA) and then digitized at 5 kHz for analysis. Data were saved on the hard disk of an IBM personal computer. The mean open probability (P_o) and lifetime of open and closed events of the Ca^{2+} release channel from records for ~2 min were calculated by 50% threshold analysis using pClamp (Version 6.0.4, Axon Instrument) software. pSTAT (Axon instrument) was used for analyzing and curve-fitting idealized data generated by FETCHAN (Axon instrument) from single channel recordings. In order to fit a non-linear equation to a data set, iterative techniques are required (Dempster, 1993). In the Levenberg–Marquadt least-squares fitting method we used, the sum of squared errors (SSE) during the fitting, the sum of the squares of the data (SS) and the number of iterations were used to evaluate the goodness of the fit. The closer the value of the coefficient of the determination, $1 - \sqrt{SSE/SE}$, is to 1 the better the fit.

The results are presented as means ± S.E. Statistical analysis was performed with a paired *t*-test or Student's *t*-test. $P < 0.05$ was regarded as significant.

3. Results

3.1. Effects of gold sodium thiomalate and $NaAuCl_4$ on muscle contraction

Exposure of a single muscle fibre to 10 μM gold sodium thiomalate significantly decreased twitch tension from 0.63 mN of the controls to 0.43 mN after 30 min ($P < 0.05$, $n = 8$), but did not affect tetanus tension (Table 1). A typical recording is depicted in Fig. 1B. An increase in gold sodium thiomalate concentration to 100 or 500 μM further decreased twitch tensions in time and dose dependently (to 81% and 53% of control values at 100 μM and to 50% and 35% at 500 μM 10 and 30 min after incubation, respectively). Even at higher concentrations, gold sodium thiomalate did not produce spontaneous tension (data not shown), an effect which differed from the response to $NaAuCl_4$ (Nihonyanagi and Oba, 1993). The effect of gold sodium thiomalate on tetanus tension seemed less intense than that on twitch tension. Gold sodium thiomalate at 500 μM significantly decreased the amplitude of tetanus tension to 82% and 73% of the control values after incubation for 10 and 30 min, respectively. The half-duration of tetanus tension was highly significantly shortened after application of 500 μM gold sodium thiomalate, while the muscle was stimulated at 100 Hz (Table 1 and see Fig. 1D for an example), indicating no retention of muscle activation. This may indicate that the fibre failed to respond to each stimulation during a long repetitive stimulation for 1 s. A rapid loss of tetanus tension was observed after subsequent application of 10 μM $NaAuCl_4$ to such gold sodium thiomalate-treated fi-

Table 1
Effects of gold sodium thiomalate on twitch and tetanus tensions

Concentrations (μM)	Incubation time (min)	Twitch tension (mN)	Tetanus tension	
			(mN)	Half duration (s)
0	–	0.631 ± 0.073	3.59 ± 0.37	1.11 ± 0.01
10	10	0.595 ± 0.099	3.79 ± 0.38	1.11 ± 0.01
	30	0.430 ± 0.072[a]	3.78 ± 0.43	1.09 ± 0.01
100	10	0.509 ± 0.104[a]	3.59 ± 0.41	1.09 ± 0.01
	30	0.336 ± 0.062[b]	3.47 ± 0.49	1.05 ± 0.01[a]
500	10	0.315 ± 0.079[b]	2.96 ± 0.43[b]	0.73 ± 0.08[b]
	30	0.222 ± 0.048[b]	2.63 ± 0.52[b]	0.59 ± 0.09[b]

Data from 8 different single fibres. Mean ± S.E.
[a] $P < 0.05$, compared with control fibres by paired *t*-test.
[b] $P < 0.01$, compared with control fibres by paired *t*-test.

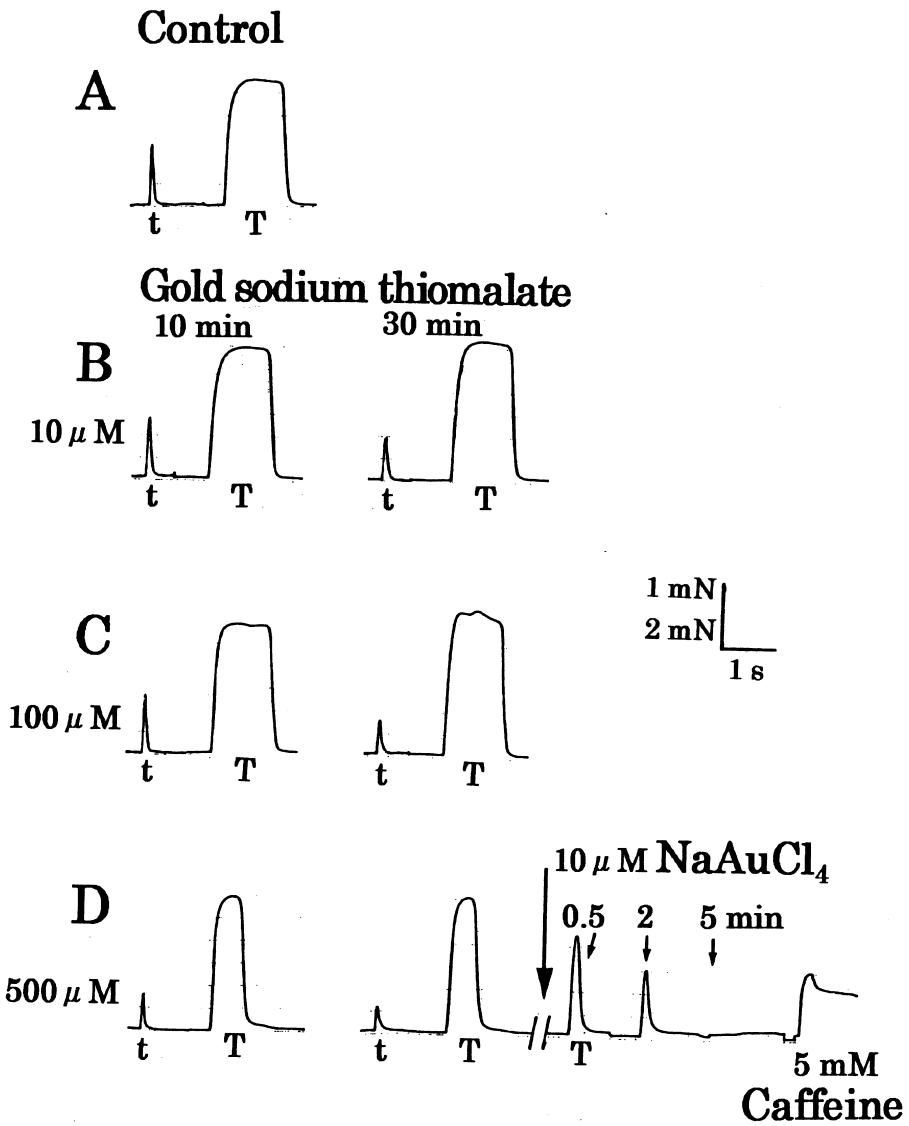

Fig. 1. Effects of sodium gold thiomalate on twitch and tetanus tensions. A: twitch (t) and tetanus (T) tensions in Ringer's solution. B, C, and D: t and T 10 and 30 min after application of 10, 100 and 500 μM gold sodium thiomalate, respectively. In D, 10 μM $NaAuCl_4$ was added to find the additive effect of $NaAuCl_4$ on the fibre which had been pretreated for 30 min with 500 μM gold sodium thiomalate. Five min later, the fibre was exposed to 5 mM caffeine. Calibration: 1 s and 1 and 2 mN in t and T, respectively.

bres (Fig. 1D). Tetanus tension was reduced from 3.12 ± 0.08 mN before application of $NaAuCl_4$ to 2.06 ± 0.06 mN and 0.03 ± 0.03 mN 30 s and 2 min after exposure to $NaAuCl_4$, respectively ($n = 4$). When we stimulated fibres after 5 min, no tension was elicited in any fibres used. Application of 5 mM caffeine to such a paralyzed fiber produced a large contracture (3.34 ± 0.49 mN, $n = 4$), indicating that the sarcoplasmic reticulum and contractile proteins were still functional.

3.2. Effects of $NaAuCl_4$ on muscle contraction in fibres pretreated with thiomalic acid

Exposure of single fibres to 10 μM $NaAuCl_4$ rapidly reduced tetanus tension. The duration of tetanus tension was markedly shortened 2 min after application of $NaAuCl_4$ in two out of 4 fibres studied, indicating no retention of muscle activation (Fig. 2A). Tetanus tension disappeared in one preparation after 2.5 min, in three after 3 min and in all preparations after 4 min. Application of 5 mM caffeine to paralyzed fibers still produced a large contracture (2.81 ± 0.25 mN, $n = 4$).

Thiomalic acid at 500 μM had little or no effect on twitch and tetanus tensions (twitch and tetanus tensions; 0.62 ± 0.07 and 3.03 ± 0.33 mN in controls to 0.55 ± 0.04 and 2.95 ± 0.31 mN 30 min after application of thiomalic acid, $n = 4$). When the fibres were pretreated with 500 μM thiomalic acid, as shown in Fig. 2, subsequent exposure to 10 or 50 μM $NaAuCl_4$ slightly, but significantly, reduced tetanus tension (2.95 ± 0.31 mN to 2.21 ± 0.10

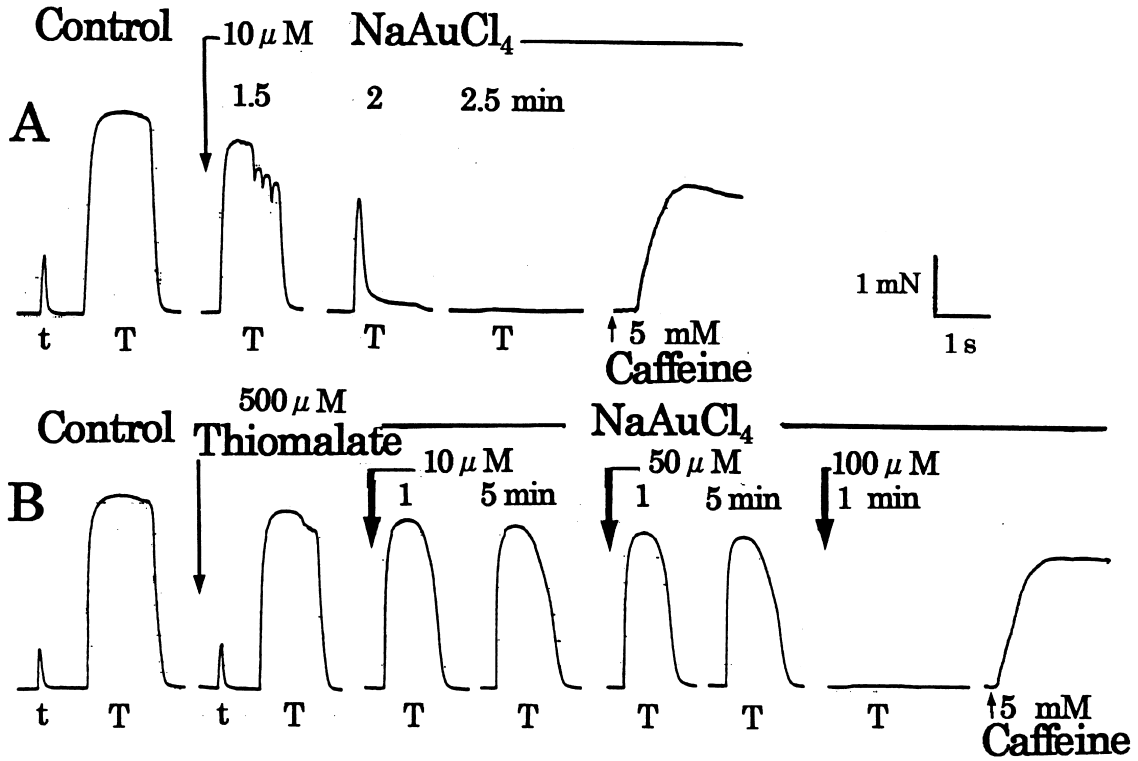

Fig. 2. Inhibition of tetanus tension by $NaAuCl_4$. A: blockade of tetanus tension (T) by 10 μM $NaAuCl_4$ in Ringer's solution. B: effect of 10, 50 and 100 μM $NaAuCl_4$ on tetanus tensions in the fibre which has been treated with 500 μM gold sodium thiomalate for 30 min. Note no inhibition even at 50 μM $NaAuCl_4$ in the presence of gold sodium thiomalate. Both paralyzed fibres contracted upon application of 5 mM caffeine. Calibration: 1 s and 1 mN.

mN and 1.86 ± 0.11 mN ($p < 0.05$) 5 min after application of 10 and 50 μM $NaAuCl_4$, respectively). This relatively mild inhibition was in marked contrast to the complete elimination of tetanus tension by 10 μM $NaAuCl_4$ alone (Fig. 2A). An increase in $NaAuCl_4$ concentration to 100–200 μM completely eliminated the tetanus tension within 30 s. Such fibres still responded to 5 mM caffeine.

3.3. Effects of gold sodium thiomalate, thiomalic acid and $NaAuCl_4$ on resting and action potentials

Our previous study showed that 50 or 100 μM $NaAuCl_4$ depolarized the frog skeletal muscle membrane within 1.5 to 2 min (Nihonyanagi and Oba, 1993). Such depolarization of the membrane potential may lead to a loss of action potential, resulting in no twitch or tetanus tensions. However, we observed muscle contraction in gold sodium thiomalate-treated fibres, as described above. Therefore, one might expect gold sodium thiomalate and $NaAuCl_4$ to have different effects on membrane potentials. This possibility was investigated by measuring intracellular membrane potentials in fibres ($n = 161$) which had been treated with each form of gold. Exposure of fibres to 500 μM gold sodium thiomalate over a range of 30 s to 22 min caused then to depolarize slightly, but significantly (-92.0 ± 0.6 mV in controls, $n = 33$, to -86.3 ± 0.6 mV, $n = 46$, $P < 0.01$) (Fig. 3). When we applied 10, 50 or 100 μM of gold sodium thiomalate to fibres, no membrane depolariza-

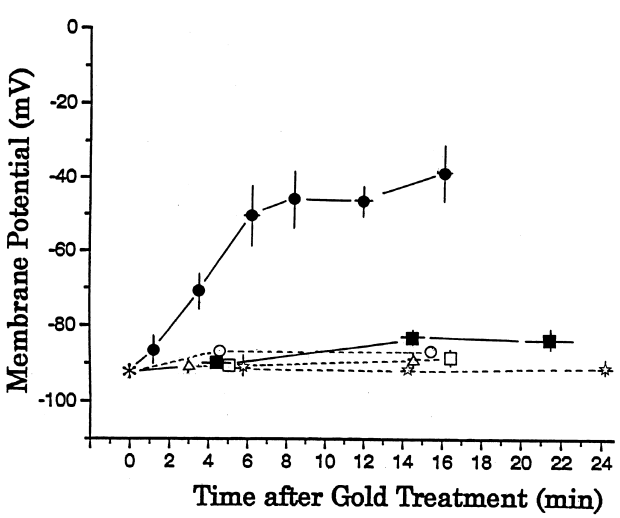

Fig. 3. Resting membrane potential after application of $NaAuCl_4$ and gold sodium thiomalate with or without thiomalic acid. Symbols show membrane potentials measured using the glass microelectrode method at the indicated time after exposure to 100 μM (●) $NaAuCl_4$ and 10 (△), 100 (□) or 500 (○) μM gold sodium thiomalate in Ringer's solution, and to 500 μM thiomalic acid (☆) and 100 μM $NaAuCl_4$ (■) 30 min after 500 μM thiomalic acid. * Represents resting membrane potential in controls. Horizontal and vertical bars in each symbol show S.E. of the mean.

tion was observed (-90.2 ± 1.1 mV ($n = 15$), -87.1 ± 1.2 mV ($n = 20$) and -90.0 ± 0.6 mV ($n = 26$) at 10, 50 and 100 μM gold sodium thiomalate, respectively). Application of 10 μM $NaAuCl_4$ slightly decreased the resting membrane potential (-88.5 ± 1.7 mV, $n = 9$, $P < 0.05$), similar to our previous finding (Nihonyanagi and Oba, 1993). An increase in $NaAuCl_4$ to 100 μM markedly depolarized the membrane potential within 2–5 min in a time-dependent manner (Fig. 3). After about 8 min, the membrane potential remained constant (-36.5 ± 1.9 mV, $n = 34$ from 4 frogs).

In the presence of 500 μM thiomalic acid, the resting potential was not affected during at least 30 min of incubation (-91.0 ± 0.8 mV in controls, $n = 13$ and -90.7 ± 0.7 mV in thiomalic acid, $n = 25$, Fig. 3). Single fibres which had been treated with 500 μM thiomalic acid for 30 min then were exposed to 100 μM $NaAuCl_4$ to determine if thiomalic acid can prevent the depolarizing effect of gold ion on skeletal muscle membrane. Pretreatment with thiomalic acid inhibited $NaAuCl_4$-induced membrane depolarization for at least 22 min (Fig. 3). The mean resting potential was -86.0 ± 1.2 mV ($n = 42$), in contrast to that in the presence of $NaAuCl_4$ without thiomalic acid ($P < 0.01$).

When we applied 500 μM gold sodium thiomalate to single fibers, we found no appreciable change in half-duration of action potential (1.73 ± 0.05 ms vs. 1.67 ± 0.02 ms in controls vs. experimental, $n = 5$, Fig. 4). We also observed no change in the maximum rate of rise (V_{max}: 327 ± 13.7 V/s in controls vs. 353.3 ± 19.7 V/s) and fall ($-V_{max}$: -69.1 ± 3.1 V/s in controls vs. -67.9 ± 4.0 V/s) or amplitude (127.3 ± 1.7 mV in controls to 124.7 ± 1.3 mV) of action potential. When we added $NaAuCl_4$ at a concentration of 100 μM, in contrast, we noted a decrease in the height of action potentials (127.3 ± 1.7 mV to 116.3 ± 10.2 mV), V_{max} (327 ± 13.7 V/s to 260.9 ± 35.1 V/s) and $-V_{max}$ (-69.1 ± 3.1 V/s to -57.6 ± 9.9 V/s) of the action potential, and prolongation of the half-dura-

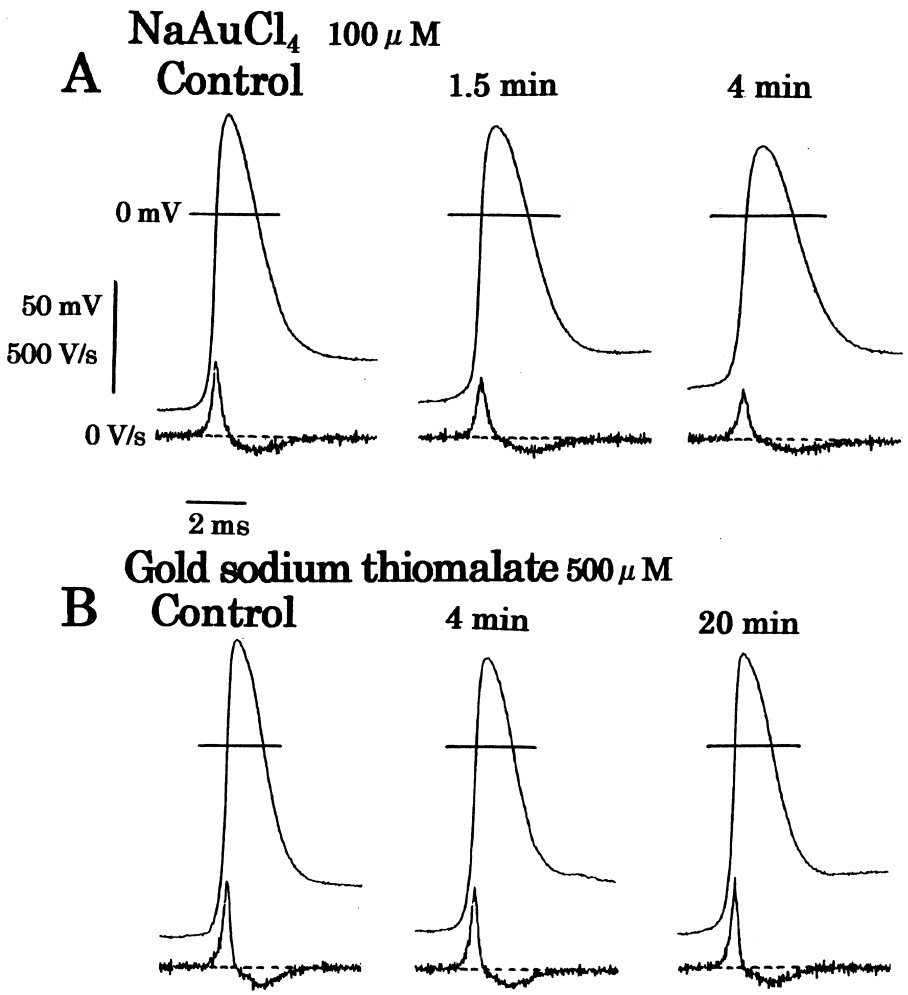

Fig. 4. Effects of $NaAuCl_4$ and gold sodium thiomalate on action potentials and its first derivatives. A: action potentials and its first derivatives obtained from 3 different fibres before and 1.5 and 4 min after application of 100 μM $NaAuCl_4$. After 4 min, no action potential was observed due to depolarization to a membrane potential less than −60 mV. B: no effect of 500 μM gold sodium thiomalate on action potentials and its first derivatives. Noisy traces indicate the first derivative of action potential. Calibration: 2 ms, 50 mV and 500 V/s.

Table 2
Effects of gold sodium thiomalate (GST) and $NaAuCl_4$ on open probability (P_o) and lifetimes of Ca^{2+}-release channel

Concentration (μM)	P_o	Open events (s^{-1})	Mean open time (ms)	Mean closed time (ms)
Control (5)	0.031 ± 0.008	15.9 ± 2.8	1.43 ± 0.23	23.58 ± 4.82
GST, 10 μM (5)	0.078 ± 0.026	36.1 ± 7.4[a]	1.77 ± 0.29	19.95 ± 5.52
Control (6)	0.032 ± 0.007	15.5 ± 2.4	1.34 ± 0.19	24.30 ± 5.21
GST, 100 μM (6)	0.105 ± 0.016[b]	49.3 ± 7.2[b]	1.84 ± 0.39	10.69 ± 2.34[a]
Control (4)	0.026 ± 0.028	15.2 ± 6.5	2.69 ± 1.16	27.46 ± 5.70
$NaAuCl_4$, 1 μM (4)	0.304 ± 0.103[a]	41.6 ± 13.9[a]	6.52 ± 1.83	10.42 ± 3.46[a]

GST and $NaAuCl_4$ were applied to the *cis* side of the Ca^{2+}-release channel. Numbers in parenthesis represent the number of preparations used. Means ± S.E.
[a] $P < 0.05$, compared with paired controls by paired *t*-test.
[b] $P < 0.01$, compared with paired controls by paired *t*-test.

tion of the action potential (1.73 ± 0.05 ms in controls vs. 2.06 ± 0.21 ms, $n = 4$), as measured just before loss of action potential due to membrane depolarization.

The addition of 500 μM thiomalic acid produced no significant effect on parameters of the action potential, such as amplitude, 130.1 ± 1.0 mV vs. 128.6 ± 1.6 mV,

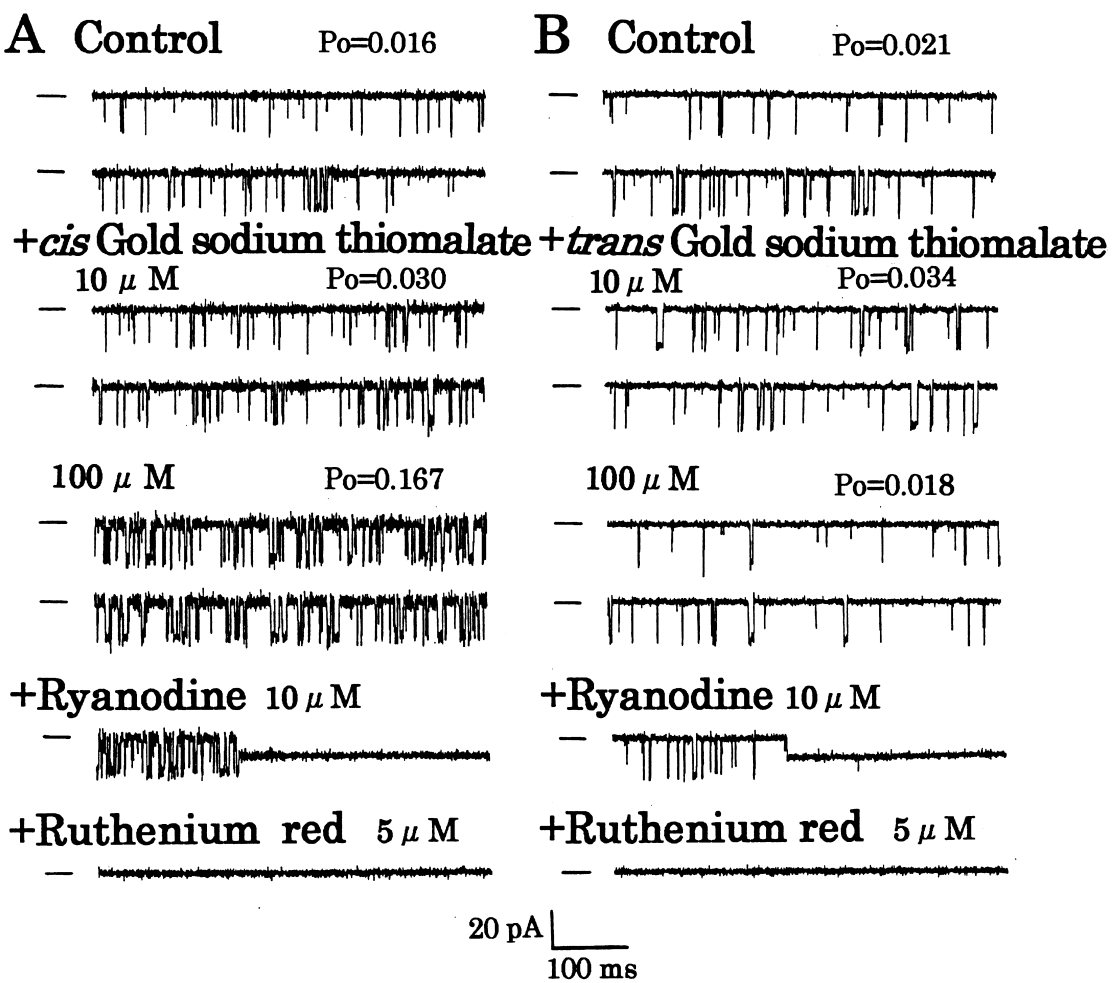

Fig. 5. Effects of *cis* (A) and *trans* (B) gold sodium thiomalate on the Ca^{2+}-release channel activity. A and B: control channel activity activated at pCa 6.4 and pCa 6.2, and after cumulative exposure to *cis* and *trans* gold sodium thiomalate at 10 and 100 μM, respectively. Channels were locked into long-lasting subconductance open state by 10 μM ryanodine and closed by 5 μM ruthenium red. Bars on the left side of each trace indicate the closed level. Calibration: 100 ms and 20 pA.

half duration, 1.92 ± 0.07 ms vs. 2.01 ± 0.04 ms, V_{max}, 334.4 ± 15.3 V/s vs. 279.6 ± 7.1 V/s and $-V_{max}$, -61.5 ± 2.2 V/s vs. -61.1 ± 2.7 V/s in controls ($n = 13$) vs. thiomalic acid-treated ($n = 24$), respectively. Exposure of such thiomalic acid-treated fibres to 100 μM $NaAuCl_4$ for at least 22 min did not affect the amplitude of the action potential (119.7 ± 4.4 mV, $n = 23$), half-duration (2.25 ± 0.05 ms) or V_{max} (299.1 ± 9.0 V/s), although $-V_{max}$ was slightly inhibited (-47.9 ± 2.1 V/s). These findings were in contrast to the action of $NaAuCl_4$ on the action potential in preparations without thiomalic acid.

3.4. Activation of Ca^{2+}-release channel / ryanodine receptor by gold sodium thiomalate and $NaAuCl_4$

To test whether gold sodium thiomalate affects Ca^{2+} release by acting on the skeletal muscle sarcoplasmic reticulum, we evaluated the effect of both gold sodium thiomalate and $NaAuCl_4$ on the Ca^{2+}-release channel incorporated into the planar lipid bilayers. When we applied 10 μM gold sodium thiomalate to the *cis* side of the bilayer in the presence of 0.5–10 μM *cis* Ca^{2+}, the open probability (P_o) of the Ca^{2+}-release channel prepared from frog skeletal muscle was increased 2.5-fold, from 0.031 in controls to 0.078, because of a marked increase in number of open events/s (Table 2), although P_o was not significant due to large variations. Exposure to 100 μM gold sodium thiomalate elicited a rapid and significant activation of the channel ($P_o = 0.032$ in controls to $P_o = 0.105$, $P < 0.01$). Such an increase in the P_o was associated with both an increase in number of open events (15.2/s in controls to 49.3/s) and a decrease in the mean closed time (27.30 ms in controls to 10.69 ms), but not with changes in the mean open time. These results were consistent with channel-gating kinetics showing no change in open time

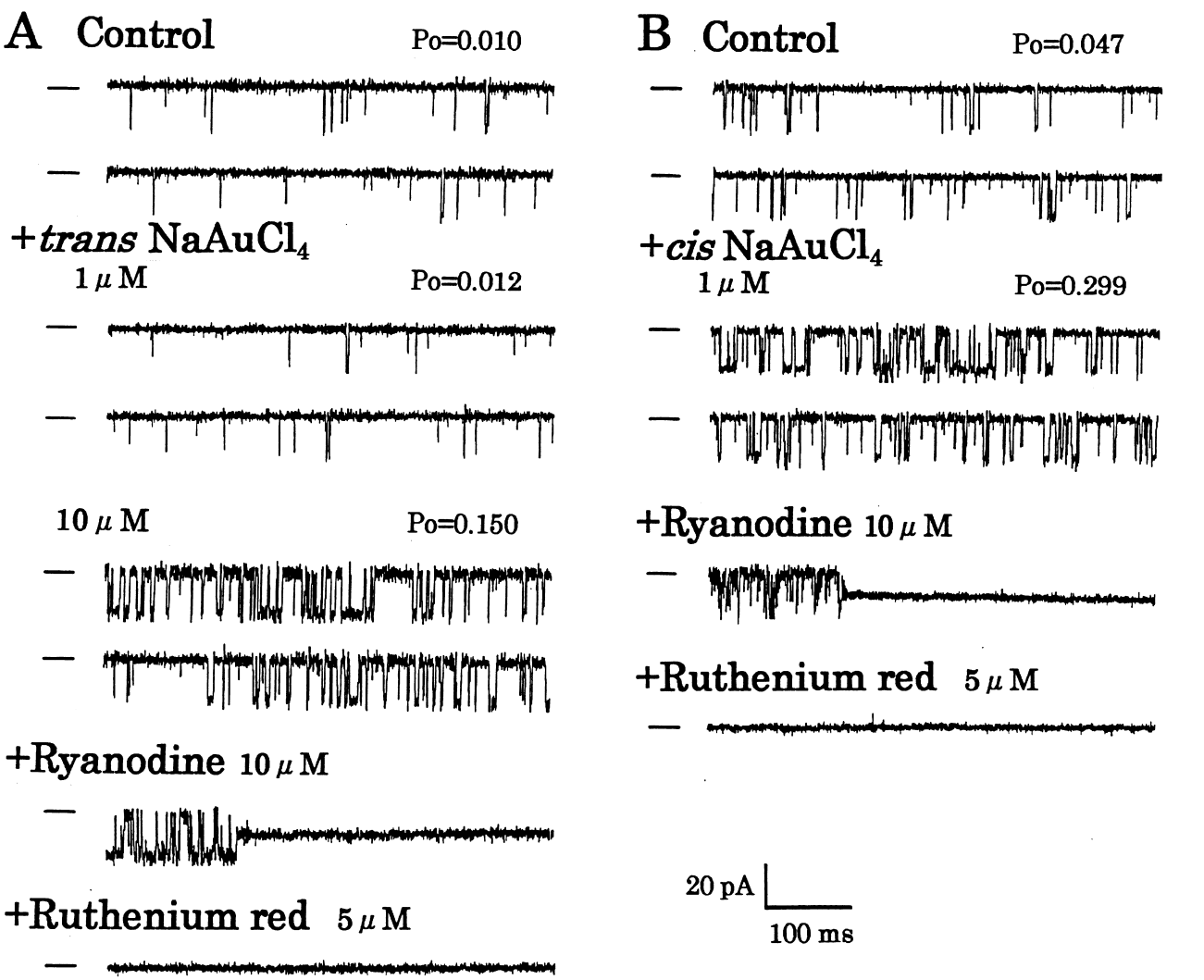

Fig. 6. Activation of the Ca^{2+}-release channel by *trans* (A) and *cis* (B) $NaAuCl_4$. A and B: control channel activity activated at pCa 6.4 and pCa 5.8 and channel activity after cumulative exposure to *trans* $NaAuCl_4$ at 1 and 10 μM, and after application of *cis* $NaAuCl_4$ at 1 μM, respectively. Channels were locked into long-lasting subconductance open state by 10 μM ryanodine and closed by 5 μM ruthenium red. Bars on the left side of each trace indicate the closed level. Calibration: 100 ms and 20 pA.

constants ($\tau_{O1} = 0.36 \pm 0.05$ ms and $\tau_{O2} = 3.49 \pm 1.06$ ms in controls and $\tau_{O1} = 0.37 \pm 0.03$ ms and $\tau_{O2} = 2.16 \pm 0.22$ ms after exposure to 100 μM gold sodium thiomalate, $n = 6$) and showing a significant decrease ($P < 0.01$) in the longest closed time constant ($\tau_{C1} = 0.70 \pm 0.20$ ms, $\tau_{C2} = 4.35 \pm 0.07$ ms and $\tau_{C3} = 27.75 \pm 3.63$ ms in controls and $\tau_{C1} = 1.02 \pm 0.27$ ms, $\tau_{C2} = 4.87 \pm 1.02$ ms and $\tau_{C3} = 15.62 \pm 1.84$ ms after exposure to 100 μM gold sodium thiomalate, $n = 6$). Similar results were observed with another isoform of the Ca^{2+}-release channel which was prepared from rabbit skeletal muscles (data not shown). A typical example showing the effect of gold sodium thiomalate on channel activity is depicted in Fig. 5A. As seen in this figure, the gold sodium thiomalate-modified channel was open-locked by application of 10 μM ryanodine and completely blocked by subsequent addition of 5 μM ruthenium red to the *cis* chamber. On the other hand, treatment of the *trans* side of the channel with 10–100 μM gold sodium thiomalate failed to activate the channel, although ryanodine and ruthenium red were still effective (Fig. 5B).

The ability of $NaAuCl_4$ to activate the ryanodine receptor seemed to be much greater than that of gold sodium thiomalate. When applied to the *cis* side of the channel, 0.1 μM $NaAuCl_4$ failed to increase the P_o (0.009 ± 0.005 in controls to 0.019 ± 0.009, $n = 3$, $P > 0.05$). Increasing the *cis* $NaAuCl_4$ concentration from 0.1 μM to 1 μM markedly increased P_o, as shown in Table 2 (also see Fig. 6B for a typical trace). Such an increase in the P_o may be attributable to the increase in number of open events (15.2/s in controls to 41.6/s) and shortening of the mean closed time (27.5 ms in controls to 10.4 ms). $NaAuCl_4$ at 1 μM produced no significant prolongation of the mean open time. The open lifetime distributions were best fitted by two exponential functions and the open time constants after $NaAuCl_4$ treatment were similar to those of the controls ($\tau_{O1} = 0.56 \pm 0.17$ ms and $\tau_{O2} = 2.83 \pm 1.10$ ms in controls and $\tau_{O1} = 0.50 \pm 0.12$ ms and $\tau_{O2} = 2.50 \pm 0.60$ ms after exposure to 1 μM $NaAuCl_4$, $n = 4$). On the other hand, the closed lifetime distributions were best fitted by three exponential functions. Closed time constants were $\tau_{C1} = 0.57 \pm 0.21$ ms, $\tau_{C2} = 3.98 \pm 0.27$ ms and $\tau_{C3} = 26.15 \pm 4.07$ ms in controls and $\tau_{C1} = 0.53 \pm 0.08$ ms, $\tau_{C2} = 2.84 \pm 1.08$ ms and $\tau_{C3} = 16.20 \pm 5.20$ ms in $NaAuCl_4$-treated channel. The $NaAuCl_4$-activated channel was open-locked by application of *cis* ryanodine and completely blocked by a subsequent addition of ruthenium red.

Exposure of the *trans* side of the Ca^{2+}-release channel to 10 μM $NaAuCl_4$ elicited a rapid and remarkable increase in channel activation ($P_o = 0.010$ in controls to $P_o = 0.150$), although 1 μM $NaAuCl_4$ failed to produce an increase in P_o, as shown in Fig. 5A. The $NaAuCl_4$-activated channel was still responsive to both ryanodine and ruthenium red. Similar results were observed in three different preparations.

4. Discussion

The present study has revealed that gold sodium thiomalate at 10–100 μM has only minor inhibitory effects on muscle contraction and membrane excitation (Figs. 1, 3 and 4), although gold sodium thiomalate activated the Ca^{2+}-release channel/ryanodine receptor when applied directly (Fig. 5). Reportedly, the maximum plasma level of 30–60 μM occurs approximately 2 h after intramuscular injection of 25–50 mg gold sodium thiomalate (Danpure et al., 1979). Gold sodium thiomalate was used in our experiments at concentrations equivalent to, or a slightly higher than, blood gold levels observed in rheumatoid patients, suggesting that this gold compound could be used for treating rheumatoid arthritis patients without severe side-effects on skeletal muscle. As shown in Fig. 1DFig. 2A, however, $NaAuCl_4$ at only 10 μM inhibited tetanus tension in Ringer's solution or in the presence of 500 μM gold sodium thiomalate, and its effect was rapid and complete. In addition, membrane potential was markedly depolarized by 100 μM $NaAuCl_4$ (Fig. 3). At this concentration of $NaAuCl_4$, the muscle failed to develop an action potential upon electrical stimulation (Fig. 4) and produced a spontaneous tonic contracture (data not shown). Such effects of $NaAuCl_4$ on skeletal muscle were consistent with our previous observations (Nihonyanagi and Oba, 1993). Our observations indicate that Au^{3+}, applied as $NaAuCl_4$ here, had quite different effects from gold sodium thiomalate on skeletal muscle and that Au^{3+} was toxic. As shown in Fig. 4A, $NaAuCl_4$ at 100 μM produced a decrease in the height and V_{max} of action potentials immediately before the loss of action potential due to membrane depolarization. It has been reported that heavy metals such as Hg^{2+} and Ag^{+}, and other sulfhydryl reagents reduced irreversibly the membrane potential by acting as sulfhydryl oxidant (Chang et al., 1970; Juang, 1976; Miyamoto, 1983; Oba and Hotta, 1985). Nihonyanagi and Oba (1993, 1996) provided evidence that Au^{3+} shares binding sites with Ag^{+} in frog skeletal muscle membranes. Therefore, Au^{3+} might inactivate the Na^{+} channel via membrane depolarization and influence the shape of action potential and finally lead to no action potential.

Two different gold compounds seem to possess apparently different actions on skeletal muscle, in spite of similar absorption of Au^{3+} from the intramuscular space into the bloodstream. Both gold sodium thiomalate and $NaAuCl_4$ can directly activate the Ca^{2+}-release channel, although the action of $NaAuCl_4$ on the Ca^{2+}-release channel was 10- to 100-fold more effective than that of gold sodium thiomalate. If this difference was applied to effects on tension and membrane potential, then a minor effect of gold sodium thiomalate on muscle functions might be expected. We assumed that the lack of effect of gold sodium thiomalate on contractile activation and muscle excitability is attributable to the presence of thiomalate in gold sodium thiomalate. As demonstrated in Figs. 2 and 3,

pretreatment of fibres with 500 μM thiomalic acid prevented a reduction in tetanus tension upon application of 10–50 μM $NaAuCl_4$ which was quite different from the effect of $NaAuCl_4$ on fibres in Ringer's solution. Therefore, we concluded that thiomalate protects muscle from the severe effects of gold. This conclusion is supported by observations that the membrane depolarization induced by 100 μM $NaAuCl_4$ in Ringer's solution was completely prevented by pretreatment with 500 μM thiomalic acid (Fig. 3). Fig. 1D indicates that 10 μM $NaAuCl_4$ rapidly inhibited the contraction in gold sodium thiomalate-treated fibres. This also indicates that the intrinsic effect of 500 μM gold sodium thiomalate as gold ion is weakened by the protective action of thiomalate, but still occurs partially as shown in Fig. 1DFig. 3. When applied subsequently, however, 10 μM $NaAuCl_4$, by acting additionally with such a partial effect of gold sodium thiomalate, had a more severe effect than that of the same concentration of $NaAuCl_4$ in Ringer's solution containing no gold sodium thiomalate.

It is worth noting that thiomalic acid at 500 μM did not influence the duration of tetanus tension and did not depolarize the membrane potential, suggesting that the gold moiety, but not thiomalate, in gold sodium thiomalate was capable of producing these effects on skeletal muscle. This is consistent with previous observations that thiomalate alone had no significant antirheumatic activity (Rudge et al., 1988) and that it also did not inhibit immunoglobulin M (IgM) production, while gold sodium thiomalate did inhibit (Hirohata, 1996). Antigen-induced blastogenesis was also inhibited by gold sodium thiomalate, but not by thiomalate (Lipsky and Ziff, 1977). It is, in any case, important that thiomalic acid, even at high concentrations, had no side-effect on functions of skeletal muscle and other organs.

The present results indicate that both gold sodium thiomalate and $NaAuCl_4$ act as activators of the Ca^{2+}-release channel (Figs. 5 and 6, and see Nihonyanagi and Oba, 1996). The threshold concentration of gold ion required to activate the Ca^{2+}-release channel was between 0.1 and 1 μM (Fig. 6). If gold ion dissociated from gold sodium thiomalate molecules entered the cell, as previously noted by Suzuki et al. (1993), then muscle may be activated. In fact, our previous observation that extracellularly applied $NaAuCl_4$ produced a tonic muscle contracture strongly suggests an influx of gold ions into the cell that released Ca^{2+} from the sarcoplasmic reticulum. As shown in Fig. 1, however, exposure of intact single muscle fibres to a high concentration of gold sodium thiomalate produced no contracture, as evidenced by the constant resting tension level. If thiomalate enters the intracellular space together with gold ion, no effect of gold ion on contractile activation would be expected, because of the protective action of thiomalate. This possibility is supported by the fact that a much higher concentration of gold sodium thiomalate than of $NaAuCl_4$ is required to activate the Ca^{2+}-release channel (compare Fig. 5 with Fig. 6). Alternatively, no contracture would be produced if the intracellular concentration of gold ion did not exceed the threshold concentration for Ca^{2+} release, even in the presence of 500 μM gold sodium thiomalate in the medium. A related finding was that gold sodium thiomalate released Ca^{2+} from permeabilized neutrophils, but had no effect on intact cells (Wong et al., 1990). However, we have observed that 500 μM gold sodium thiomalate directly activated Ca^{2+}-release channels isolated from skeletal muscle, when applied to the trans side of the channel incorporated into planar lipid bilayers ($n = 2$). Further study will be required to elucidate these possibilities.

It has been reported that in erythrocytes strong binding of gold ion to the membrane occurs via thiol pairs rather than through isolated sulfhydryl groups (Campbell et al., 1992). Inhibition of interleukin-2 release from T cells may also be mediated by formation of an adduct between gold ion and two thiol groups and this inhibitory effect of gold ions might contribute to the therapeutic effect of gold compounds in rheumatoid arthritis (Griem et al., 1995). Previously, we discussed the mechanism by which $NaAuCl_4$ elicits phasic and tonic contractures (Nihonyanagi and Oba, 1993) and suggested a significant role of sulfhydryl groups in the L-type Ca^{2+} channel (dihydropyridine receptor) of the transverse-tubular membrane and on the Ca^{2+}-release channel in the sarcoplasmic reticulum. Gold sodium thiomalate also acts as an activator of the Ca^{2+}-release channel, as described above. Many previous reports provide evidence that sulfhydryl groups in the Ca^{2+}-release channel modify the channel-gating mechanism (Boraso and Williams, 1994; Favero et al., 1995; Oba et al., 1996, 1998; Aghdasi et al., 1997; Eager et al., 1997). Gold sodium thiomalate and $NaAuCl_4$, therefore, may share functions via thiol pairs in the Ca^{2+}-release channel and in the L-type Ca^{2+} channel. This possibility should be investigated further.

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