Acetaldehyde alters Ca\(^{2+}\)-release channel gating and muscle contraction in a dose-dependent manner

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Running title: acetaldehyde and calcium release

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Abstract

We studied whether acetaldehyde, which would be produced by alcohol consumption, impacts ryanodine receptor (RyR) activity and muscle force. Exposure to 50–200 µM acetaldehyde enhanced channel activity of frog RyR and rabbit RyR1 incorporated into lipid bilayers. An increase in acetaldehyde to 1 mM modified channel activity in a time-dependent manner, with a brief activation and then inhibition. Application of 200 µM acetaldehyde to frog fibers increased twitch tension. The maximum rate of rise of tetanus tension was accelerated to 1.5 and 1.74 times the control rate on exposure of fibers to 50 and 200 µM acetaldehyde, respectively. Fluorescence monitoring using Fluo-3 demonstrated that 200-400 µM acetaldehyde induced Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) in frog muscles. Acetaldehyde at 1 mM inhibited twitch tension by ~12% with an increased relaxation time after a small and transient twitch potentiation. These results suggest that moderate concentrations of acetaldehyde can elicit Ca\(^{2+}\) release from the SR by increasing the open probability of the RyR channel, resulting in increased tension. However, the effects of acetaldehyde at clinical doses (1-30 µM) are unlikely to mediate alcohol-induced acute muscle dysfunction.

keywords: ryanodine receptor, single-channel current, Flou-3 fluorescence, Ca\(^{2+}\) release, Ca\(^{2+}\) uptake
Acute intoxication due to alcohol consumption elicits reversible skeletal muscle dysfunction including muscle weakness (1,25,29). The major oxidized product of ethanol, acetaldehyde, may function as a toxic substance underlying the alcohol-induced muscle dysfunction (14). This assumption, however, has not received convincing experimental support necessary to be fully validated. In cardiac myocytes, it has been reported that acetaldehyde in excess of several mM depressed contraction, decreased the intracellular Ca\(^{2+}\) transient, and blocked the L-type Ca\(^{2+}\) channel (3,17,26,27). Acetaldehyde at <1 mM, however, affected neither the resting intracellular Ca\(^{2+}\) level nor the Ca\(^{2+}\) transient (3,26). In skeletal muscles, on the other hand, acute exposure to 0.9-1.8 mM acetaldehyde markedly potentiated twitch tension without changing tetanus force (10). On the contrary, our previous study has demonstrated that channel activity of the ryanodine receptor (RyR1) purified from rabbit skeletal muscles was inhibited by exposure to 1 mM acetaldehyde (21). Because acetaldehyde is metabolized to acetate by aldehyde dehydrogenase (ALDH, EC 1.2.1.3) in the liver, the blood concentration of acetaldehyde is maintained at low levels (<0.5 \(\mu\)M) after intoxication with 0.5g/kg alcohol. In alcoholics, blood acetaldehyde level is relatively high (~2 \(\mu\)M), probably due to hepatic inhibition of ALDH activity (13). Blood concentration of acetaldehyde, on the other hand, remains high (30–500 \(\mu\)M) after alcohol ingestion in Asian persons with genetically low activity of ALDH (7,33). However, the blood level of acetaldehyde has not been reported to reach mM concentrations after alcohol intoxication. To our knowledge, there is no study on the acute effect of low or moderate concentrations of acetaldehyde (<~200 \(\mu\)M) on skeletal muscle function. Therefore, it is interesting to study whether clinical concentrations of acetaldehyde (1-30\(\mu\)M) function as a primary toxin underlying skeletal muscle dysfunction. To elucidate this issue, we examined effects of acetaldehyde on channel activity of two kinds of RyRs, intracellular Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR), and muscle contraction. The results indicate that the effect of acetaldehyde on muscle functions was concentration- and time-dependent. Exposure to 50-200 \(\mu\)M acetaldehyde enhanced RyR channel activity, elicited Ca\(^{2+}\) release \textit{in vivo}, potentiated
twitch tension and accelerated the rate of rise of tetanus. However, such treatment did not affect maximum force and caffeine contracture. Low concentrations of acetaldehyde at $\sim 30 \mu M$ did not affect muscle functions. These observations suggest that acetaldehyde at or near clinical concentrations is unlikely to play a crucial role as the primary factor in skeletal muscle acute dysfunction.

Materials and Methods

Preparations of single intact and mechanically skinned muscle fibers and ryanodine receptors

The institution’s guidelines for the care and use of laboratory animals were followed. Single toe muscle fibers (musculus flexor brevis digiti) from bullfrogs (*Rana catesbeiana*) were dissected 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 (20). The solution was gassed with 95% O$_2$ and 5% CO$_2$.

To examine the direct action of acetaldehyde on the Ca$^{2+}$ release channel, heavy SR vesicles were isolated from leg skeletal muscles of *Rana catesbeiana* (12). The RyR1 was purified from rabbit skeletal muscle (19) and was kindly provided by Dr. T. Murayama (Juntendo University, School of Medicine). Our previous results have demonstrated that channel activity of the skeletal muscle RyRs depended on the redox states of the channel protein (18,22,23), consistent with previous reports (5,8,15). We used only RyR1 channels with mean open probability ($P_o$) $\geq 0.05$ at pCa 4 for the present study. The frog RyR, in which the channel activity displayed a bell-shaped curve of $P_o$ against cytoplasmic (cis) Ca$^{2+}$ concentration similar to that in the RyR1 channel, was examined. The preparations were quickly frozen in liquid N$_2$ and stored at $-80$ °C until use.

Single channel experiments
Dose-dependent effects of acetaldehyde on RyR channel activity were explored by incorporating the frog RyR or the rabbit RyR1 into lipid bilayers, as previously reported (21,22). Lipid bilayers consisting of a mixture of L-α-phosphatidylethanolamine, L-α-phosphatidyl-L-serine and L-α-phosphatidylcholine (5:3:2 wt/wt/wt) in n-decane (40 mg/ml) were formed across a hole of ∼250 μm in diameter in a polystyrene partition separating cis and trans chambers. The cis (1 ml)/trans (1.5 ml) solutions consisted of 250/50 mM CsCH$_3$SO$_3$, 10 mM CsOH (pH 7.4 by HEPES), 0.1 mM CaCl$_2$ for the frog RyR channel and 250/50 mM KCl, 20 mM HEPES/Tris (pH 7.4) and 0.1 mM CaCl$_2$ for the RyR1 channel. After the channel incorporation, single-channel current was recorded in a symmetrical solution containing 250mM Cs$^+$ or K$^+$ adjusted by adding 3 M CsCH$_3$SO$_3$ or KCl to the trans chamber. The cis (cytoplasmic) side was voltage-clamped at −40 mV and the trans (intraluminal) side was held at ground potential. Then, the channel activity was measured upon cumulative application of acetaldehyde to the cis compartment. In another experiment, 1 mM acetaldehyde was exposed to the cis chamber to examine the time-dependent effect on channel activity of the frog RyR.

Single-channel currents were amplified by a patch clamp amplifier (Axopatch 1D, Axon Instrument, Foster City, CA), filtered at 1 kHz using an eight-pole low-path Bessel filter (Model 900, Frequency Devices, Haverhill, MA) and then digitized at 5 kHz for analysis. Data were saved on the hard disk of an IBM personal computer. Po and lifetime of open and closed events of the Ca$^{2+}$-release channel from records of >2 min were calculated by 50% threshold analysis using pClamp software (Version 6.0.4, Axon Instrument, CA).

**Fluorescent monitoring of Ca$^{2+}$ using Fluo-3**

A bundle containing 2-4 fibers was isolated from the frog toe muscle and fixed to the bottom of a 35-mm culture dish. Fiber movement was prevented by stretching to 1.5 times the slack length (3.1 to 3.3 μm of the sarcomere length, as estimated by a laser diffraction method (20)). The fibers were incubated in Ringer’s solution containing 10 μM Fluo-3
acetoxymethylester (Fluo-3 AM) and 0.04% Pluronic F127 (Molecular Probes) for 20 min at room temperature to load Fluo-3. The preparation was set on an inverted microscope (Axiovert 200M, Carl Zeiss, Inc.). Fluorescent intensity was measured to estimate intracellular Ca\(^{2+}\) release using a confocal imaging system (LSM 5 Pascal, Carl Zeiss, Inc.) equipped with an Argon-ion laser. The 488-nm line of the laser was used to excite the Fluo-3, and the emitted fluorescence was collected at wavelengths of >505 nm using a Plan-Neofluar x40 objective (Carl Zeiss, Inst.). Image acquisition was carried out using the full-frame (xy) mode (0.82 ms/line, 512 pixels/line) and data were analyzed by a software (LSM image browser, Carl Zeiss, Inc.). Two-dimensional fluorescence images were obtained by scanning 230.3 µm (x dimension) x 230.3 µm (y dimension) of the area. This procedure resulted in an image-acquisition rate of one frame per 983 ms. The optical resolution of the system was 0.45 µm in the x and y dimensions and 0.8 µm in the z dimension. The fibers were superfused (1.74 ml/min flow rate) with Ringer’s solution containing 50-400 µM acetaldehyde by a circulation pump. The confocal images of intracellular Ca\(^{2+}\) were collected starting two seconds before exposure to acetaldehyde, and then 120 frames were serially recorded. Each of the xy images obtained 2 min after exposure to 200 µM acetaldehyde and 4 min after 400 µM exposure was subtracted from the control image before acetaldehyde application to more readily detect the actual Ca\(^{2+}\) release events. The Ca\(^{2+}\) image was shown as pseudocolors corresponding to the fluorescence intensity.

In another experiment, the Ca\(^{2+}\) transient (492 ms/image, 0.41 ms/line, 512 pixels/line) produced by a single electrical stimulation (0.05-ms pulse duration, supramaximal current through a pair of Pt electrodes) was recorded and compared with acetaldehyde-induced spontaneous Ca\(^{2+}\) release.

**Isometric contraction measurement**

One tendon of the fiber was fixed to a chamber containing 4 ml Ringer’s solution and
the other led to an isometric transducer. Twitch and tetanus tensions (100 Hz for 1 s, 0.05 ms pulse duration; SEN-3301, Nihon-Koden, Tokyo) were checked in oxygenated Ringer’s solution. After the fiber was exposed to 0, 2, 20, 50 or 200 µM acetaldehyde for 2 min, twitch and tetanus tensions were recorded again. At the end of each experiment, a caffeine (20 mM) contracture was elicited to estimate releasable Ca\(^{2+}\) content in the SR. To determine the time-dependent effect of a high concentration of acetaldehyde on twitch tension, a single fiber was exposed to 1 mM acetaldehyde, and then the amplitude and the half-duration of twitch tension (0.1 Hz, 0.05 ms pulse duration) were monitored for 10 min. The releasable Ca\(^{2+}\) content in the SR of such fibers was estimated by applying 20 mM caffeine.

To investigate the effect of acetaldehyde on Ca\(^{2+}\) uptake by the SR, single fibers of frog semitendinosus muscle were mechanically skinned in ice-cold relaxing solution \(H\) (in mM: KCl, 100; total MgCl\(_2\), 4; total ATP, 4; EGTA, 5; pCa=9; Tris-maleate 20, pH 7.0). After removal of Ca\(^{2+}\) remaining in the SR by 10 mM caffeine (\(Solution\ F\): KCl, 100; total MgCl\(_2\), 1; Tris-maleate 20; caffeine, 10 mM), skinned fibers were actively Ca\(^{2+}\)-loaded by immersing in \(Solution\ U\) for 2 min. The \(Solution\ U\) was equivalent to \(Solution\ H\), with the exception that pCa=7.08. Free Ca\(^{2+}\) concentration of the solution was calculated using a \(K_{app}\) for EGTA of 2.51 x 10\(^{-6}\)M\(^{-1}\). The releasable Ca\(^{2+}\) sequestered by the SR was estimated from the peak value of the caffeine contracture. Then, the fiber was washed once with \(solution\ H\) and twice with \(solution\ L\) (equivalent to \(solution\ H\), with the exception that EGTA was 1mM) to remove Ca\(^{2+}\) within the SR. The fiber was incubated for 5 min in \(solution\ N\) (equivalent to \(solution\ F\), with the exception that no caffeine was added) with or without 1 mM acetaldehyde. Ca\(^{2+}\) was taken up again in \(solution\ U\) with or without 1 mM acetaldehyde for relatively brief loading periods (0-30 s). The releasable Ca\(^{2+}\) content in the SR was estimated again from the caffeine contracture. Experiments were done at room temperature (20–22°C).

The results are presented as means ± SD. Statistical analysis was done with ANOVA followed by Fisher’s least-significant difference method or Student’s \(t\) test. Values of \(P<0.05\)
were regarded as statistically significant.

Chemicals.

Caffeine (100 mM stock solution; Sigma Chem., St. Louis, MO) was dissolved in hot distilled water immediately before application to the fiber. Ryanodine (1 mM stock solution; Wako Pure Chemical, Osaka, Japan) and ruthenium red (1 mM stock solution; Sigma) were dissolved in ethanol and ultrapure water, respectively, and stored at –20°C. Acetaldehyde (98% stock solution; Sigma) was diluted to appropriate concentrations with buffer solutions immediately before use. Flou-3 AM (Dojindo, Kumamoto, Japan) was prepared immediately before use.

Results

Activation of RyRs induced by acetaldehyde

Exposure of frog RyRs to 10-30 µM acetaldehyde did not affect channel activity, when the channel was incorporated into lipid bilayers. Increase in acetaldehyde to 50 µM increased the mean open probability (Po) about 3-fold, from 0.044 in the control in pCa 6 to 0.142 (Fig.1A). The number of open events increased from 38.1/s in the control to 67.6/s in 50 µM acetaldehyde, and mean closed time decreased from 41.0 ms to 7.6 ms, but mean open time remained unchanged (1.51 ms in the control vs. 1.36 ms). The threshold concentration required to open the channel was between 30 and 50 µM. A further increase in acetaldehyde to 1 mM, however, markedly decreased the channel activity to Po=0.034 and the number of open events to 34.9/s. Experiments were repeated with 6 separate channels, and the result is summarized in Fig. 1B. A significant increase in Po was observed only when the channel was exposed to acetaldehyde over a range between 50 and 300 µM. As expected from these results, a single application of acetaldehyde at high concentrations modulated the channel gating in a time-
dependent manner. A typical example is depicted in Fig. 2. In this channel, 1 mM acetaldehyde increased Po two-fold, from 0.017 in the control to 0.033, after 10 s and 5-fold, to 0.087, after 30 s. Thereafter, the channel activity drastically decreased (Po=0.013 at 1-min and Po=0.005 at 15-min). Similar results were obtained for 4 separate channels.

Acetaldehyde produced a similar effect on the rabbit RyR1 channel. Exposure to 100 µM acetaldehyde significantly increased channel activity from Po=0.025±0.007 (n=6) in the control to Po=0.089±0.04 (p<0.05) (Fig.1B). In contrast to the frog RyR channel, however, the channel activity in the RyR1 was not enhanced significantly by applying 50 µM or 300 µM acetaldehyde (0.025±0.009 or 0.028±0.014, respectively).

Acetaldehyde-induced Ca\textsuperscript{2+} release from the sarcoplasmic reticulum

Brief local events of Ca\textsuperscript{2+} release from the SR appear as Ca\textsuperscript{2+} sparks in skeletal and cardiac muscles under Ca\textsuperscript{2+} fluorescence monitoring (4,11,31). We explored whether acetaldehyde can cause the release of Ca\textsuperscript{2+} from the SR under in vivo conditions. No significant increase in intracellular Ca\textsuperscript{2+} concentration was observed in response to exposure to 50-100 µM acetaldehyde, compared with controls before application of acetaldehyde (data not shown). Figure 3 C and D show the spontaneous Fluo-3 fluorescence xy images on application of 200 and 400 µM acetaldehyde, respectively. As clearly shown in Fig. 3E-1 or E-2, in which actual Ca\textsuperscript{2+} release events were detected by subtracting the control image (Fig.3B) from the Ca image in Fig. 3C or D, acetaldehyde increased Ca\textsuperscript{2+} release events in all 3 fibers in a dose-dependent manner, although responsiveness to acetaldehyde varied widely among fibers. The Ca\textsuperscript{2+} release was much less after acetaldehyde application than after a single electrical stimulation (Fig.3A) or after exposure to 20 mM caffeine (data not shown), indicating that acetaldehyde only weakly stimulates Ca\textsuperscript{2+} release from the SR in vivo. The action of acetaldehyde was also time-dependent. The Ca\textsuperscript{2+} release began to be observed 5.6 s after exposure to 400 µM acetaldehyde, and Ca\textsuperscript{2+} release events reached maximal about 8 s later. A relatively high concentration of
cytoplasmic Ca\(^{2+}\) was retained for at least 4 min.

**Effects of acetaldehyde on muscle contraction**

The observation that 200-400 µM acetaldehyde can induce the release of Ca\(^{2+}\) from the SR *in vivo*, as evidenced by laser confocal fluorescence microscopy (Fig. 3), suggests that exposure of intact fibers to acetaldehyde may affect mechanical force by activating RyR channels. Application of 2-50 µM acetaldehyde for 2 min did not affect the amplitude of twitch tension (Fig. 4A). An increase in acetaldehyde to 200 µM significantly increased twitch tension 1.22-fold (p<0.05 from control, n=5). The maximum rate of rise of tetanus tension was enhanced by 49.2% in the presence of 50 µM acetaldehyde and 73.6% in 200 µM. Amplitudes of maximum tetanus and caffeine contracture, however, were not affected at each concentration of acetaldehyde used. Concentration-mechanical response relationships are summarized in Fig. 4, expressed as percentage change from respective control values before application of acetaldehyde.

Since 1 mM acetaldehyde had a time-dependent effect on RyR channel activity (Fig.2), we expected that this concentration of acetaldehyde would potentiate the twitch tension transiently and thereafter inhibit it. Electrical stimulation at 0.1 Hz for 10 min had no effect on twitch tension amplitude before application of acetaldehyde (Fig.5A). As shown in Fig.5B, the tension was enhanced (7%) only at the first stimulation after treatment with 1 mM acetaldehyde and then decreased to reach a steady level (87.7 ± 2.0%, p<0.05 from control, n=5). When estimated after 10 min, the half duration of twitch tension was increased to 146.7 ± 4.1% of the control value (p<0.01). The prolongation and the amplitude decrease of twitch tension were restored to control levels by washing fibers with normal Ringer’s solution containing no acetaldehyde (Fig.5C).

**Effect of 1 mM acetaldehyde on Ca\(^{2+}\) uptake by the sarcoplasmic reticulum**
As shown above, a high concentration of acetaldehyde prolonged the twitch tension (Fig. 5), suggesting an inhibition of Ca\(^{2+}\) uptake. This possibility was explored by estimating Ca\(^{2+}\) taken up by the SR of skinned fibers in the presence of acetaldehyde. In controls, the SR was maximally loaded with Ca\(^{2+}\) by incubating the fiber in a solution containing pCa=7.08 for 20 or more seconds, whereas a longer loading period was required in the presence of acetaldehyde (Fig. 6). In addition, the maximal Ca\(^{2+}\) content observed after a 2-min loading of Ca\(^{2+}\) in the acetaldehyde-treated SR was inhibited by 11.2±0.06% (p<0.05, n=5), compared with that in controls. The time required to develop a half maximal Ca\(^{2+}\) accumulation (T\(_{1/2}\)) was 1.8 s for controls and 2.8 s for acetaldehyde-treated fibers, indicating that acetaldehyde slightly inhibited Ca\(^{2+}\) uptake by the SR.

Discussion

The present results are the first to demonstrate that acute exposure of the mammalian skeletal muscle RyR1 and frog RyR to acetaldehyde alters the channel gating mechanism in concentration- and time-dependent manners. Acetaldehyde at the blood concentrations (~100 µM) that would be found in Asian persons with genetically lower activity of ALDH enhanced the channel activity. Acetaldehyde over a similar concentration range (~50-400 µM) evoked also frog RyR channel activation and Ca\(^{2+}\) release from the SR in frog skeletal muscle fibers \textit{in vivo}, as evidenced by Fluo-3 fluorescence experiments. An acetaldehyde concentration of 200 µM was required to potentiate the twitch tension, but at 50 µM the compound failed to change the twitch amplitude. Increase in acetaldehyde to 1 mM inhibited the twitch tension after transient potentiation. These observations suggest that acetaldehyde at blood concentrations (less than 10 µM) in normal subjects or in alcoholics may be unlikely to play a crucial role as the primary factor for acute alcoholic effects on skeletal muscle. However, the present study does not exclude the possibility that acetaldehyde may display some effect on muscle.
dysfunction in Asian subjects who genetically lack ALDH.

The concentration of acetaldehyde required to activate the RyR channel (∼100 µM) appears to be 1-2 order higher than that occurring in the blood during alcohol consumption in normal subjects, as described above. Acetaldehyde concentration in the medium solution should decrease with time, because of its volatility in open air. This may explain why acetaldehyde at concentrations less than ∼30 µM failed to induce any noticeable effect on the RyR channel gating and mechanical force of the fiber. This possibility, however, seems to be unlikely. In the present experiments, exposure of the frog RyR to 50 µM acetaldehyde kept channel activity high for at least 2 min. No time-dependent effect of acetaldehyde on channel activity was observed at a concentration of 30 µM; Po=0.018±0.004 for 30 s immediately after exposure and Po=0.030±0.011 for 30 s after a 2-min exposure (p>0.05, n=6). These results strongly suggest that exposure to acetaldehyde at concentrations over the threshold level is essential to stimulate the Ca²⁺-release channel, and channel activation lasts for several minutes, even though acetaldehyde volatilizes from the medium in a time-dependent manner. The Fluo-3 fluorescence observation that the cytoplasmic Ca²⁺ concentration of a single fiber exposed to 200-400 µM acetaldehyde remained relatively high for a long time (Fig. 3), also supports the long-lasting effect of acetaldehyde.

The action of acetaldehyde as a Ca²⁺-release channel activator may largely contribute to the twitch potentiation and the increased rate of rise of the tetanus tension. In addition, the enhanced effect of acetaldehyde at low to moderate concentrations on the maximum rate of rise of tetanus tension (Fig.4) strongly suggests that acetaldehyde does influence cross-bridge cycling. In this regard, a previous study has shown that exposure of frog skeletal muscle to acetaldehyde at millimolar levels, but not near 100 µM, causes no significant change in the initial rate of rise of the tetanus tension (10). In cardiac muscles, 1 to 3 mM acetaldehyde has been extensively reported to reduce the contraction or cell shortening, but not to affect the maximal shortening velocity (6, 26, 28). The present study, therefore, is the first to demonstrate
that moderate concentrations of acetaldehyde affect cross-bridge kinetics in skeletal muscles. It is of interest to know whether the observation that application of 50 µM acetaldehyde to single fibers accelerates the maximum rate of rise of the tetanus tension can be extrapolated to mammalian muscle fibers. Further studies are required to elucidate this issue. Muscle contractile function also has been accepted to be regulated by other factors, such as myofilament Ca\(^{2+}\) sensitivity and the rate of Ca\(^{2+}\) accumulation by the SR, in addition to the Ca\(^{2+}\) release from the SR and cross bridge kinetics. It is not clear whether clinical concentrations of acetaldehyde affect myofilament Ca\(^{2+}\) sensitivity or Ca\(^{2+}\) uptake rate to enhance the contractile force in skeletal muscle. We did not examine these issues in the present study in detail. As shown in Fig. 6, however, 1mM acetaldehyde elicited a slight inhibition of Ca\(^{2+}\) uptake by the SR, thereby producing the prolonged tension curve seen in Fig. 5.

There is an earlier report that addition of 0.9-1.8 mM acetaldehyde to isolated frog skeletal muscles caused marked twitch potentiation without affecting the tetanus force, whereas at 18 mM, acetaldehyde inhibited twitch and tetanus tensions (10). This observation suggests a concentration-dependent effect of acetaldehyde on skeletal muscle function, consistent with the present study. In our experiments, however, twitch tension was increased at 0.2 mM acetaldehyde but inhibited at 1 mM (Figs. 4 and 5), indicating a shift of one order of magnitude in the effective dose of acetaldehyde compared with the results of Khan (10). The discrepancy remains to be explained, but may be due to the different muscles used: the semitendinosus of *Rana temporaria* and the toe muscle of *Rana catesbeiana*. Acetaldehyde has been reported to exert biphasic effects in heart muscles (26-28). A positive effect was produced at concentrations <3 mM due to β-adrenergic activation and a negative one (such as depression of contraction, shortening and intracellular Ca\(^{2+}\) transient), observed at concentrations >3 mM, was mediated via myogenic mechanisms. In isolated heart muscle cells, acetaldehyde at concentrations of 0.1-1 mM failed to change the intracellular Ca\(^{2+}\) transient and cell shortening. Together, these results suggest that acetaldehyde has only inhibitory action in cardiac muscle. Therefore, the previous
and present findings indicate that the enhanced effects of acetaldehyde at low concentrations on skeletal muscle function are mediated by a mechanism different from that on cardiac muscle, although the inhibitory effects at high concentrations are similar. This discrepancy may be explained by different mechanisms underlying excitation-contraction (E-C) coupling. In skeletal muscle, a voltage sensor on the dihydropyridine receptor (DHPR or L-type Ca\textsuperscript{2+} channel) located in the T-tubule responds to depolarization, leading to Ca\textsuperscript{2+} release from the SR (9). Thus, two proteins, the DHPR as the voltage sensor and the RyR as the SR Ca\textsuperscript{2+}-release channel, are the major components of E-C coupling. It has been proposed that signal transmission from the T-tubule to the SR is mediated by mechanical coupling between the DHPR molecule and the RyR one. Thus, the inhibition of Ca\textsuperscript{2+} influx via the L-type Ca\textsuperscript{2+} channel does not affect skeletal muscle contraction. On the other hand, E-C coupling in cardiac muscle is dependent on trans-sarcolemmal Ca\textsuperscript{2+} influx via the L-type Ca\textsuperscript{2+} channel. Ca\textsuperscript{2+} flow into the myocyte activates further release of Ca\textsuperscript{2+} from the SR in a process termed Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) (9). Acetaldehyde has been demonstrated to block the L-type Ca\textsuperscript{2+} channel (17, 27), thereby depressing muscle shortening and intracellular Ca\textsuperscript{2+} transient in cardiac myocytes. These different roles of L-type Ca\textsuperscript{2+} channels in skeletal muscle and heart muscle could help explain why acute exposure to acetaldehyde can produce only muscle depression in the heart and a biphasic effect in skeletal muscle. In recent research with mouse cardiac muscle, interestingly, overexpression of alcohol dehydrogenase elevated acetaldehyde production and exacerbated the defect of ethanol-induced contractile functions such as cell shortening and the intracellular Ca\textsuperscript{2+} transient (6). These results suggest that chronic exposure to acetaldehyde, even at a low concentration, can induce cardiac contractile dysfunction. Whether this holds true in skeletal muscle is not clear and deserves further study.

The frog RyR channel was activated transiently and immediately after exposure to 1 mM acetaldehyde and thereafter inhibited to reach the steady-state channel activity (Fig. 2), consistent with our previous study dealing with the effect of acetaldehyde on the RyR1 channel.
This concentration- and time-dependent action of acetaldehyde suggests the occurrence of two distinct binding sites on the RyR channel protein. Binding to a high-affinity site contributes to channel activation and binding to a low-affinity site contributes to channel inhibition. It has been well demonstrated that FK506-binding protein (FKBP12) is tightly associated with the RyR1 with a stoichiometry of one mol per mol of the RyR1 monomer to stabilize channel activity (2,30,32). In addition, SR vesicles contain other endogenous proteins including triadin and calsequestrin, and these modulators also affect RyR channel activity (16,24). High concentrations of acetaldehyde may bind to these endogenous associating proteins and develop complicated concentration- and time-dependent effects on the channel protein. As shown in Fig. 1, the concentration-dependent effect of acetaldehyde on channel activity was observed in purified RyR1 preparations, as well as in fragmented SR. Our previous results have demonstrated that the purified RyR1 was free from FKBP12 and other proteins (18). Therefore, it is reasonable to consider that the binding site(s) of acetaldehyde is on the Ca\textsuperscript{2+}-release channel itself, but not on the modulating proteins. The possibility is also eliminated that the action of acetaldehyde is mediated by some mechanism associated with the interaction between the channel and FKBP12. The present observations that acetaldehyde alters the number of open events and mean closed time in concentration- and time-dependent manners, without any changes in unitary conductance and mean open time, suggest that acetaldehyde affects the channel gating mechanism by changing the transition rate between the closed and the open states. Although interesting, we did not conduct further biochemical studies of the binding site(s) or the structural changes of the channel.

In summary, acetaldehyde modified skeletal muscle function in a dose-dependent manner. At blood concentrations of 50 to several hundred µMs, as found in Asian persons who genetically lack ALDH, acetaldehyde acts as a mammalian and frog RyR channel activator. However, it acts as an inhibitor at millimolar concentrations, never found in human blood even after heavy alcohol drinking. The present results suggest that acetaldehyde at or near clinical
blood concentrations in normal subjects or alcoholics may play no role as the primary factor for skeletal muscle dysfunction produced by acute alcohol intoxication. Further studies will be required to elucidate whether acetaldehyde at clinical concentrations may contribute to the chronic dysfunction of skeletal muscle.
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References


Figure legends

Figure 1. Effects of acetaldehyde on single-channel activity of the ryanodine receptor (RyR). A; frog RyR channel. After activation in pCa 6 (control), acetaldehyde was cumulatively applied to the cis chamber. Acetaldehyde at 50 µM induced a marked increase in channel open probability (Po), whereas an increase to 1 mM provoked a decrease in Po. Channel activity ascribed to the RyR was confirmed by the responses to ryanodine and ruthenium red. Downward deflection of channel current indicates channel opening. B; Dose-dependent effects of acetaldehyde on Po in frog RyR (●, n=6) and single rabbit RyR 1 (○, n=6) channel in pCa 6. Significantly different from corresponding control single-channel activity in channels not treated with acetaldehyde: *p<0.05.

Figure 2. Time-dependent effect of acetaldehyde on frog RyR channel activity. A; control in pCa 6, B; time course of channel activity after application of 1 mM acetaldehyde to the cis side. Note channel activation immediately after addition of acetaldehyde, and subsequent marked inhibition. Downward deflection of channel current indicates channel opening. Calibration, 20 pA and 1 s.

Figure 3. Ca²⁺ fluorescence images from three Fluo-3-loaded skeletal muscle fibers. A; an xy image at single electrical stimulation (white arrow, 0.05-ms pulse duration and supramaximal current). Transient fluorescence fluctuations, representing Ca²⁺ release events, are indicated by the pseudocolored peak. The number 1, 2, and 3, shows three different fibers. Acquisition time of the full-frame image was 492 ms. B; resting control scan. Note that different fluorescent intensities between fibers indicate the difference of resting Ca²⁺ level. C; Ca²⁺ fluorescence fluctuation 2 min after exposure to 200 µM acetaldehyde. D; Ca²⁺ fluorescence fluctuation 4 min after exposure to 400 µM acetaldehyde. Inset; E-1, Ca²⁺ release events obtained by subtracting B from C (E-1), indicating actual Ca²⁺ events by 200 µM acetaldehyde. E-2, Ca²⁺
release events obtained by subtracting B from D (E-2), indicating actual Ca\textsuperscript{2+} events by 400 µM acetaldehyde. Acquisition time of full-frame images from B to E-2 was 983 ms. Dotted lines in each scan indicate the border of the fiber.

Figure 4. Concentration-dependent responses to acetaldehyde of single intact fibers of frog toe muscle. A; twitch tension. B; tetanus tension amplitude (●) and its maximum rate of rise (○). C; caffeine contracture amplitude. Each response is indicated as % change of control value before application of acetaldehyde. *p<0.05 vs respective control.

Figure 5. Effects of 1 mM acetaldehyde on twitch tension in single intact fibers. Fibers were electrically stimulated at a frequency of 0.1 Hz for 10 min. The final twitch tension was expanded to examine the rate of rise of twitch and half duration. A; control. B; 1 mM acetaldehyde at an arrow. Note a slight increase in twitch tension immediately after exposure to acetaldehyde and subsequent decrease. C; recovery from the twitch inhibition by acetaldehyde. Calibration; 0.5 mN and 1 min, except for expanded traces (100 ms).

Figure 6. Acetaldehyde-induced inhibition of Ca\textsuperscript{2+} uptake by the SR in frog skinned fibers. The ability of the SR to uptake Ca\textsuperscript{2+} was estimated from a 20 mM caffeine contracture relative to that after active loading of Ca\textsuperscript{2+} for 2 min in solution U without acetaldehyde. The amounts of Ca\textsuperscript{2+} uptake with (●, n=19) or without (○, n=20) 1 mM acetaldehyde were plotted against the loading time (s) by the least-square method using Origin 6.0 (Macrocal Software).
Fig. 1

A

Control, pCa 6

+1 mM Acetaldehyde

P₀ = 0.044

+50 µM Acetaldehyde

P₀ = 0.034

+10 µM Ryanodine

+5 µM Ruthenium red

20 pA

100 ms

B

Open probability

Control 10⁻⁶ 10⁻⁵ 10⁻⁴ 10⁻³ 10⁻²

Acetaldehyde [M]

Fig. 1
Fig. 2

A  Control  \( P_o = 0.017 \)  

\[
\begin{array}{c}
\text{1 min} \quad \text{Po} = 0.013 \\
\text{15 min} \quad \text{Po} = 0.005 \\
\end{array}
\]

B  1 mM Acetaldehyde  

\[
\begin{array}{c}
\text{10 s} \quad \text{Po} = 0.033 \\
\text{30 s} \quad \text{Po} = 0.087 \\
\end{array}
\]

\[
\begin{array}{c}
\text{15 min} \quad \text{Po} = 0.005 \\
\end{array}
\]
Fig. 3
A. Twitch

B. Tetanus

C. Caffeine contracture

Fig. 4
Fig. 5

A Control

B 1 mM Acetaldehyde

C After washout

0.5 mN

1 min    100 ms