Cellular redox state protects acetaldehyde-induced alteration in cardiomyocyte function by modifying Ca\textsuperscript{2+} release from sarcoplasmic reticulum

Toshiharu Oba\textsuperscript{1}, Yoshitaka Maeno\textsuperscript{2}, Masataka Nagao\textsuperscript{2}, Nagahiko Sakuma\textsuperscript{3}, and Takashi Murayama\textsuperscript{4}

Departments of \textsuperscript{1}Cell Physiology, \textsuperscript{2}Forensic Medicine, \textsuperscript{3}Internal Medicine and Pathophysiology, Nagoya City University Graduate School of Medical Sciences, Mizuho-ku, Nagoya 467-8601, Japan
\textsuperscript{4}Department of Pharmacology, Juntendo University School of Medicine, Tokyo 113-8421, Japan

Running head: redox potential and acetaldehyde-induced Ca\textsuperscript{2+} release

Correspondence:
Toshiharu Oba, PhD.
Department of Cell Physiology
Nagoya City University Graduate School of Medical Sciences
Mizuho-ku, Nagoya 467-8601
Japan
e-mail; tooba@med.nagoya-cu.ac.jp
Tel; 52-853-8130
Fax;52-842-0863
Abstract

Recent studies indicate that low concentrations of acetaldehyde may function as the primary factor in alcoholic cardiomyopathy by disrupting Ca\(^{2+}\) handling or disturbing cardiac excitation-contraction coupling. By producing reactive oxygen species (ROS), acetaldehyde shifts the intracellular redox potential from a reduced state to an oxidized state. We examined whether the redox state modulates acetaldehyde-induced Ca\(^{2+}\) handling by measuring Ca\(^{2+}\) transient using a confocal imaging system and single RyR2 channel activity using the planar lipid bilayer method. Ca\(^{2+}\) transient was recorded in isolated rat ventricular myocytes with incorporated Fluo3. Intracellular reduced glutathione (GSH) level was estimated using the monochlorobimane fluorometric method. Acetaldehyde at 1 and 10 \(\mu\)M increased Ca\(^{2+}\) transient amplitude and its relative area in intact myocytes, but acetaldehyde at 100 \(\mu\)M decreased Ca\(^{2+}\) transient area significantly. Acetaldehyde showed a minor effect on Ca\(^{2+}\) transient in myocytes in which intracellular GSH content had been decreased against challenge of diethylmaleate (DEM) to a level comparable to that induced by exposure to about 50 \(\mu\)M acetaldehyde. Channel activity of the RyR2 with slightly reduced cytoplasmic redox potential from near resting state (-213 mV) or without redox fixation was augmented by all concentrations of acetaldehyde (1-100 \(\mu\)M) used here. However, acetaldehyde failed to activate the RyR2 channel, when the cytoplasmic redox potential was kept with a reduced (-230 mV) or markedly oxidized (-180 mV) state. This result was similar to effects of acetaldehyde on Ca\(^{2+}\) transient in DEM-treated myocytes, probably being in oxidized redox potential. The present results suggest that acetaldehyde acts as an RyR2 activator to disturb cardiac muscle function, and redox potential protects the heart from acetaldehyde-induced alterations in myocytes.

Keywords; Ca\(^{2+}\) transient, single RyR2 channel activity, intracellular glutathione level, Intracellular redox state
Introduction

The possibility that alcohol use adversely affects cardiac muscle has been recognized for over a century (37). Heavy alcohol drinking causes acute but reversible myocardial muscle depression. However, the underlying mechanism whereby these processes are caused by alcohol intoxication still remains unknown, although several hypotheses have been proposed, including generation of acetaldehyde-protein adduct, disturbance of oxidant-antioxidant balance and inhibition of intracellular Ca²⁺ mobilization (6, 29). Ethanol is metabolized mainly in the liver to produce acetaldehyde, which is ten times more toxic than ethanol (4). Since plasma concentrations of acetaldehyde increase markedly after alcohol consumption, one may assert that alcohol-induced decrease in cardiac inotropy is attributable to acetaldehyde. However, acetaldehyde is oxidized quickly to acetate by aldehyde dehydrogenase (ALDH), leading to a very low blood concentration (several μM after moderate alcohol intoxication, except for persons genetically deficient in ALDH isoenzyme2) (18, 19, 27, 38). Early studies have shown that acetaldehyde concentrations in excess of several mM are required to decrease the intracellular Ca²⁺ transient in rat cardiac muscles (5, 36). We have to keep in mind that effective concentrations of acetaldehyde used in these studies would not usually be encountered even after heavy alcohol drinking. However, recent studies have supported the hypothesis that acetaldehyde plays a crucial role in the development of alcoholic cardiomyopathy, in a transgenic mouse model of cardiac overexpression of ADH, ALDH2 or catalase (6, 15, 44). In rat ventricular myocytes, incubation with 10 μM acetaldehyde for 4-6 h has been reported to decrease cell shortening on electrical stimulation (1). These observations suggest that physiological concentrations of acetaldehyde which cause adverse effect on cardiac muscle function may lead to disrupted Ca²⁺ handling or may disturb cardiac excitation-contraction coupling. Such abnormality may function as the primary factor in alcohol cardiomyopathy. With regard to this assumption, we found that acetaldehyde
at 1 to 3 μM activates directly the ryanodine receptor type 2 (RyR2), a Ca\(^{2+}\)-release channel in the rabbit cardiac SR (29).

Glutathione is considered to be the major cytosolic redox buffer in cardiomyocytes, although not the sole redox buffer. Under normal physiological conditions, the [GSH]/[GSSG] ratio is more than 10; however, it can decrease under pathological conditions such as ischemia-reperfusion (31) or hypoxia (42). The maintenance of high [GSH]/[GSSG] ratio is very important for antioxidant defense of myocytes. Previous results provide evidence that alteration of the redox state of SH groups of two neighboring cysteine residues leads to formation and breaking of disulfide bonds and affects the structure and function of ion channels (32, 43; see also 14 for review). Acetaldehyde produces reactive oxygen species (ROS) including •O\(_2\)\(^-\), H\(_2\)O\(_2\) and •OH\(^-\) (15, 26, 44) without any effect on glutathione peroxidase and glutathione reductase (33). H\(_2\)O\(_2\) oxidizes GSH to produce GSSG and shifts the intracellular redox potential toward the oxidized redox state. It has been shown that ryanodine receptor type 1 (RyR1) molecule functions as a redox sensor (7, 41), and the redox state of the molecule is demonstrated to be a decisive factor in determining the Ca\(^{2+}\) dependence of the channel activity (21, 30). Our previous results show that the shift of the cytoplasmic redox potential toward a reduced state inhibited RyR1 channel activity and the shift toward oxidized state activated the channel (30). If the RyR2 channel also serves as a redox sensor in a way analogous to that for the RyR1, excessive ROS production or an impaired antioxidant state which would be elicited by acetaldehyde might modulate development and/or progression of alcoholic cardiomyopathy. Therefore, we investigated whether direct effects of acetaldehyde on RyR2 channel activity and Ca\(^{2+}\) release from the SR in single myocytes are modified by intracellular redox state or decrease in intracellular GSH content.

In this study, we found that acetaldehyde at 1-10 μM activated the RyR2 channel and increased Ca\(^{2+}\) transient amplitude and its relative area in myocytes, whereas acetaldehyde at 100 μM was inhibitory. Interestingly, such alteration of the
Ca\(^{2+}\) release from the SR by acetaldehyde was attenuated, when the intracellular redox potential was shifted toward either reduced or markedly oxidized potential. These results suggest that intracellular redox states act as a crucial modifier against acetaldehyde-induced physiological or mechanical alteration in cardiac myocytes.

**Materials and Methods**

**Isolation of rat ventricular myocyte and purification of RyR2 from rabbit heart muscle**

All of the experiments were carried out by the principles and guidelines of the Japanese Council of Animal Care and Use and were in accordance with the Institutional Animal Care and Use Committee at the Nagoya City University Graduate School of Medical Sciences. After excision from male Wistar rat (7 weeks old) under pentobarbital (50mg/kg, ip, Sigma, St Louis, MO) sedation, the heart was superfused with Krebs-Henseleit buffer solution composed of 118mM NaCl, 2.6mM KCl, 1.2mM MgSO\(_4\), 1.2mM KH\(_2\)PO\(_4\), 11mM glucose and 25mM NaHCO\(_3\) (pH7.4) gassed with 95% O\(_2\) and 5% CO\(_2\). Cardiomyocytes were isolated by a collagenase digestion procedure described previously (13, 20). Freshly isolated myocytes were suspended in Medium 199 (Sigma) containing 0.2% bovine serum albumin, and then seeded on laminin-coated glass coverslip bottom dishes, and incubated at 37°C until used. Cardiac myocytes were used within 6 h after isolation and experiments were done at room temperature (18-20°C).

The RyR2 was purified from ventricular muscles of rabbit hearts as described previously (24) with following modifications. After partial purification by sucrose gradient ultracentrifugation, the RyR2-containing fractions were applied onto a Mono-Q column that had been equilibrated with a buffer containing 0.2 M NaCl. After washing with the buffer, RyR2 was eluted with a linear gradient of NaCl (0.2-0.4 M).
RyR2 was eluted around 0.25 M NaCl. The preparations were quickly frozen in liquid N2 and stored at –80°C until use.

**Single channel current recording**

Single channel currents were recorded by incorporating the purified RyR2, as described previously (30). Briefly, lipid bilayers (a mixture of L-α-phosphatidyl-ethanolamine, L-α-phosphatidyl-L-serine and L-α-phosphatidylcholine 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. Channel currents were recorded in symmetrical solution of 250 mM KCl buffered with 20 mM HEPES-Tris (pH 7.4). The cis (cytoplasmic) side was voltage-clamped at -40 mV, unless noted, and the trans (intraluminal) side was held at ground potential. Acetaldehyde was applied cumulatively to the cis side of the single channel. Experiments were carried out at room temperature (18-20 °C). Channel currents amplified by an Axopatch 1D patch-clamp amplifier (Axon Instrument, CA) were filtered at 1 kHz using an eight-pole low-pass Bessel filter (Frequency Devices, MA) and digitized at 5 kHz for analysis. Mean open probability ($P_o$) and lifetime of open and closed events were calculated from the records of duration > 1 min by 50 % threshold analysis using pClamp (version 6.0.4) software.

To elucidate whether redox state of the RyR2 molecule affects the channel activity activated by acetaldehyde, some experiments were done under redox control of the cis and/or trans compartments using a [GSH]/[GSSG] buffer solution. We first tried to check the exact GSSG concentration in the cis buffer solution containing dissolved commercially available GSH by using the liquid chromatography/mass spectrometric (LC/MS) method of Guan et al. (9), as shown below. One mM GSH solution was contaminated by 6.5 μM GSSG (Table 3). Therefore, redox potential was calculated from the Nernst equation (11) taking account of this contamination. We used -0.24V as the standard redox potential as reported previously (30). It has been reported that
intracellular redox potential in resting muscle fibers is maintained in a reduced state of about -220mV (11), but the intraluminal side of the SR in an oxidized state (about -180mV; see 7). Therefore, we fixed at -180mV for the intraluminal redox potential and at potentials between -230mV and -180mV for the cytoplasmic solution.

**Measurement of GSSG and GSH in cis buffer solution**

To test whether the [GSH]/[GSSG] ratio is changed by acetaldehyde, [GSH] and [GSSG] were measured in presence of acetaldehyde using the LC/MS method (9). Briefly, 100 μl of 1mM GSH in cis buffer solution was incubated with 1, 10 and 100 μM acetaldehyde at room temperature for 5 min. Then, glutathione ethylester was added to 50 μl of the mixture, as an internal standard (I.S.) (1.25mM, 5 μl), and Ellman’s reagent (10mM, 100 μl). The reaction mixture was vortex-mixed, followed by the addition of 30 μl of 20% 5-sulfosalicylic acid. Using an autosampler, 10 μl of each mixture was injected into a Nova-Pak C18 column (150mm x 2mm; Waters, Japan). LC/MS analysis was performed using a Waters 2690 separation and Platform LC Z (Waters, Japan). Gradient elution was applied to discriminate GSSG, the Ellman’s reagent-derivative GSH (GSH-Ellman), and I.S (I.S.-Ellman). The mass spectrometer was set to simultaneously monitor ions with m/z of 613, 505 and 533, which correspond to the protonated molecular ions of GSSG, GSH-Ellman, and I.S.-Ellman, respectively (Fig. 3). [GSSG] was calculated from the peak area ratio of GSSG to the I.S.-Ellman. The standard curve for [GSSG] was constructed over a range of 6.5-65 μM.

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

The single ventricular myocyte was incubated in Krebs-Henseleit buffer solution containing 10 μM Fluo 3-acetoxyethyl ester (AM) and 0.04% Pluronic F127 (Molecular Probes, Eugene, OR) for 20 min at 37 °C to load Fluo 3. The preparation was set on an inverted microscope (Axiovert 200M; Carl Zeiss, Thornwood, NY). Fluorescence intensity change was stored in the hard disc of a personal computer to
estimate Ca$^{2+}$ transient with a confocal imaging system (LSM 5 Pascal; Carl Zeiss) equipped with an argon ion laser, as reported previously (28). Fluo 3 was excited by light at 488-nm, and the emitted fluorescence was collected at wavelengths of >505 nm with a Plan-Neofluar x40 objective (Carl Zeiss). Image acquisition was carried out with a line-scan (xt) mode (0.82 ms/line, 512 pixels/line) to measure the Ca$^{2+}$ transient after electrical field stimulation of the myocytes (1 ms pulse duration and supramaximal current through a pair of Pt electrodes). The myocytes were superfused (1.74 ml/min flow rate) with buffer solution containing 1, 10 or 100 μM acetaldehyde by a circulation pump. The solution was gassed with 95% O$_2$ and 5% CO$_2$. The data were analyzed with a software (LSM image browser; Carl Zeiss).

**Fluorescent monitoring of intracellular reduced GSH using in mBCl**

A membrane-permeable fluorescence probe, monochlorobimane (mBCl, Molecular Probes) has been used as a sensitive and specific probe to analyze intracellular GSH content in living cells (8, 12). To evaluate whether acetaldehyde decreases the intracellular GSH level by functioning as an oxidative factor, cardiac myocytes were exposed to 100 μM mBCl in presence or absence of acetaldehyde. Upon entering the cell, mBCl forms a fluorescent adduct with GSH specifically and preferentially over other thiols via the reaction catalyzed by glutathione S-transferase (8, 12). Intracellular GSH concentration was measured by a confocal imaging system (LSM 5 Pascal: Carl Zeiss). After 1 h of seeding on laminin-coated glass coverslip bottom dishes, isolated myocytes were incubated with a loading solution containing (in mM): 140 NaCl, 6.0 KCl, 1.0 MgCl$_2$, 2.0 CaCl$_2$, 10 glucose, 5.0 HEPES and 100 μM mBCl at room temperature. Then the fluorescence image was recorded for 15min. The fluorescent GSH-bimane adduct was exited by light at 458-nm, and the emitted fluorescence was collected at wavelengths of 475-nm with a Plan-Neofluar x40 objective (Carl Zeiss). Image acquisition was carried out with a full-flame (xy) mode (0.82 ms/line, 512 pixels/line), as reported previously (28). 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 1 frame/492ms. The optical resolution of the system was 0.45 μm in the x and y dimensions and 0.8 μm in the z dimension.

Next, we studied the effect of decreased GSH levels on the Ca^{2+} transient in cardiac myocytes treated with acetaldehyde. Intracellular GSH level was decreased by preincubation with 0.4mM diethyl maleate (DEM) for 15min at room temperature. This method was similar to a previous way reported for intact hepatocytes (8). The (F-Fo)/Fo ratio and the decay rate constant (λ) of Ca^{2+} transient were analyzed before and after application of acetaldehyde. Fo represents basal fluorescence intensity.

Results are presented as means ± S.E. Statistical analysis was done with one-way ANOVA and the post hoc Bonferroni’s test for multiple comparisons, or Student’s t-test for simple two-sample test. Values of P<0.05 were regarded as statistically significant.

Chemicals

Acetaldehyde (98% stock solution; Wako Pure Chemical, Osaka, Japan) was diluted to appropriate concentrations with buffer solutions and Fluo 3-AM (Dojindo, Kumamoto, Japan) immediately before use. Caffeine (100 mM stock solution; Sigma, St Louis, MO) was dissolved in hot distilled water immediately before application. Monochlorobimane (mBCl, 10 mM stock solution) was purchased from Molecular Probes Inc. (Tokyo, Japan) and diethylmaleate (DEM, 20 mM stock solution) from Sigma. Glutathione (reduced and oxidized forms, Sigma) was dissolved in nanopure water (stock solution, 0.5 M and 0.25 M, respectively) and stored at -20ºC.
Results

Acetaldehyde-induced Ca\(^{2+}\) release from sarcoplasmic reticulum of intact myocytes

Cardiac myocytes release Ca\(^{2+}\) transiently from the SR (termed Ca\(^{2+}\) transient) upon excitation of the surface membrane. We explored whether acetaldehyde alters the Ca\(^{2+}\) transient under *in vivo* condition. The amplitude of the Ca\(^{2+}\) transient was estimated as a relative fluorescence intensity ((F-Fo)/Fo; F is fluorescence intensity at time \(t\), Fo is baseline fluorescence intensity). As shown in Fig. 1A, acetaldehyde at 1 \(\mu\)M enhanced Ca\(^{2+}\) transient amplitude compared with that in controls before exposure to acetaldehyde. The Ca\(^{2+}\) transient amplitude was kept high even after exposure to 10 \(\mu\)M acetaldehyde. On the contrary, Ca\(^{2+}\) transient was decreased markedly in presence of 100 \(\mu\)M acetaldehyde to a level below that of the control. Experiments were repeated with 43 separate myocytes and pooled data are depicted in Fig. 1B. Clearly, a significant increase in Ca\(^{2+}\) transient was obtained only when myocytes were pretreated with low or moderate concentrations of acetaldehyde. At 10 \(\mu\)M acetaldehyde, the relative area of Ca\(^{2+}\) transient was increased significantly from controls, whereas an increase of acetaldehyde to 100 \(\mu\)M decreased markedly the relative area below the level of controls (Fig. 1C). The myoplasmic Ca\(^{2+}\) released from the SR has been reported to be rapidly reduced to a resting level less than 0.1 \(\mu\)M through re-uptake by the SR via action of Ca\(^{2+}\)-ATPase and efflux to extracellular space by the Na\(^+\)/Ca\(^{2+}\) exchanger or Ca\(^{2+}\) pump (3). If acetaldehyde affects Ca\(^{2+}\)-ATPase activity and/or Ca\(^{2+}\) efflux, the decay time constant of Ca\(^{2+}\) transient (\(\lambda\)) would be influenced. Fig.1D shows no significant change in the decay time constant at all doses of acetaldehyde used here.

The decrease in Ca\(^{2+}\) transient amplitude noted in presence of 100 \(\mu\)M acetaldehyde may be elicited by depletion of Ca\(^{2+}\) content in the SR. This possibility was examined by estimating amounts of caffeine-induced Ca\(^{2+}\) release. Exposure of
myocytes to 20 mM caffeine produced a large Ca\(^{2+}\) release (termed caffeine transient). The maximum fluorescence intensity of caffeine transient was slightly, but not significantly, reduced in 100 \(\mu\)M acetaldehyde-treated myocytes ((F-Fo)/Fo=2.34 ± 0.28, n=7 in controls; 2.02 ± 0.24, n=7 in 100 \(\mu\)M acetaldehyde group, p>0.05). However, the ratio of caffeine transient/Ca\(^{2+}\) transient was decreased significantly from 0.69 ± 0.07 in controls to 0.58 ± 0.04 in presence of acetaldehyde (p<0.05). These results are shown in Table 1. The result indicates that an acute bolus application of 100 \(\mu\)M acetaldehyde reduced Ca\(^{2+}\) content in the SR by ~15\%, probably resulting in decreased Ca\(^{2+}\) transient observed in myocytes exposed to 100 \(\mu\)M acetaldehyde.

**Activation of RyR2s induced by acetaldehyde**

As expected from above results, acetaldehyde may function as a modulator of the RyR2 channel. We checked the effect of acetaldehyde on channel activity of the purified RyR2, which is free from FK506 binding protein (FKBP12) or other associated proteins such as calmodulin and triadin (23). Exposure of RyR2 to 1 \(\mu\)M acetaldehyde activated channel activity from Po=0.033 in the control to Po=0.116, when the channel protein was incorporated into lipid bilayers (Fig.2A). Increase in acetaldehyde to 10 or 100 \(\mu\)M increased Po to 0.267 or 0.329, respectively. The effect of acetaldehyde on channel gating kinetics was examined by analyzing the open and closed time. The open and closed time constants, and its relative areas calculated by using data described in Fig. 2A, are represented in Fig. 2B. Open time constants of the mean open lifetime were best fit with two exponential components in controls (\(\tau_01=0.64\)ms, and \(\tau_02=3.41\)ms). The relative areas of the fast and slow components were 88.1\% and 11.9\%, respectively. When 1 \(\mu\)M acetaldehyde was added in the cis chamber, the fast component increased three-fold with a similar relative area (\(\tau_01=1.73\)ms and 81.0\%) and the slow component also increased two-fold (\(\tau_02=7.10\)ms, and 19\%), being reflected in a prolongation of the mean open time (1.7ms to 3.3ms). The closed time constants were best fit with three similar exponentials between
controls and acetaldehyde-treated RyR2s. Acetaldehyde produced a pronounced increase in numbers of open events (65.2 ± 14.0/s in controls and 98.9 ± 19.0/s after 10 μM acetaldehyde). Pooled data (n=8) of Po, mean open and closed times, and numbers of open events are summarized in Table 2. These effects of acetaldehyde probably contributed to the increase in Ca^{2+} transient amplitude seen in Fig. 1.

**Effects of acetaldehyde on channel activity in RyR2 under fixation of redox potential**

We checked first whether acetaldehyde reacts with GSH in cis buffer solution to form GSSG. As shown in Figure 3B using the mass-spectrometer, a small peak of GSSG was observed in the presence of 100 μM acetaldehyde and the concentration was estimated to be 6.5 μM, similar to that in a control solution without acetaldehyde (Fig. 3A and Table 3). GSH concentration remained unchanged after application of 1, 10 or 100 μM acetaldehyde, indicating no decomposition of GSH by acetaldehyde at concentrations used here and resulting in no alteration of redox potential by acetaldehyde applied in cis buffer solution.

When redox potential in trans compartment was defined at -180 mV using glutathione buffer (see MATERIALS AND METHODS), RyR2 channel activity was inhibited, as shown in Fig. 4. Subsequent fixation of the cis potential at -213 mV (a slight oxidation from resting redox potential) restored the channel activity to undefined control level (Po=0.033 from Po=0.025 in control). Under this redox condition (cis/trans redox potential=-213mV/-180mV), exposure to acetaldehyde stimulated the RyR2 channel in a dose-dependent manner (Po=0.052 at 1 μM and Po=0.083 at 10 μM) (Fig. 4A). Similar results were observed using six separate channels, and pooled data are summarized in Table 4. Further increase in acetaldehyde to 30 μM or 100 μM further enhanced the channel activity, dose-dependently (Po=0.137 ± 0.051 at 30 μM and Po=0.291 ± 0.095 at 100 μM). When cis redox potential was shifted from -213mV toward a further oxidative state (cis/trans redox potentials=-180 mV/-180mV), the
channel activity was markedly enhanced from Po=0.004 at trans potential=-180 mV with no cis redox fixation to Po=0.174 (Fig. 4B and see Table 4; Po=0.243 ± 0.071 in 6 preparations). When the RyR2 channel was fixed symmetrically at an oxidized redox potential of -180mV, channel activity was only slightly augmented even after exposure to high concentrations of acetaldehyde, as shown in Table 4 (Po=0.074 ± 0.023 in control and Po=0.126 ± 0.034 at 100 μM, n=6). Representative traces are depicted in Fig. 4B.

When the cytoplasmic redox potential was shifted from -213mV (fixed at trans redox potential = -180mV) toward -220 mV, the RyR2 channel activity at pCa 5 was slightly inhibited (Po=0.373 to 0.263) (Fig. 5, left traces). Even under the condition where the redox potential was fixed, the channel responded well on alteration of cytoplasmic Ca2+ concentration (data not shown). A reduction of the cis redox potential to -230 mV markedly decreased the channel activity (Po=0.089). Exposure of this channel to 1, 10 or 100 μM acetaldehyde never augmented the channel activity (Fig. 5, right traces and Table 4). Under these highly oxidized or reduced redox conditions, the RyR2 channel failed to activate the activity upon application of acetaldehyde, unlike the RyR2 channel fixed at cis/trans redox potential=-213 mV/-180mV (Fig. 4A and Table 4).

**Decrease in cytoplasmic GSH content by acetaldehyde**

The above results indicate that an intracellular redox state modulates RyR2 channel activity and Ca2+ release from the SRs in cardiac myocytes. Acetaldehyde may produce ROS within cells to serve as an inducer for oxidative stress, as reported in other tissues (see 29 for review). Therefore, we used a fluorometric method to determine whether acetaldehyde can oxidize GSH in intact heart cells. Incubation of single myocytes with a buffer solution containing 100 μM mBCl increased the fluorescence intensity emitted by formation of GSH-bimane adduct in a time-dependent manner (Fig. 6A and see Fig. 8, closed circle). The maximum
fluorescent intensity was calculated by fitting data to a single exponential curve. We estimated the extent of the decrease in the GSH content from the maximum fluorescent intensity. The results indicate that the mBCl fluorometric method can be used to measure the GSH content in intact cardiac myocytes, as previously reported in hepatocytes (8, 12). Using this method, we investigated the effect of acetaldehyde on intracellular GSH content. The fluorescent intensity at 30 sec after exposure to 10 or 100 μM acetaldehyde (Fig. 6B, C and Fig. 8 closed triangle or inverted closed triangle) was similar to that in myocytes not treated with acetaldehyde, suggesting that acetaldehyde does not affect penetration of mBCl across the cell membrane and the rate of GSH-bimane formation inside the myocyte. The maximum fluorescence intensity observed after application of 10 μM acetaldehyde was 60% of that in controls. With a further increase in acetaldehyde to 100 μM, the fluorescence intensity increased more slowly and the maximum intensity was decreased to 40% of the control peak value. These results indicate that acetaldehyde has the ability to decrease the cytoplasmic GSH content in a dose-dependent manner.

Effect of acetaldehyde on Ca\(^{2+}\) transient in cardiac myocytes with decreased myoplasmic GSH content

Response of the RyR2 channel to acetaldehyde was modified by cytoplasmic redox states, as shown in Figs. 4 and 5. Glutathione is the main non-protein thiol in many cells and plays a crucial role in redox regulation (see Introduction). Therefore, we examined effects of acetaldehyde on Ca\(^{2+}\) release from the SRs of cardiac myocytes in which the intracellular GSH content had been decreased. It has been reported that exposure of isolated hepatocytes to 0.4 mM diethyl maleate (DEM) decreased cytoplasmic GSH content to ~10% of control value with a minor change in mitochondrial GSH content (8). After 0.4 mM DEM was applied to cardiac myocytes for 15 min, myoplasmic GSH content was estimated by the fluorometric method using 100 μM mBCl (Fig. 7). The maximum fluorescence intensity was decreased to ~50%
of that in controls without DEM treatment (Fig. 8, open circles vs. closed circles). Ten separate experiments were repeated and pooled data are summarized in Fig 8. This observation provides evidence that DEM treatment decreased cytoplasmic GSH content to an extent similar to that after exposure of cardiac myocytes to \( \sim 50 \) \( \mu \)M of acetaldehyde.

When examined in cardiac myocytes depleted cytoplasmic GSH by pretreatment with 0.4 mM DEM, the amplitude of \( Ca^{2+} \) transient was larger than that in intact myocytes without DEM application ((F-Fo)/Fo= 4.0 ± 0.2 in Fig. 9B, n=17 vs. 2.8 ± 0.1 in Fig.1B, n=43). The decay rate constant of the \( Ca^{2+} \) transient (\( \lambda \)=0.32) was similar to that with no DEM pretreatment (\( \lambda \)=0.37). A representative trace is depicted in Fig. 9A. These suggest that intracellular GSH depletion enhances the \( Ca^{2+} \) release from the SR upon depolarization of cell membranes. In such GSH-depleted myocytes, application of 10 \( \mu \)M acetaldehyde increased only slightly the \( Ca^{2+} \) transient amplitude by \( \sim 4\% \). Exposure to 100 \( \mu \)M acetaldehyde failed to elicit a further alteration in \( Ca^{2+} \) transient. The decay time constant was not affected by acetaldehyde in myocytes pretreated with DEM. Experiments were repeated using 17 separate myocytes and pooled data are summarized in Fig. 9B and C.

**Discussion**

The present study provides evidence that clinical concentrations of acetaldehyde adversely regulate the cardiac muscle SR functions and intracellular redox potential modulates the acute effects of acetaldehyde. The novel findings were that (i) acetaldehyde has a biphasic action on \( Ca^{2+} \) transient in cardiac myocytes in a dose-dependent manner, whereas acetaldehyde showed only a minor effect on \( Ca^{2+} \) transient in myocytes whose intracellular GSH content had been decreased against challenge of DEM, (ii) acetaldehyde does not react with GSH in a buffer solution, (iii) channel activity of the RyR2 with fixation of redox potential at/near resting state or
without redox fixation was augmented by all concentrations of acetaldehyde (1-100 μM) used here, and (iv) acetaldehyde attenuated markedly activation of the RyR2 channel activity, when the cytoplasmic redox potential was kept with a considerable reduced or markedly oxidized state.

Recent remarkable studies by Dr. J. Ren’s group (University of Wyoming) and others using transgenic mice overexpressing alcohol dehydrogenase (ADH), catalase, or aldehyde dehydrogenase-2 (ALDH2) provide evidence supporting some contribution of a clinical dose of acetaldehyde to ethanol-induced cardiomyopathy. A moderate amount of alcohol (3 g/kg)-treated ADH transgenic mice produced ~17 μM acetaldehyde and developed hypertrophic hearts with extensive ultrastructural changes (17). In ventricular myocytes isolated from ADH transgenic mice, acute exposure to ethanol over a range of 240-640 mg/dl augmented a depression of cell shortening and a decrease in intracellular Ca²⁺ concentration observed on challenge to ethanol in wild mice (6). An increase in acetaldehyde level from 7 μM before chronic alcohol intake to 214 μM after intake exacerbated mechanical and intracellular Ca²⁺ defects in ADH cardiac myocytes (10). These results suggest that acetaldehyde at clinical doses may play a crucial role in the development of alcoholic cardiomyopathy. This assumption is supported from a recent observation that overexpression of ALDH2 transgene in human cardiac myocytes prevented acetaldehyde-induced cell injury (15).

Reportedly, disturbance of cardiac excitation-contraction coupling may serve as the most important factor for the underlying mechanism by which acetaldehyde induces alcoholic cardiac myopathy (6). Consistent with our preliminary observation (29), the present findings that acetaldehyde at 1 or 10 μM increased Ca²⁺ transient in isolated intact myocytes and activated the purified RyR2 channel activity support the conclusion that acetaldehyde plays a crucial role for cardiomyopathy by modifying excitation-contraction coupling. Interestingly, however, we observed the fact that a high dose of acetaldehyde (100 μM), which would not usually be encountered even after heavy alcohol ingestion in healthy persons, elicited a decrease in Ca²⁺ transient...
(Fig. 1), in spite of keeping the single RyR2 channel activity high (Fig. 2). This inhibition of Ca\(^{2+}\) transient is in line with previous results that exposure to high concentrations of acetaldehyde reduced Ca\(^{2+}\) transient (10) and a long-term incubation (4-6 h) with a low concentration of acetaldehyde (10 \(\mu\)M) decreased the cell shortening (1). Upon exposure to a high acetaldehyde concentration (100 \(\mu\)M), we found SR Ca\(^{2+}\) content to be reduced slightly, as estimated from caffeine-induced Ca\(^{2+}\) transient (Table 1). However, no change in Ca\(^{2+}\) content was obtained after acute challenge to 1-10 \(\mu\)M acetaldehyde (data not shown). Therefore, high concentrations of acetaldehyde for a bolus application or probably low concentrations of acetaldehyde for a long-term treatment may be required to produce a reduction of Ca\(^{2+}\) content in the SR and contractile dysfunction, which have been observed in previous studies. However, we carried out no experiments on the long-term effect of acetaldehyde on Ca\(^{2+}\) release from cardiomyocytes. Therefore, a direct link between acute effects of acetaldehyde on Ca\(^{2+}\) release observed here and chronic effects is not clear. Further studies will be required to extrapolate acute effects of acetaldehyde to chronic effects, which would be acquired by using transgenic mice overexpressing ADH or ALDH-2 knockout mice.

It has been reported that Na\(^+\)/Ca\(^{2+}\) exchange (NCX) and SR Ca\(^{2+}\)-ATPase are the main mechanism of Ca\(^{2+}\) removal from intracellular space in cardiac myocytes. In rat ventricle, 7% of cardiac relaxation is due to NCX and 92% due to SR Ca\(^{2+}\)-ATPase, although the extent of dependency for each system is dependent on species (2, 3, 25). These observations indicate that Ca\(^{2+}\) extrusion from an intracellular space in rat ventricular muscle is mainly attributable to Ca\(^{2+}\) uptake by the SR (3, 39). If acetaldehyde blocks SR Ca\(^{2+}\)-ATPase and/or NCX, the decay of Ca\(^{2+}\) transient would be expected to become slow and in turn may lead to the increase in the amplitude of Ca\(^{2+}\) transient. However, the decay time constant of Ca\(^{2+}\) transient in the present study was not affected in presence of acetaldehyde (Fig. 1A and D). These results suggest that acetaldehyde at concentrations used here would have no ability to inhibit activities of Ca\(^{2+}\)-ATPase and NCX. In heart muscle, consequently, Ca\(^{2+}\) extrusion from a cell
inside is not so much during systole. Therefore, acetaldehyde serves only as a RyR2 channel activator, and promotes the Ca\(^{2+}\) release from the SR in response to depolarization of cell membranes.

Recent studies have demonstrated that acetaldehyde produces intracellularly reactive oxygen species (ROS) including \(\bullet \text{O}_2^-\), \(\text{H}_2\text{O}_2\) and \(\bullet \text{OH}^-\) (15, 26, 44). \(\text{H}_2\text{O}_2\) oxidizes reduced glutathione (GSH) to produce oxidized glutathione (GSSG), probably resulting in a shift of the intracellular redox potential toward the oxidized state. Consistent with these studies, we observed an acetaldehyde-induced marked decrease in the intracellular GSH content in cardiac myocytes (Figs. 6 and 8), although acetaldehyde did not directly react with GSH in \textit{cis} buffer solution (Fig. 3 and Table 3). Therefore, acetaldehyde would change cytoplasmic redox potential from a reduced state to an oxidative state. Similar to our previous observations with the RyR1 channel (30), the present study indicates that the RyR2 channel activity is modulated markedly by cytoplasmic redox potential; shift of redox potential from near/at resting redox potential to -230 mV inhibited the channel activity with a decreased number of open events, whereas a marked oxidation from -213 mV to -180 mV activated the channel with an increased number of open events (Figs. 4 and 5). These results suggest that some cysteine residue(s) in both RyR1 and RyR2 channels serves as redox sensor, although we did not identify in the present study which cysteine residue(s) functions as the sensor. In skeletal muscle RyR1, an interesting model has been proposed that Ca\(^{2+}\) release or force production is a function of intracellular redox balance, resting redox balance is in a slightly reduced state, and muscle activation is maximal when redox balance shifts toward slightly oxidized state (34, 35). This model has been confirmed at a molecular level of RyR1 channel (40). The authors demonstrated that RyR1 channel activity correlates with the redox state on the molecule, i.e., oxidation of \(\sim 10\) thiols had little effect on channel activity, the loss of \(\sim 25\) thiols activated the channel in a number-dependent manner, and further loss of thiols irreversibly inactivates the channel. On the other hand, several lines of evidence establish in the heart muscle that
oxidant balance has a crucial role in protecting the heart and in allowing normal contractile performance (see 22 for review). More recently, Li and Ren (16) also have reported attenuation of alcohol-induced contractile dysfunction in cardiomyocytes by overexpression of antioxidant metallothionein. Our observations in the present study that shift of redox potential to a slightly oxidized state (RP=-213 mV) stimulated RyR2 channel more than that at RP=-220 mV near resting potential and that reduction state produced a channel inhibition may be explained by the above model. If so, the explanation would be that response to acetaldehyde at the slightly oxidized potential (for example, -213mV) observed here is more sensitive than the response at other redox potentials, and the effect on the RyR2 channel was similar to effects on the Ca\(^{2+}\) transient in intact myocytes (Fig. 1). In addition, a marked response of RyR2 channel activity to acetaldehyde in redox potential-undefined RyR2 may indicate that the channel is under a slightly oxidized state, although we have no supporting evidence. These observations suggest strongly that the action of acetaldehyde on Ca\(^{2+}\) release depends on the intracellular redox potential and may be maximal at/near resting redox potential.

When redox potential was fixed at a redox state slightly oxidized from a resting condition (cis/trans redox potential = -213mV/-180mV), acetaldehyde increased the Po in a dose-dependent manner (Fig 4A and Table 4), and the extent of channel activation induced by 10 \(\mu\)M acetaldehyde (Table 2) was quite similar to that in the RyR2 channel with no fixation of redox potential (Table 4). On the other hand, shifts of redox potential from either -213 mV to -230 mV (Fig. 5) or -180 mV (Fig 4B) failed to activate markedly the RyR2 channel on exposure to a high concentration of acetaldehyde (100 \(\mu\)M). These results suggest that cytoplasmic redox potential plays a role as a modulator for response of the RyR2 channel to acetaldehyde. We investigated whether this assumption is the case by using myocytes in which intracellular GSH content had been decreased markedly by pretreating with 0.4 mM DEM for 15 min. We found that application of DEM decreases intracellular GSH content to values similar to
that in the presence of ∼50 μM acetaldehyde (Figs. 8). Decrease in GSH content would shift the redox state toward oxidative potential, although we could not calculate the intracellular redox potential, because of no direct measurement of GSH and GSSG concentrations. Interestingly, at such a low cytoplasmic GSH level, exposure of myocytes to 100 μM acetaldehyde increased only slightly, but not significantly, the amplitude of Ca\(^{2+}\) transient (Fig 9A and B). This observation is in agreement with a minor effect of acetaldehyde observed in purified RyR2 channels which redox potentials in cis and trans chambers were fixed symmetrically at -180 mV (Table 4, p>0.05 among groups treated with different doses of acetaldehyde, and also see Fig. 4 for original traces).

Since our purified RyR2 channels lack accessory proteins such as triadin, calsequestrin, junctin, and 12 kDa FK506 binding protein (23), the site of action of acetaldehyde is thought to be on the RyR2 channel itself. The present observations that acetaldehyde alters the number of open events and mean closed time, without any changes in unitary conductance and mean open time (Table 2), show that acetaldehyde affects the channel gating mechanisms by changing the transition rate between the closed and the open states. However, we did not conduct further studies of the binding site(s) or the structural changes of the channel.

In summary, acetaldehyde activated cardiac Ca\(^{2+}\) channel upon exposure to low concentrations of 1 to 10 μM as evidenced by measuring Ca\(^{2+}\) transient in intact myocytes and single RyR2 channel activity. On the other hand, acute application of 100 μM acetaldehyde inhibited Ca\(^{2+}\) transient in intact myocytes, although the channel activity was kept high. Acetaldehyde functions as an activator of the RyR2 channel. A bolus application of high concentration or probably a long-term exposure to low concentrations of acetaldehyde may decrease Ca\(^{2+}\) content in the SR and result in contractile dysfunction (alcoholic cardiomyopathy), although further experiments will be required. Such actions of acetaldehyde were modified by intracellular (cis side of the chamber) redox potentials. The maximal release of Ca\(^{2+}\) from the SR was produced
under a condition in which the redox potential was fixed at or near the resting state, and the channel activation was weakened under considerable redox states or a marked oxidative stress. These observations were similar to the effects observed when acetaldehyde was applied to intact myocytes, and to intracellular GSH-depleted myocytes. Therefore, intracellular redox states are suggested to play an important role to protect the heart from acetaldehyde-induced physiological or pathophysiological alteration in cardiac muscles.
Grants

This work was supported by Grants-in-Aid for Scientific Research, Japan Society for Promotion of Science (to T.O. and T.M.) and The Nakatomi Foundation (to T.O.).
References


41. Xia R, Stangler T, Abramson JJ. Skeletal muscle ryanodine receptor is a redox sensor with a well defined redox potential that is sensitive to channel modulators. J Biol Chem 275:36556-36561, 2000.


**Figure legends**

Figure 1. Effect of acetaldehyde on Ca^{2+} transient in ventricular myocytes. A: representative line-scan (xt) images of Ca^{2+} transient before (control) and within 3 min after exposure to 1, 10 or 100 μM acetaldehyde. Each Ca^{2+} transient is indicated by the pseudocolored peak. Note an increase in fluorescence intensity amplitude at lower concentrations of acetaldehyde and a decrease at 100 μM. B, C and D: pooled data of acetaldehyde effects on (F-Fo)/Fo, relative area of Ca^{2+} transient and decay time constant (λ) of Ca^{2+} transient, respectively. The data are presented as means ± S.E., n=10-43. * and **; significantly different from both control and 100 μM acetaldehyde at p<0.05 and p<0.01, respectively. †; p>0.05 from control.

Figure 2. Effect of acetaldehyde on single Ca^{2+}-release channel activity in the purified RyR2. A: activation of channel activity by acetaldehyde cumulatively applied to the cis chamber after measurement of control channel activity in cis pCa 6.8. Channel open probability (Po) is shown at the right corner. Downward deflection of channel current indicates opening of the channel. B: effects of 1 μM acetaldehyde on open and closed time distribution of RyR2 channel activity. Open and closed time constants, their relative areas, and mean open and closed time were calculated from corresponding data in A.

Figure 3. Mass chromatograms at m/z 613, 533 and 505, showing protonated molecular ions of GSSG, I.S.-Ellman and GSH-Ellman, respectively, in control condition (A) and in presence of 100mM AcA (acetaldehyde) and 1mM GSH in the cis buffer solution (B). Note difference in units of relative abundance (%) in each trace. The x-axis indicates the time in min after the onset of recording.
Figure 4. Differential effects of acetaldehyde on purified RyR2 channel activation in myocytes under redox control. Redox potential was estimated using a [GSH]/[GSSG] redox buffer as described in MATERIALS AND METHODS. After trans redox potential was defined to -180 mV, cis potential was set to -213 mV in A (slightly oxidized redox state) and to -180 mV in B (markedly oxidative redox state). Under such redox control, acetaldehyde at 1 and 10 μM was cumulatively applied to cis chamber. Note that the effect of acetaldehyde on channel activation was almost null under highly cytoplasmic oxidative redox control.

Figure 5. Effect of acetaldehyde on channel activation in the purified RyR2 controlled at reduced redox potentials. Redox potentials in the cis chamber were changed cumulatively from -213 mV to -230 mV. Redox potential in the trans side was kept at -180 mV. Cytoplasmic pCa is decreased from 6.6 in control to 5.0 after fixation of redox potential. Under cis/trans redox control at -230 mV/-180 mV, acetaldehyde at 1, 10 and 100 μM was cumulatively applied to cis chamber. Note no effect of acetaldehyde on channel activation.

Figure 6. Confocal fluorescence xy images of cytoplasmic GSH and inhibition of GSH content by acetaldehyde. Acquisition time of the full-flame image was 492 ms. Fluorescence intensity emitted by forming GSH-bimane adduct is indicated by the pseudocolored peak. A: control fluorescence intensity in myocytes 30 s and 15 min after application of 100 μM mBCl. Note almost uniform staining within a single myocyte, although the mean fluorescence intensity varied among cells. B, C: fluorescence intensities 30 s and 15 min after exposure to 10 μM and 100 μM acetaldehyde, respectively. Each frame in A, B and C contains 7 to 9 separate myocytes.
Figure 7. Depletion of cytoplasmic GSH by diethyl maleate (DEM). A: confocal fluorescence xy images of cytoplasmic GSH. After preincubation of 0.4 mM DEM for 15 min, 100 μM mBCl was applied to myocytes. Fluorescence intensity of GSH-bimane adduct was observed using the same method as Figure 6. B: resting control scan immediately before mBCl treatment. C: three dimensional fluorescence intensity 15 min after exposure to mBCl.

Figure 8. Time courses of relative fluorescence intensity of GSH-bimane adduct in presence of DEM or acetaldehyde. Relative F/Fo is calculated as the ratio of time (t) after application of mBCl to the basal fluorescence intensity amplitude immediately before exposure to mBCl (Fo). A horizontal dotted line represents Fo level. Each curve was best fit with a single exponential function. Mean ± SE, n=10.

* p<0.05 from 100 μM acetaldehyde, ** p<0.01 from values for DEM or acetaldehyde.

Figure 9. Effect of acetaldehyde on Ca^{2+} transient in myocytes pretreated with DEM for 15 min. A: confocal fluorescence xy images of Ca^{2+} transient and its time course before and after application of 10 μM or 100 μM acetaldehyde. B: (F-Fo)/Fo, Note that DEM increased the maximum amplitude of Ca^{2+} transient compared with no treatment of DEM as shown in Figure 1 A ((F-Fo)/Fo=4.00 ± 0.15, n=17 vs. 2.76 ± 0.10 in control, n=43, p<0.01). C: decay time constant (λ) of Ca^{2+} transient. The data are presented as means ± S.E., n=15-23.
Table 1. Effect of 100 μM acetaldehyde on Ca$^{2+}$ content in myocyte sarcoplasmic reticulum estimated by caffeine (20 mM)-induced Ca$^{2+}$ transient

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>100 μM acetaldehyde</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca$^{2+}$ transient (n=7)</td>
<td>3.40 ± 0.31*</td>
<td>3.58 ± 0.50</td>
</tr>
<tr>
<td>Caffeine transient (n=7)</td>
<td>2.34 ± 0.28</td>
<td>2.02 ± 0.24</td>
</tr>
<tr>
<td>Caffeine transient/Ca$^{2+}$ transient</td>
<td>0.69 ± 0.07</td>
<td>0.56 ± 0.04**</td>
</tr>
</tbody>
</table>

mean ± S.E.

** p<0.05 from control
Table 2. Dose-dependent activation of purified rabbit RyR2 channel by acetaldehyde under undefined redox potential

<table>
<thead>
<tr>
<th>Acetaldehyde (µM)</th>
<th>No. of open events (/s)</th>
<th>Mean open time (ms)</th>
<th>Mean closed time (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.052 ± 0.010</td>
<td>26.8 ± 5.8</td>
<td>6.6 ± 1.6</td>
</tr>
<tr>
<td>1</td>
<td>0.077 ± 0.018</td>
<td>39.0 ±  9.2*</td>
<td>9.4 ± 2.1</td>
</tr>
<tr>
<td>3</td>
<td>0.146 ± 0.018*§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.194 ± 0.030†§</td>
<td>52.0 ± 11.0†§</td>
<td>8.4 ± 1.1</td>
</tr>
<tr>
<td>30</td>
<td>0.218 ± 0.015†‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0.306 ± 0.083†‡¶‖</td>
<td>73.3 ± 15.9†‡¶‖</td>
<td>9.6 ± 1.3</td>
</tr>
</tbody>
</table>

Values are means ± S.E.; n=8 preparations. * and †; P<0.05 and P<0.01 from corresponding control group, respectively. § and ‡; P<0.05 and P<0.01 from 1µM corresponding acetaldehyde group, respectively. ¶; P<0.05 from 10µM corresponding acetaldehyde group.
Table 3. Changes in GSH and GSSG concentrations after application of acetaldehyde to 1 mM GSH solution

<table>
<thead>
<tr>
<th>Acetaldehyde (μM)</th>
<th>GSH (mM)</th>
<th>GSSG (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>6.43, 6.60*</td>
</tr>
<tr>
<td>1</td>
<td>1.03 ± 0.01**</td>
<td>6.51 ± 0.05</td>
</tr>
<tr>
<td>10</td>
<td>1.07 ± 0.01</td>
<td>6.41 ± 0.03</td>
</tr>
<tr>
<td>100</td>
<td>1.06 ± 0.01</td>
<td>6.47 ± 0.08</td>
</tr>
</tbody>
</table>

*: duplicated detection, **: Mean ± S.E. (n=3). No significant difference among groups.
Table 4. Effects of acetaldehyde on open probability (Po) of purified rabbit RyR2 channels defined at various redox potentials

<table>
<thead>
<tr>
<th>Group</th>
<th>Acetaldehyde (μM)</th>
<th>Redox potential (cis/trans) (mV)</th>
<th>Po</th>
<th>Po</th>
<th>Po</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-180/-180</td>
<td>-213/-180</td>
<td>-230/-180</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0</td>
<td>0.031 ± 0.006</td>
<td>0.034 ± 0.011</td>
<td>0.066 ± 0.016</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0</td>
<td>0.243 ± 0.071§§</td>
<td>0.117 ± 0.039§</td>
<td>0.038 ± 0.026</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0</td>
<td>0.074 ± 0.023*,†</td>
<td>0.066 ± 0.020*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.059 ± 0.011</td>
<td>0.076 ± 0.024</td>
<td>0.031 ± 0.015</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.078 ± 0.028</td>
<td>0.134 ± 0.030</td>
<td>0.049 ± 0.020</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.126 ± 0.034</td>
<td>0.291 ± 0.095¶</td>
<td>0.040 ± 0.018</td>
<td></td>
</tr>
</tbody>
</table>

A; Redox potential was not defined and Po was calculated from single channel current recorded at cis pCa 4. Po values are means ± S.E.; n=6 preparations.

B; Redox potential was fixed at each potential and pCa 4. C; *, pCa was increased to 6.5-7.0 without changing redox potential. Po at redox potential=-230/-180 mV was used in the value of B as controls without changing pCa. Effects of 1, 10 100 μM acetaldehyde were observed under these conditions.

§, §§; P<0.05 and P<0.01 from corresponding group A. †; P<0.05 from corresponding group B. ¶; P<0.05 from 0 or 1 μM acetaldehyde group.
Fig. 1

A. 

Control | 1 µM | 10 µM | 100 µM

(B) 

(F-Fo)/Fo

Control | 1 | 10 | 100

Acetaldehyde (µM)

Relative area of Ca²⁺ transient

Control | 1 | 10 | 100

Acetaldehyde (µM)

C.

D.

λ

Control | 1 | 10 | 100

Acetaldehyde (µM)
**A**

Control  
\( pCa \ 7 \)  
\( P_o = 0.033 \)

Acetaldehyde

1 μM  
\( P_o = 0.116 \)

10 μM  
\( P_o = 0.267 \)

100 μM  
\( P_o = 0.329 \)

---

**B**

Control

\( \tau_{O1} = 0.64 \text{ ms}, \ 88.1\% \)
\( \tau_{O2} = 3.41 \text{ ms}, \ 11.9\% \)

\( \tau_{C1} = 4.17 \text{ ms}, \ 21.4\% \)
\( \tau_{C2} = 36.31 \text{ ms}, \ 72.5\% \)
\( \tau_{C3} = 157.44 \text{ ms}, \ 6.1\% \)

Open time  
\( = 1.7 \text{ ms} \)

Closed time  
\( = 12.5 \text{ ms} \)

1 μM Acetaldehyde

\( \tau_{O1} = 1.73 \text{ ms}, \ 81.0\% \)
\( \tau_{O2} = 7.10 \text{ ms}, \ 19.0\% \)

\( \tau_{C1} = 2.67 \text{ ms}, \ 27.6\% \)
\( \tau_{C2} = 16.41 \text{ ms}, \ 57.1\% \)
\( \tau_{C3} = 53.34 \text{ ms}, \ 15.3\% \)

Open time  
\( = 3.3 \text{ ms} \)

Closed time  
\( = 19.3 \text{ ms} \)

---

Log dwell time (ms)
Fig. 3

A. Control (1 mM GSH)

B. 1 mM GSH + 100 μM Acetaldehyde

Relative abundance (%) vs. Time (min)

- m/z 613: GSSG
- m/z 533: I.S.-Ellman
- m/z 505: GSH-Ellman

Copyright Information
**A**  
Control  
pCa=6.5  
Po=0.025  
Redox potential  
trans=-180mV  
Po=0.006  
cis=-213mV, trans=-180mV  
Po=0.033  
2 min  
1 min  
+Acetaldehyde  
Po=0.052  
1 μM  
Po=0.083  
10 μM  

**B**  
Control  
pCa=7  
Po=0.031  
Redox potential  
trans=-180mV  
Po=0.004  
cis=-180mV, trans=-180mV  
Po=0.174  
2 min  
1 min  
+Acetaldehyde  
Po=0.130  
1 μM  
Po=0.209  
10 μM
Control

\[ p_{Ca} = 6.6 \quad P_0 = 0.034 \]

Redox potential

\[ \text{cis} = -213 \text{mV}, \text{trans} = -180 \text{mV} \]
\[ p_{Ca} = 5.0 \quad P_0 = 0.373 \]

\[ \text{cis} = -220 \text{mV}, \text{trans} = -180 \text{mV} \]
\[ P_0 = 0.263 \]

\[ \text{cis} = -230 \text{mV}, \text{trans} = -180 \text{mV} \]
\[ P_0 = 0.089 \]

+ Acetaldehyde

1 \( \mu \)M

\[ P_0 = 0.057 \]

10 \( \mu \)M

\[ P_0 = 0.049 \]

100 \( \mu \)M

\[ P_0 = 0.040 \]

20 pA

250 ms
A  Control

B  10 μM Acetaldehyde

C  100 μM Acetaldehyde
A 0.4 mM DEM + 100 μM mBCl

Fig. 7
Fig. 9

A

0.4 mM DEM

<table>
<thead>
<tr>
<th>Acetaldehyde</th>
<th>10 μM</th>
<th>100 μM</th>
</tr>
</thead>
</table>

B

(F-Fo)/Fo

C

λ (S⁻¹)

λ

Acetaldehyde (μM)

Acetaldehyde (μM)

Copyright Information