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Effects of t-Butyl Hydrogen Peroxide on Single SR Calcium Release Channels

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Using lipid bilayer reconstitution technique, we investigated the oxidation effect of t-butyl hydrogen peroxide (tBHP) on the single channel activity of the sarcoplasmic reticulum (SR) calcium release channels isolated from canine latissimus dorsi muscles. When 0.7% tBHP was added in the cytosolic side, the channel activity became suppressed (n=7), and it was recovered by changing the solution to the control solution. The suppression was due to the change in the gating mode of the channel: before tBHP the channel opened to four sub-conductance levels, but it opened to only one level after tBHP. These effects by tBHP were different from the previous finding using hydrogen peroxide (H_2O_2), which may be explained by different oxidation patterns between the two oxidants.

Key Words: Lipid bilayer, canine latissimus dorsi muscle, SR calcium release channel, t-butyl hydrogen peroxide

In skeletal muscles, electrical signals from the motor neuron terminal are transduced into the release of calcium from intracellular calcium storage, sarcoplasmic reticulum (SR) (Endo, 1977; Fleischer and Inui, 1989), and the elevation of calcium induces the muscle contraction. This transduction process, termed 'excitation-contraction (E-C) coupling', is mediated by two proteins: dihydropyridine-sensitive receptor (DHPR) in the plasma membrane which senses the electrical signals (Rios and Brum, 1987) and calcium channel in the SR membrane, which releases calcium from SR. The SR Ca^{2+} channel binds with high affinity to ryanodine, an alkaloid extracted from plants, and it is also known as ryanodine-sensitive receptor/channel (RyR). Three different isoforms have been cloned: RyR1 (skeletal muscle specific) (Takeshima *et al.* 1989), RyR2 (cardiac muscle specific) (Otsu *et al.* 1990), and recently RyR3 from neuronal cells (Hakamata *et al.* 1992).

Reactive oxygen species (ROS) mediate various pathological conditions in a variety of tissues (Freeman and Crapo, 1982). These ROS such as hydrogen peroxide (H_2O_2), singlet oxygen, hypochlorous acid, superoxide radical, and the hydroxyl radical may be generated either by electron reduction or energy activation during ischemia and/or reperfusion. Effects of the ROS on whole tissue or isolated sarcoplasmic reticulum (SR) from skeletal muscle have been extensively studied (Xiong *et al.* 1992; Boraso and Williams, 1994; Favero *et al.* 1995), and the ROS effects at the level of single SR calcium channel have been also reported. The effects on the single SR calcium channel were found to be diverse depending upon the ROS applied.

In this study, we recorded the single SR calcium channel activities from SR heavy microsomes purified from the canine latissimus dorsi muscle, and

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investigated the effects of t-butyl hydrogen peroxide (tBHP) on the channel activities.

MATERIALS AND METHODS

Purification of SR heavy microsomes

An adult dog (~20 kg) was anesthetized by i.v. injection of 300 mg sodium pentobarbital (Hanlim Pharm., Seoul). A total of 20 g of the latissimus dorsi muscle was collected from the animal and stored in liquid nitrogen. SR heavy microsomes were purified according to Coronado *et al.* (1992). Briefly, the tissue was broken into small pieces in liquid nitrogen, transferred to ice-cold homogenization solution (0.3 M sucrose, 0.5 mM EGTA, 20 mM $Na_4P_2O_7$, 20 mM NaH_2PO_4, 1 mM $MgCl_2$, 1 μM pepstatin, 1 mM iodoacetamide, 0.1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 mM benzamidine, pH 7.1), and homogenized for 2 min using a waring blender at maximum speed. The homogenate was spun for 15 min at 9000 rpm in a Centrikon A6.14 rotor (10,000 g). The supernatant was filtered through 4 layers of gauze and centrifuged at 18,000 rpm for 30 min in a Centrikon A8.24 rotor (30,000 g). Pellets were resuspended to a final volume of 5 ml in the homogenization solution, gently homogenized to dissolve clusters, and layered on top of a step sucrose gradient composed of 10 ml of 27% (w/v) sucrose, 10 ml of 32% sucrose, 15 ml of 38% sucrose, in 20 mM $Na_4P_2O_7$, 20 mM NaH_2PO_4, 1 mM $MgCl_2$, 1 μM pepstatin, 1 mM iodoacetamide, 0.1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 mM benzamidine, pH 7.1. Gradients were centrifuged for 16 hr at 28,000 rpm in a Centrikon TST28.38 rotor (100,000 g). The heavy microsome fraction was collected from the 32~38% sucrose interface. After diluting 4-fold with homogenization solution without sucrose, a pellet was obtained by centrifugation at 30,000 rpm for 40 min in a Centrikon TFT70.38 (65,000 g). Microsomes were resuspended in 0.3 M sucrose, 5 mM HEPES-KOH, pH 7.0 (final protein concentration: ~1 mg/ml), aliquoted to 20 μl each, and stored at $-80^{\circ}C$ for up to 3 months.

Lipid bilayer reconstitution technique

Phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS) (Avanti Polar Lipids, Alabaster, AL) were mixed at the ratio of 3:1 and dissolved in n-decane (20 mg/ml). A polystyrene cup with a hole of 250 μm was placed in a chamber. Both inside and outside the cup were filled with a solution of (in mM) 50~500 KCl, 1 MgCl2, 0.5 CaCl2, 1 EGTA, 10 mM HEPES, pH 7.2 (see figure legends for detail). The lipid was applied to the hole. After a bilayer with a capacitance of 200~300 pF was formed, SR microsome was added to the solution outside the cup (cis-side).

Currents under various voltages applied between cis and trans were amplified by an Axopatch 200A amplifier (Axon Instruments, Inc., Foster City, CA, USA). The signal ground was connected to the cis solution. The currents were filtered with 1 kHz low-pass filter built in the amplifier and stored in digital audio tape using DTR-1204 (Biologic Science Instruments, Claix, France). Later, the stored data were replayed and sampled at 5 kHz using an Axotape v 2.0.2 software (Axon Instruments, Inc.). The analysis of channel activities was performed with a pClamp v 6.0.1 software (Axon Instruments, Inc.).

RESULTS

When the SR heavy microsome was added to the cis solution, the channel activities were observed as shown in Fig. 1 (A). We reasoned that the channel activity in Fig. 1 (A) was from the activity of the SR calcium release channel based upon three criteria. First, 4 sub-conductance levels were observed from the channel activity (Smith *et al.* 1988; Brillantes *et al.* 1994). In Fig. 1 (A), the dotted line on the top represents the closed level of the channel. When the channel opens, there are 4 levels of the opening as indicated by 4 subsequent dotted lines. The 4 open levels are more evident when a histogram of the current amplitude is constructed as in Fig. 1 (B). Second, the conductance of the channel was around 500 pS (Smith *et al.* 1988; Shin *et al.* 1996). Third, 50 μM of ryanodine induced a long

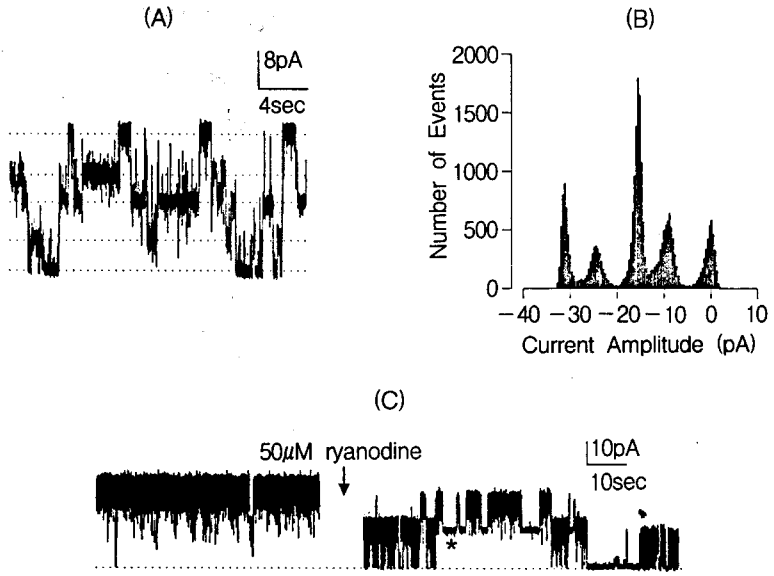

Fig. 1. *Single channel recordings show typical characteristics of SR calcium release channels. (A) Single channel activities of SR calcium release channel are plotted. The dotted line on the top represents the closed level of the channel and downward deflections are the openings of the channel. Four subsequent lines represent 1/4-open, 1/2-open, 3/4-open, and fully-open levels, respectively. The cis solution contained (in mM) 500 KCl, 1 MgCl2, 0.5 CaCl2, 1 EGTA, 10 HEPES (pH 7.2), and the trans 50 KCl, 1 EGTA, 10 HEPES (pH 7.2). The holding potential was −40 mV. (B) The histogram of the current amplitude in the trace (A) has five Gaussian curves superimposed, confirming four sub-conductance states. (C) 50 μM of ryanodine added to the cis solution induced a long opening at the intermediate level as marked with an asterisk(*) and subsequently blocked the channel activity. The dotted line represents the closed level. All the recording conditions were the same as in (A) except that the holding potential was +80 mV.*

opening at the intermediate level and subsequently blocked the channel activity as shown in Fig. 1 (C) (Lai *et al.* 1989).

When 0.7% of tBHP was applied to the cis solution, the channel activity decreased within a minute and remained decreased for more than 20 minutes as shown in Fig. 2. When the cis solution was changed back to the control solution, the activity returned to the control level (n=3). The tBHP effect was observed differentially between channels. Latency of the activity suppression after the tBHP application was observed to vary in the range of two minutes. In one experiment, the transient increase in activity was observed after the first tBHP application, and a further application then suppressed the channel activity. However, the channel activity became suppressed after the first tBHP application in most channels (n=7).

Fig. 3 illustrates the channel activities before and

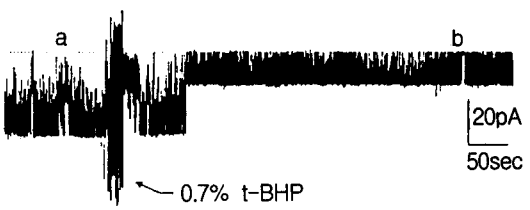

Fig. 2. *t-BHP suppresses the activity of the SR calcium channel. The activities of the SR calcium channel are plotted. t-BHP was added to the cis solution to make a final concentration of 0.7% as indicated. The increased current artifact while adding t-BHP is due to stirring the cis solution. The dotted line represents the closed level. The composition of the cis and trans solution is the same as in Fig. 1. The holding potential was −40 mV. See Fig. 3 for the explanation of a and b.*

after tBHP application of Fig. 2 in more detail. While the channel opens between sub-conductance levels in control trace, after tBHP application the

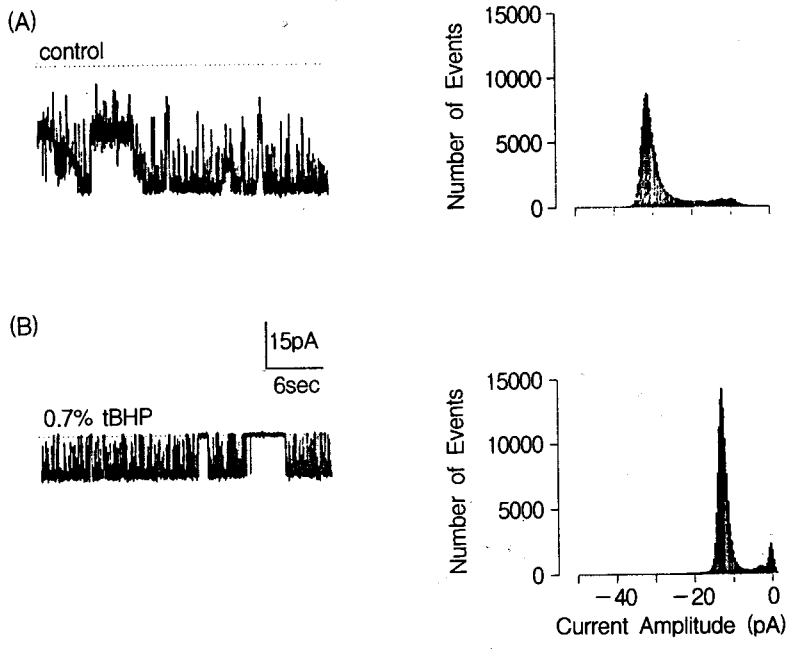

Fig. 3. t-BHP effect was to stabilize the 1/4 sub-conductance level. The representative traces of (A) before and (B) after tBHP are selected from Fig. 2 (denoted as 'a' and 'b') and plotted in a slower time scale. The histograms of the current amplitude are plotted to show the transition of the open level after tBHP application.

channel opens and closes with a different gating mode. The channel now opens to only one level. By comparing the size of the current, the open level after tBHP is between 1/4-open and 1/2-open. From the kinetic analysis, the mean open times were fitted to 20.92 ms (35.8%) and 132.37 ms (64.2%) and the mean closed times were fitted to 6.34 ms (82.2%) and 266.68 ms (17.8%).

DISCUSSION

Redox modulation of the channel activity has been reported in various channels (Sullivan *et al.* 1994; Han *et al.* 1996; DiChiara and Reinhart, 1997). It is important since the redox modulation of the channel activity can provide a link between metabolic states and electrical property in a cell, thereby mediating physiological functions and pathological conditions.

In this study, we investigated the activity modulation of the SR calcium release channel by tBHP. tBHP at 0.7% concentration suppressed the single SR channel activity. The activity remained suppressed for more than 20 minutes, and returned to the prior level when the control solution was reperfused. The onset of the activity suppression was observed to be diverse between channels in the range of a couple of minutes. In one experiment, the channel activity even increased transiently after the tBHP application, which was subsequently suppressed by further application of tBHP. This effects were not due to the stability of bilayer structure since the tBHP application without heavy SR microsomes did not affect the bilayer stability for more than 30 minutes (Boraso and Williams, 1994). These suppression by tBHP was due to a change in the gating mode of the channel. Before the tBHP application, the channel opened to 4 sub-conductance levels as described elsewhere for a typical SR calcium channel. However, after the tBHP application the channel opened to only 1 level. The size of the level was between 1/4 and 1/2 sub-conduc-

tance levels, and from the kinetic analysis the mean open times were fitted to 20.92 ms (35.8%) and 132.37 ms (64.2%), and the mean closed times were fitted to 6.34 ms (82.2%) and 266.68 ms (17.8%).

Our findings basically agree to the results of Favero *et al.* (1995). They reported that H_2O_2 stimulates the activity of the skeletal SR calcium channel. They found that the H_2O_2 effect is bi-phasic from [^{3}H]ryanodine binding assay and single channel recording. At a low concentration of H_2O_2 around 100 μM, the activity increases but becomes suppressed when more than 1mM H_2O_2 is applied. However, they did not find the change in gating mode of the channel. One possible explanation for the discrepancy is the different oxidation patterns between H_2O_2 and tBHP as oxidants. Keck reported that oxidation patterns at methionine (Met) residues of interferon γ protein are observed differently between H_2O_2 and tBHP (Keck, 1996). While all five Met residues are oxidized by H_2O_2, only two Met residues are oxidized by tBHP. Interestingly, two Met residues are located at the surface of the molecule as suggested from X-ray crystallographic studies. Keck suggested that tBHP can be used as a probe for 'exposed' Met oxidation. More experiments are necessary to confirm the tBHP-specific effects at the molecular level.

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