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Fast and Slow Gating Types of SR Ryanodine Receptor/Channel Purified from Canine Latissimus Dorsi Muscle

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The ryanodine receptor/channel (RyR) mediates the release of calcium from the sarcoplasmic reticulum (SR) in both skeletal and cardiac muscle cells. There are three isoforms of the RyR: RyR1, RyR2, and RyR3. RyR1 is specifically expressed in skeletal muscles and RyR2 in cardiac muscles. RyR3 is yet another isoform found in non-muscle cells such as neuronal cells. Single channel recordings of RyR1 and RyR2 reconstituted in artificial lipid bilayer show that the characteristics of two isoforms are very distinct. RyR1 has a shorter mean open time and is activated at a higher concentration of Ca^{2+} than RyR2. In this study, we isolated the heavy SR membranes from canine latissimus dorsi muscles and investigated the single channel activities from the heavy SR membrane fraction using Cs^{+} as a charge carrier. Two different types of activities were observed. The fast-gating type (FG) with the mean open time of 0.9 ms was more frequently recorded (n=12) than the slow-gating type (SG) with the mean open time of 269.2 ms. From the I-V relation, the slope conductance of the FG was calculated to be 514.7 pS and the SG, to 625.6 pS. The activity of the fast gating type increased by raising the concentration of Ca^{2+} in the cis-solution up to 100 μM. The appearance of the SG in the canine heavy SR membrane fraction suggests a possibility that two types of RyR isoform are co-expressed in mammalian skeletal muscle as well as in avian, amphibian, and piscine fast twitch muscles.

Key Words: Canine latissimus dorsi muscle, sarcoplasmic reticulum, ryanodine receptor/channel, lipid bilayer reconstitution, dynamic cardiomyoplasty

In striated muscles, electrical signals from the motor neuron terminal are transduced into the release of calcium from the intracellular calcium storage, sarcoplasmic reticulum (SR) (Endo, 1977; Fleischer and Inui, 1989), and the elevation of calcium induces muscle contraction. This transduction process termed 'excitation-contraction (E-C) coupling' is mediated by two proteins: the dihydropyridine-sensitive receptor (DHPR) in the plasma membrane which senses the electrical signals (Rios and Brum, 1987) and the calcium channel in the SR membrane. The interaction between these two proteins is distinct among different muscle types. In cardiac muscle, the extracellular calcium enters into the cell through the DHPR (Bean, 1989), and activates the SR Ca^{2+} channel to release more Ca^{2+} from the SR (Nabauer *et al.* 1989). However, in skeletal muscles, the active form of DHPR directly activates the SR Ca^{2+} channel through pro-

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tein-protein interaction (Schneider, 1994). Other studies have shown that different isoforms of both DHPR and SR Ca^{2+} channel are expressed in a tissue-specific manner.

The SR Ca^{2+} channel binds with high affinity to ryanodine, an alkaloid extracted from plants, and is generally called a 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). From ^{3}[H]ryanodine binding assay (Chu *et al.* 1990), $^{45}Ca^{2+}$ flux assay (Rousseau *et al.* 1986), and single channel recording (Smith *et al.* 1986), each isoform is known to have characteristics different from the others (Meissner, 1994a). The expression of RyR in other non-muscle cells (Hakamata *et al.* 1992; Tunwell and Lai, 1993) suggests that RyR has a role not only in E-C coupling but also in cellular calcium signaling with an IP3-sensitive calcium channel (Simpson *et al.* 1995).

In this study, we investigated the single channel activities from SR heavy microsomes purified from the canine latissimus dorsi muscle. Two different types of activities were observed: one with a faster gating (fast-gating subtype) and the other (slow-gating subtype). Co-expression of two types of RyR (αRyR and βRyR) was reported using skeletal muscles of other vertebrates such as avian (Percival *et al.* 1994), piscine (Murayama and Ogawa, 1992), and amphibian (Olivares *et al.* 1991), but it is generally agreed that only one type of RyR exists in mammalian skeletal muscles. Recently, however, the heterogeneity of RyR from the rabbit skeletal muscle was reported (Ma, 1995). Our results confirmed that two types of the RyR activities can be found from the skeletal muscle of other mammals.

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 an ice-cold homogenization solution (0.3 M sucrose, 0.5 mM ethylene glycol-bis-[β-aminoethyl ether]N,N,N',N'-tetraacetic acid (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 (PMSF), 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 9,000 rpm in a Centrikon A6.14 rotor (10,000 g). The supernatant was filtered through four 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 PMSF, 1 μM leupeptin, and 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 is 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 N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES)-KOH, pH 7.0 (final protein concentration:~1 mg/ml), aliquoted to 20 μl each, and stored at −80℃ for up to 3 months.

Lipid bilayer reconsitution technique

Phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), and cholesterol (all lipids from Avanti Polar Lipids, Alabaster, AL, USA) were mixed at the ratio of 9:3:1 and dissolved in n-decane (20 mg/ml). A polystyrene cup with a hole having a diameter of 250 μm was placed in a chamber. Both the in-

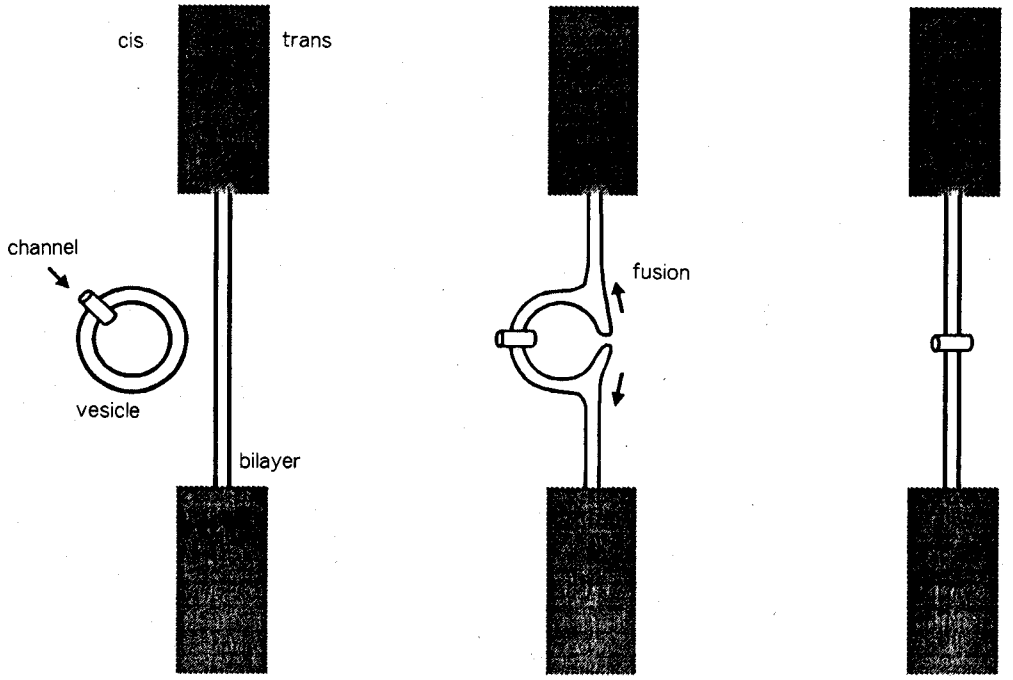

Fig. 1. *A diagram of lipid bilayer reconstitution.*
This is a cross sectional view of lipid bilayer reconstitution method. Lipid is applied to the hole having diameter of around 250 μm to form a bilayer (left). Conventionally, a partition where the vesicles containing channels are added is called 'cis', and the other 'trans'. The vesicle is fused into the bilayer (center and right). (modified from fig. 2 of White (1986)).

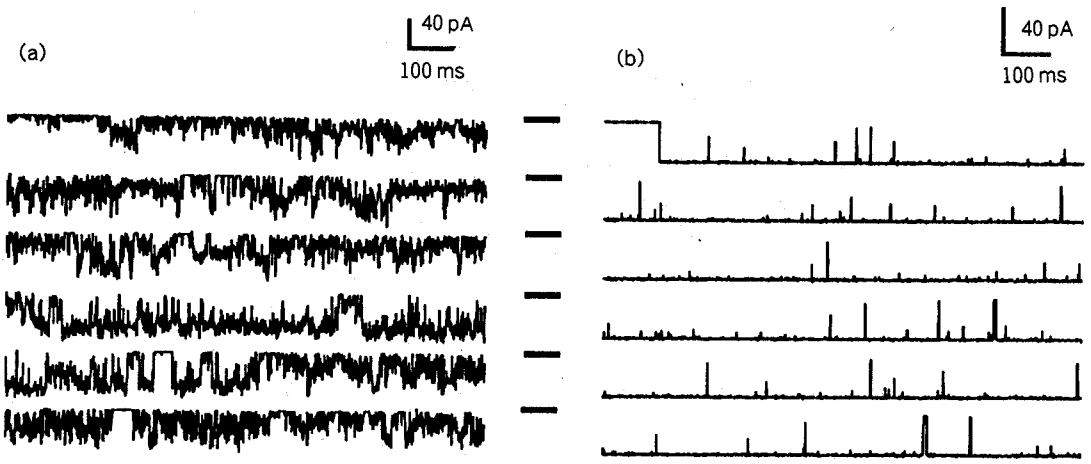

Fig. 2. *Two types of the SR calcium channel activities.*
(a) Fast-gating type (FG). V_H = -90 mV; $[Cs^+]_{cis,trans}$=250 mM; pCa=5 (b) Slow-gating type (SG). V_H = 0 mV; $[Cs^+]_{cis}$=500 mM; $[Cs^+]_{trans}$=50 mM; pCa=7. Thick lines in the middle of the traces represent the levels where channels close. FG (n=12) was more frequently observed than SG (n=6).

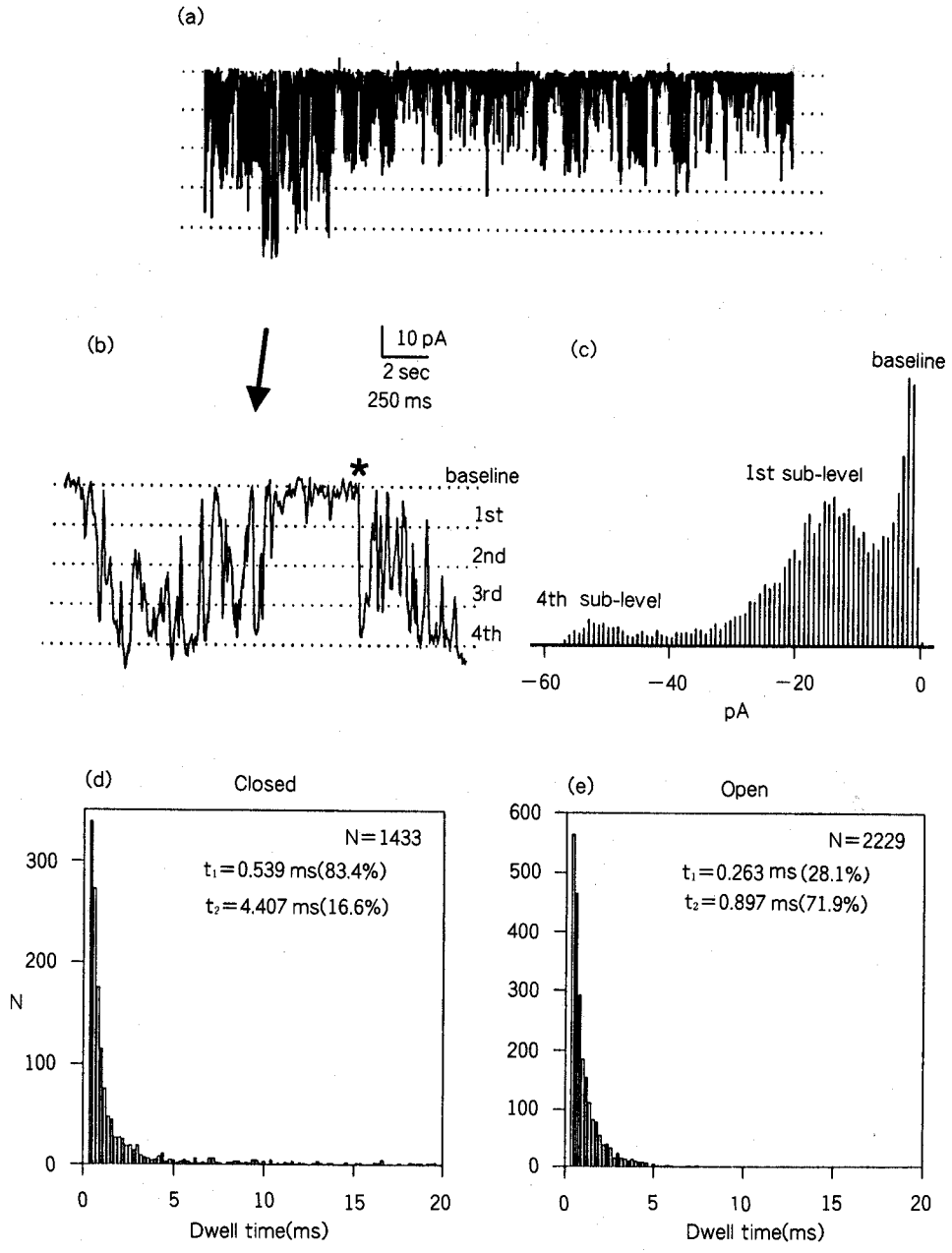

Fig. 3. *Fast-gating type (FG).*

(a), (b) The recording was under the same condition as in Fig. 2(a). An event of the transition from the closed level to the 3rd sub-open level is marked with an asterisk (see text). (c) A histogram of current amplitude reveals three peaks. From the recording, the histograms of (d) closed time and (e) open time were constructed. The histograms were fitted with two-exponential curve to obtain the mean closed time and mean open time.

side and outside of the cup were filled with a solution of 50~500 mM Cs piperazine-N,N'-bis [2-ethanesulfonic acid] (PIPES), 10 mM HEPES-KOH, pCa 7, pH 7.2. The lipid was applied to the hole. After a bilayer with a capacitance of 200-300 pF was formed, 3 μl of SR microsome was added to the solution outside the cup (cis-side) (Fig. 1). During the experiments, pCa was changed by adding a calculated amount of 100 mM $CaCl_2$ (Fabiato, 1981) and stirring for 1 min.

Currents under various voltages applied between cis and trans were amplified by an Axopatch 200A amplifier (Axon Instruments, Inc., Foster City, CA, USA), filtered with 1 kHz low-pass Bessel filter, and stored together with the applied voltages in digital audio tapes using a DTR-1204 (Biologic Science Instruments, Claix, France). Later, the stored data were replayed and sampled at 5 kHz using an Axotape v2.0.2 software (Axon Instruments, Inc., Foster City, CA, USA). The analysis of channel activities was performed with a pClamp v6.0.1 software (Axon Instruments, Inc., Foster City, CA, USA).

RESULTS

When a bilayer was formed around the hole and became stable, 3 μl of the SR heavy microsome fraction was added to the cis-solution. We used CsPIPES for two reasons: (1) Cs^+ ion blocks most of K^+ channels and (2) PIPES ion is too big to pass through Cl^- channels

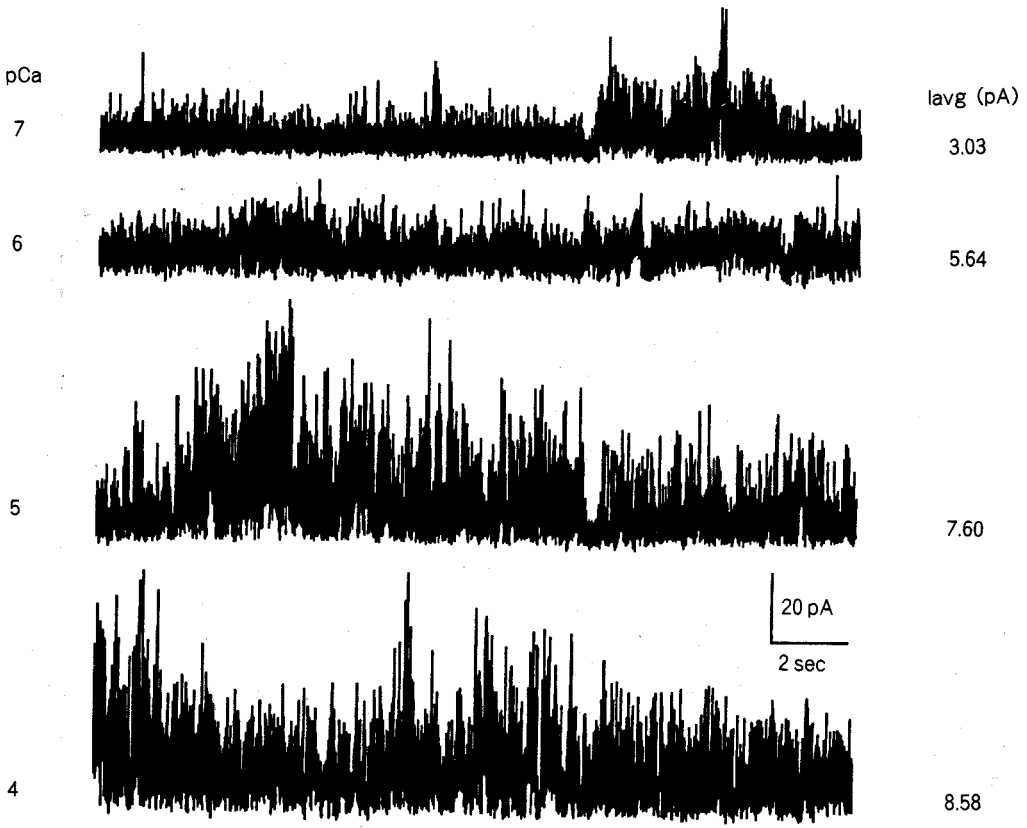

Fig. 4. *FG activity increased by free calcium concentration of cis-solution* V_H=0 mV; $[Cs^+]_{cis}$=50 mM; $[Cs^+]_{trans}$= 250 mM.

(Sitsapesan and Williams, 1994). Therefore, activities other than K^+ and Cl^- channels can be observed. Among the activities, two different types having a conductance larger than 500 pS were observed (Fig. 2). The channel type with a faster gating will be referred to the fast-gating type (FG) (Fig. 2(a)), and the other the slow-gating type (SG) (Fig. 2(b)). FG (n=12) was observed more often than SG (n=6).

Fig. 3(a) is a typical trace of the FG activity. When the trace was expanded with time, four sub-conductance levels were manifested (Fig. 3(b)). The transition between lev-

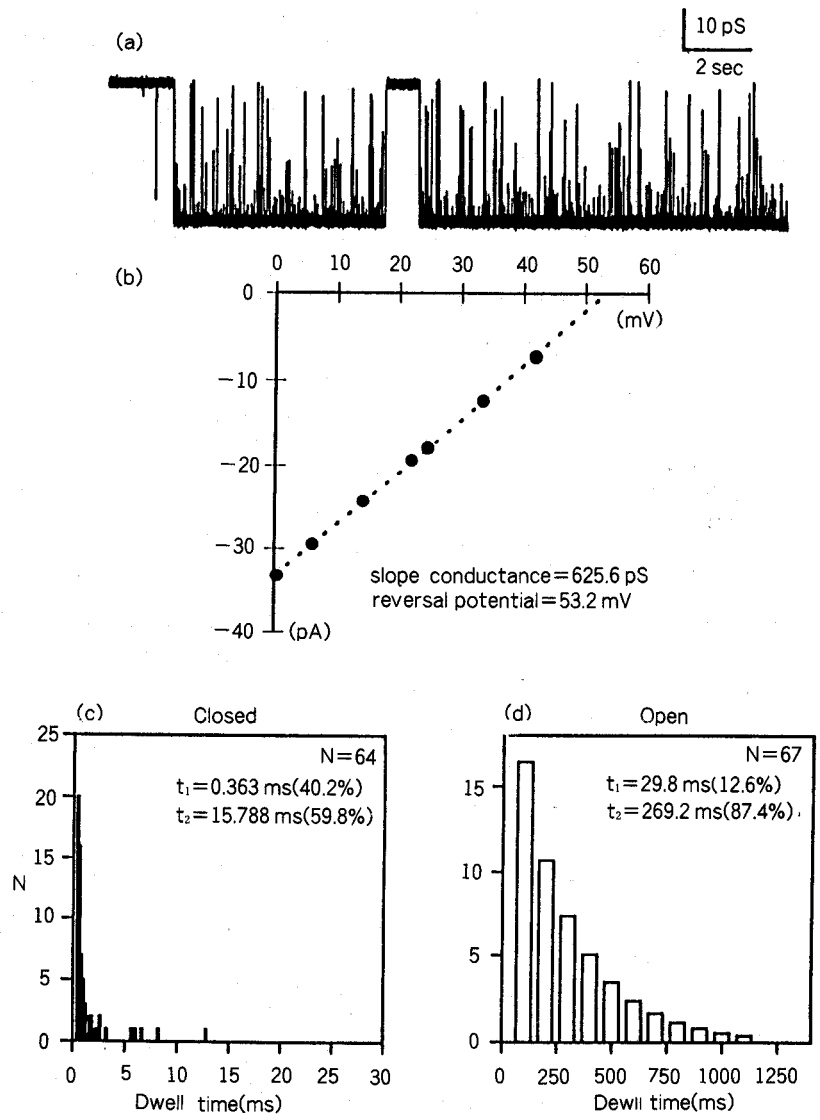

Fig 5. *Slow-gating type (SG).*

(a) The recording was under the same condition as in Fig. 2(b). (b) Channel currents at various voltages shows a linear relationship (dotted line). (c) The mean closed time and (d) the mean open time were obtained by the same method as in Figure 3(d) and (e).

els did not always occur subsequently. One example of the non-subsequent transition is the transition from the closed level to the 3rd sub-opened level (marked with an asterisk), and there are many more. Therefore, it is very unlikely that the activity observed is a superposition of the activities of four independent channels. A histogram of the current (Fig. 3(c)) shows three peaks corresponding closed level, 1st sub-opened level, and fully opened level, confirming the existence of four sub-conductance levels. The slope conductance of FG was 514.7 pS (data not shown), and the mean closed time (Fig. 3(d)) and the mean open time at the 1st open level (Fig. 3(e)) were 0.54 ms and 0.9 ms, respectively. FG activity was dependent on the free calcium level in the cis-solution. It increased with a sequential change of pCa from 7 to 4. These electrophysiological characteristics of FG were similar to those observed from other mammalian skeletal muscle SR RyR1.

The other type, SG, was also observed from the SR microsome. The channel gating of SG was much slower than FG. SG has only one open level (Fig. 5(a)). The slope conductance and the reversal potential were calculated to be 625.6 pS and 53.2 mV, respectively (Fig. 5 (b)). The mean open time and mean closed time were 269.2 mS and 15.79 ms (Fig. 5(c) and (d)).

DISCUSSION

Two types of SR RyR isoforms co-exist in avian, piscine, and amphibian skeletal muscles (Olivares *et al.* 1991; Murayama and Ogawa, 1992; Percival *et al.* 1994): αRyR and βRyR. These two isoforms have different mobility on gels (Airey *et al.* 1990; Olivares *et al.* 1991), and have been shown to relate immunologically to the mammalian skeletal and cardiac isoforms, respectively (Lai *et al.* 1992). They also have different electrophysiological characteristics. O'Brien *et al.* (1995) suggested a hypothesis for the co-expression of two isoforms in the skeletal muscles. In these muscles, calcium is released from SR by (1) the direct interaction between DHPR and αRyR as in the mammalian skeletal muscle and (2) calcium either already released from SR or entering from the outside of the cell (two-component model of calcium release). One benefit of having two pathways can be to provide greater flexibility in signal transduction such as activation of the muscle over a wide temperature range and modulation of the amount of calcium released and degree of fiber activation (O'Brien *et al.* 1995). Interestingly, only αRyR was found to be expressed in the extraocular muscle and the swim bladder muscle which are among the fastest contracting muscles (O'Brien *et al.* 1993).

It has been generally accepted that only one type of SR RyR is expressed in mammalian skeletal muscle (Meissner, 1994a; Meissner, 1994b). However, there are some reports where two types of activities have been observed. Brilllantes *et al.* (1994) observed that FK506-binding protein, a proline isomerase, affects the RyR activities. When the FK506-binding protein was added, four levels of sub-conductance disappeared leaving only one fully-opened level and the gating became slower. Ma (1995) reported the heterogeneity of RyR activities from rabbit skeletal muscle. Two types differ in ATP-sensitivity, desensitization of channel activity by high voltage, and time course of reaction with ryanodine. Ma (1995) suggested three possibilities for the heterogeneity. First, two isoforms of RyR are co-expressed as in a non-mammalian skeletal muscle. This has not been confirmed biochemically. Secondly, one isoform is expressed, but the post-translational modification such as phosphorylation modifies the RyR activity (Hain *et al.* 1994). Third, the interaction between RyR and some regulatory protein like FK506-binding protein affects the channel activities.

In this study, we purified the SR microsome from the canine latissimus dorsi muscle, and investigated the calcium channel activity from the SR microsome. Two types with different gating modes, FG and SG, were observed. FG has a conductance of 514.7 pS, and SG 625.6 pS. FG has four levels of sub-conductance, but SG has only one open level. The two types are very distinct in terms of gating.

The mean open times of FG and SG were 0.9 ms and 269.2 ms, and the mean closed time 0.54 ms and 15.79 ms. The same possibilities as stated above can be applied to explain the two types of the activities: ① different isoforms, ② the post-translational modification, and ③ some regulatory proteins. More experiments would be needed to differentiate among these possibilities.

A surgical technique called dynamic cardiomyoplasty has been developed to assist impaled cardiac muscles with transformed skeletal muscles. There have been many successful cases since Carpentier and Chachques (1985). One of the essential parts in the technique is to electrically train skeletal muscle tissues, usually from the latissimus dorsi muscle, for some period of time (Acker *et al.* 1986). There is a body of evidence suggesting that the chronic stimulation induces a change in muscle type from type II to type I at the histologic and molecular level (Leberer *et al.* 1989; Dux *et al.* 1990). Therefore, it would be interesting to investigate whether the chronic stimulation also induces a change in expression of SR RyR, and our results have established a basis in elucidating the question.

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