

Original Paper

Caffeine Induced Ca-release Dependence on the Bound Ca on the Outersurface Membrane of the Sarcoplasmic Reticulum Skeletal Muscle

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Abstract

It is known that the Ca release channels/RyR binding sites of skeletal muscle sarcoplasmic reticulum is modulated by caffeine. Caffeine potentiates Ca sensitivity in the Ca induced Ca release (CICR) channels. The aim of this experiment was to investigate whether Ca uptake in the FSR and Ca release from the FSR were changed by the bound Ca on the outer surface of the FSR membrane. When FSR was treated with low concentrations of caffeine, Ca release from the FSR was activated and the Ca bound on the outer surface membrane of the FSR was suppressed. However, when FSR was treated with low concentrations of procaine, Ca release from the FSR by caffeine was inhibited. Also, the Ca bound on the outer surface membrane of procaine treated FSR was not suppressed by caffeine and EGTA. It was suggested that the Ca release sites/RyR binding sites were accelerated with the increases of the stored Ca^{2+} and the bound Ca on the outer surface membrane of the SR, and that the inhibition of CICR by procaine was induced by the lock of the bound Ca on the outer surface membrane of the SR.

Introduction

In skeletal muscle cells, depolarization of the transverse (T) tubular system induced Ca release from the sarcoplasmic reticulum (SR), the internal Ca^{2+} store site. The Ca^{2+} released from the SR causes muscle contraction (1-4). This initial step of skeletal muscle excitation contraction coupling does not seem to require

a second messenger such as Ca^{2+} (5). It is believed that the physiological Ca release from the SR, primarily regulated by depolarization of the T membranes is the richest source of voltage sensitive Ca channels (3). It is not yet clear, however, how a change in potential across the T membranes leads to Ca release from the SR. The current notion about this process is that the voltage sensitive

receptor (Ca-channel) in the T membranes senses a change in membrane voltage and undergoes a molecular rearrangement that is postulated to directly the Ca release channel gate in the SR membrane (6). The Ca release channel protein has been purified as the ryanodine receptor (RyR) which spans the junction between the SR and T membranes (7. 8. 9). The Ca release channel can be activated by an increase in cytoplasmic free Ca^{2+} (Ca induced Ca release-CICR) (1. 5). However there is no strict functional evidence that this CICR channel protein functions by physiologically depolarizing the gated Ca release channel (10. 11). Caffeine is known to potentiate CICR such that resting intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) triggers Ca release from the SR (3. 4. 5). It is established that the action site of caffeine is at the RyR (12. 13). The present study shows that the Ca^{2+} bound on the outer surface membrane of the SR participates in the initial phase of Ca release from the SR induced by caffeine.

Material and Methods

1) Preparation of the SR membrane

Fragmented of the sarcoplasmic reticulum (FSR) membrane was separated from the leg skeletal muscle of the Japanese toad (*Bufo vulgaris japonicus*) as described in Nishijima et al (1972) (14). The leg skeletal muscle was homogenized in a waring blender for 10 seconds in 5 volumes of a mixture of ice cold 40 mM Tris-HCl buffer (pH 7.2) and 80 mM KCl. Myofibrils, nuclei and debris were separated by centrifugation at 4500rpm (AV. 4200×G) for 20min. using rotor No 12 of Hitachi centrifuge RP 20. The supernatant was recentrifuged at 8500rpm (AV. 8570×G) for separation of the mitochondria. The resultant supernatant was filtered through a No. 5B filter, and the filtrate was centrifuged for 60

min., at 17500rpm in rotor No. 18 (AV. 36500×G). The sediment was washed twice with a 50mM Tris maleate buffer (pH 6.8). The precipitate was then suspended in the same buffer solution and stored at 4 °C. The protein concentration of the FSR membrane was determined by the Lowry's method (15).
2) Ca-uptake and Ca-release measurements.

The external reaction solution was composed of 100 mM KCl, 1.0mM MgCl_2 , 20mM Tris maleate buffer (pH 6.8) and 0.1mM $^{45}\text{Ca}-\text{CaCl}_2$. After the FSR was incubated for 20 min. at 20 °C., the Ca-uptake in FSR was started by the addition of 1.0mM ATP and stopped at definite time intervals by separating the FSR membrane from the reaction mixture using a millipore filter membrane (pore size 0.22 μ). For Ca bound on the FSR, the reaction was started by the addition of $^{45}\text{Ca}-\text{CaCl}_2$ after incubation in a reaction solution without CaCl_2 . The specific radioactivity of $^{45}\text{Ca}^{2+}$ containing in the FSR and in the reaction mixture and the filtered reaction solution were measured by a liquid radioactive spectrometer. The total amounts of Ca^{2+} in the filter and each solutions were measured by atomic spectrophotometer (Hitachi). From these results, the amounts of Ca^{2+} in the FSR was calculated.

Results and Discussion

1. Ca-uptake rate and capacity of the caffeine treated FSR

It is known that the Ca uptake capacity of FSR separated from toad skeletal muscle is $200-450 \times 10^{-9}$ moles/mg protein (16), and that the Ca-uptake rate and capacity of FSR are inhibited and the Ca-induced Ca release (CICR) is activated by caffeine (1. 3).

As shown in Fig. 1a the Ca uptake capacity of FSR using in this experiment reached the maximum value at 30sec. after the addition of ATP, and this capacity held for 300sec.

The maximum Ca uptake capacity was $238 \pm 3.6 \times 10^{-9}$ moles/mg protein. When the FSR were treated with various concentration of caffeine, the amount of Ca uptake in the initial phase (at 15sec after the addition of ATP) and the maximum amounts of Ca uptake dependent on the caffeine concentration.

As shown in Fig. 1b the Ca uptake in initial phase of the caffeine treated FSR depressed exponentially, while the maximum capacities of Ca uptake depressed linearly with the increase of caffeine concentration. FSR immediately after preparation is in a markedly inactive state, because the amount of Ca^{2+} in the FSR is $30-8 \times 10^{-9}$ moles/mg protein (16). Therefore, it was shown that the Ca uptake rate depended on the amount of Ca^{2+}

in the FSR and the caffeine concentration. However, the mechanism by which caffeine inhibited the Ca uptake on the FSR in the inactivated state is unknown. On the other-hand, the linear suppression of maximal Ca uptake in the FSR treated with various concentrations of caffeine, reflected a dependence on caffeine concentration. It is well established that caffeine potentiates CICR and that the resting $[\text{Ca}^{2+}]_i$ triggers Ca release from the SR, and that the action site of caffeine is at the ryanodine receptor (RyR) (11, 12). In Fig. 1, if the linear suppression of Ca uptake activities on the caffeine treated FSR was induced by the action of RyR, Ca release from the FSR may be linearly dependent on the caffeine concentration.

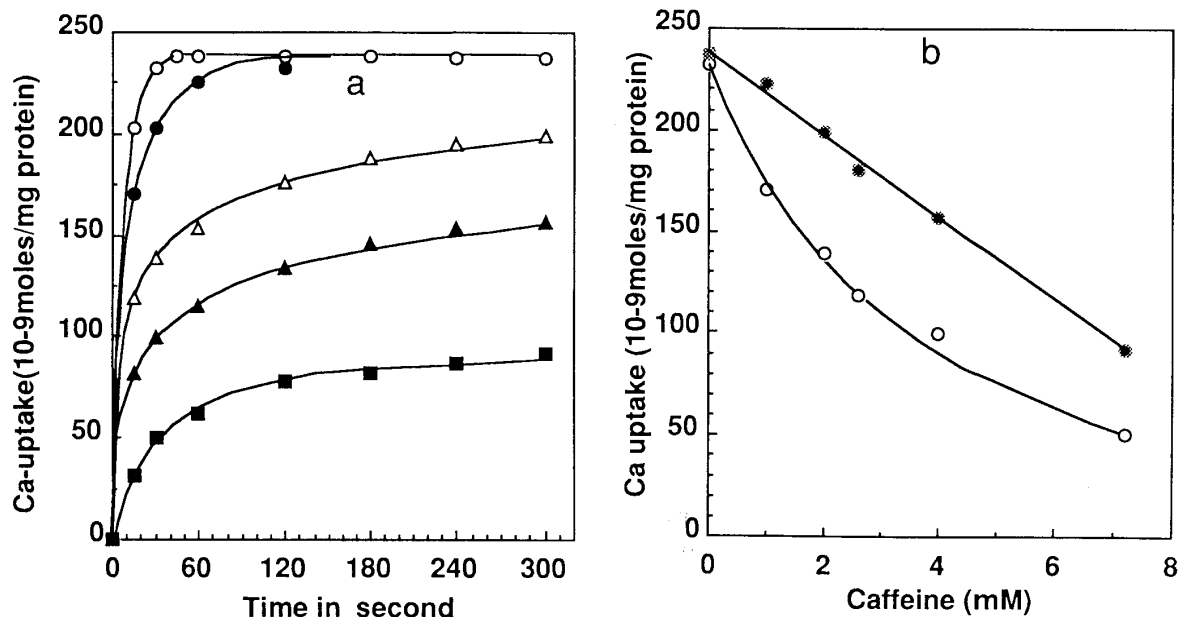


Fig 1 Changes of Ca uptake rate and capacity in the caffeine treated FSR.

FSR($200 \mu\text{g} \cdot \text{ml}^{-1}$) was incubated at 20°C in a reaction solution composed of 100 mM KCl, 1.0 mM MgCl_2 , 20 mM Tris maleate buffer (pH 6.8), 0.1 mM $^{45}\text{Ca}-\text{CaCl}_2$, and was incubated for 2 min. with various concentrations of caffeine. After 1.0 mM ATP was added in to the reaction solution, the reaction was stopped by the filtration method at an appropriate time. The Ca uptake in FSR used in this figure reached the maximum amounts at 30 sec. after the addition of ATP, and this capacity held for 300 sec. The maximum Ca uptake capacity was $238 \pm 3.6 \times 10^{-9}$ moles/mg protein. Figure a shows the changes of Ca uptake on non treated FSR (\circ), 1.3mM (\bullet), 2.6 mM (\triangle), 6.0mM (\blacktriangle) caffeine treated FSR. Figure b shows the amount of Ca^{2+} taken up at 30 sec (\circ) after the reaction start and the maximum amounts (\bullet) of Ca^{2+} taken up in FSR treated with various concentration of caffeine. Data are the means of 6-8 samples, standard errors are between 3 and 6×10^{-9} moles/mg protein.

2. Ca release from the FSR by caffeine

As shown in Fig. 2a when various concentrations of caffeine were applied to the FSR fully charged with Ca^{2+} , Ca^{2+} in the FSR was released at different rates depending on the caffeine concentration. In this experiment, the amount of released Ca^{2+} from the FSR with 0.93mM caffeine was 8–10% of the total amount in the FSR at 30sec after the

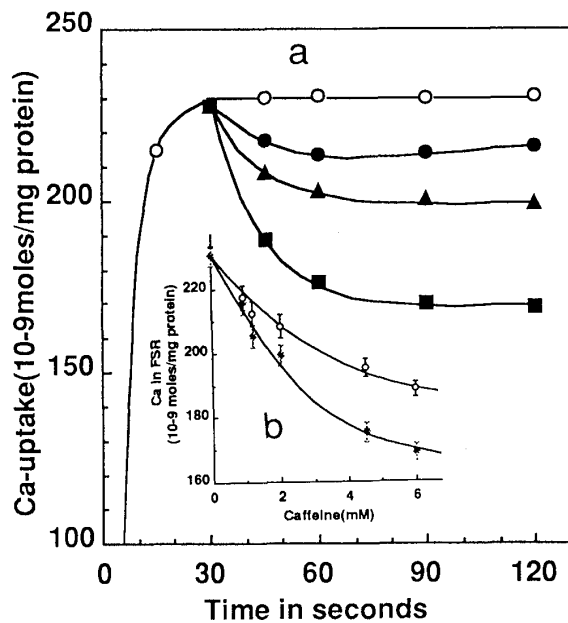


Fig 2 Ca release from the FSR by caffeine. The maximum Ca uptake capacity of FSR in this experiment was $212 \pm 3.4 \times 10^{-9}$ moles/mg protein. When the maximum amounts of Ca^{2+} taken up at 30 sec after the addition of ATP, the various concentrations of caffeine added to the reaction mixture. Figure a shows the changes of Ca^{2+} taken up in non treated FSR (○), and shows the changes in the release from FSR with 0.94 mM (●), 2.03 mM (△), 6.0 mM (▲) caffeine. Data are the means of 6–8 samples, standard errors are between 3 and 6×10^{-9} moles/mg protein. Figure b. shows the amounts of Ca^{2+} released at 15 sec (○) after the addition of various concentration of caffeine and the maximum amounts (●) of released Ca. Data are the means of 6–8 samples, standard errors are between 4 and 7×10^{-9} moles/mg protein.

addition of caffeine, and the released Ca^{2+} was reuptaked in the FSR after a period of time.

The amounts of released Ca^{2+} in the initial phase after the addition of caffeine, as shown in Fig. 2b increased exponentially depending on the caffeine concentration. Also, the maximum amounts of released Ca^{2+} from the FSR also increased exponentially depending on the caffeine concentration. The maximum amounts of released Ca^{2+} from the FSR by low concentrations of caffeine were in the initial phase. The difference between the amounts of released Ca^{2+} in the initial phase and the maximum amounts of released Ca^{2+} increased depending on the caffeine concentration. However, when the FSR was treated with the high concentrations (6.3mM) of caffeine, the maximum amounts of Ca^{2+} released from the FSR was less than 30% of the total amounts of Ca^{2+} in the FSR. The maximum amounts of Ca^{2+} released by caffeine from FSR fully charged with Ca^{2+} were different from the maximum amount of Ca^{2+} taken up by the FSR in an inactivated state as shown in Fig 1.

As shown in Fig. 1a the linear inhibition in the initial phase of Ca uptake by various concentrations of caffeine showed that RyR receptors on the FSR membrane were not activated by myoplasmic Ca^{2+} and caffeine, and that the activities of RyR manifested with an increase of Ca^{2+} in the FSR. However, the difference between the maximum amounts of released Ca^{2+} by caffeine from the FSR fully charged with Ca^{2+} and the maximum amounts of Ca^{2+} taken up by the FSR in the inactivated state remain an unsolved problem. If Ca released from the FSR is activated by myoplasmic Ca^{2+} , the Ca release by caffeine in this experiment would induce the activation of bound Ca on the outer surface of the FSR membrane.

3. The relationship between the Ca^{2+} activity and the passive Ca^{2+} binding.

As shown in Fig. 3, when the FSR in the inactivated phase were treated with 0.5mM procaine, the maximum volume of Ca uptake

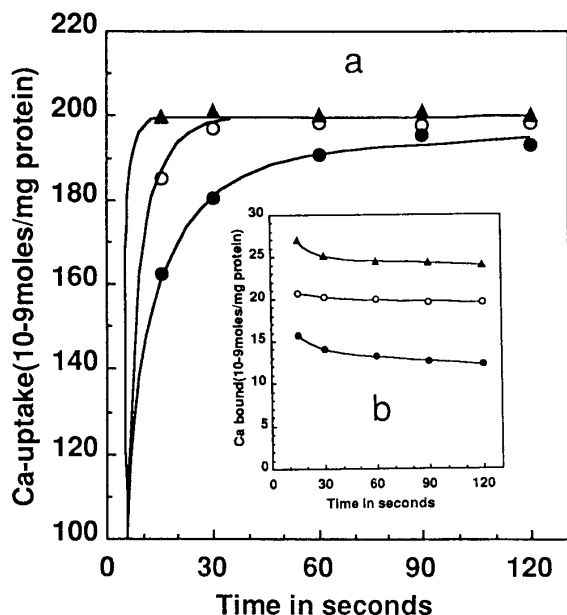


Fig 3 Changes of Ca uptake in procaine treated FSR.

^{45}Ca - CaCl_2 uptake was measured as described in Figure 1. FSR was incubated for 2 min. in a reaction solution including 0.5 mM procaine and 0.93 mM caffeine. After 1.0 mM ATP was added to the reaction solution, the reaction was stopped by the filtration method at an appropriate time. The Ca uptake on FSR used in this figure reached the maximum value at 30 sec. The maximum Ca uptake capacity was $198 \pm 3.1 \times 10^{-9}$ moles/mg protein. The Ca uptake rate on procaine treated FSR was faster than that of control FSR. But the maximum capacities on procaine treated FSR was not significantly greater than that of control FSR. Figure a. shows the changes of Ca uptake in non treated FSR (○), 0.93 mM caffeine (●), 0.5 mM procaine (▲) treated FSR. Data are the means of 6-8 samples, standard errors are between 3 and 6×10^{-9} moles/mg protein. Figure b. shown the changes in bound Ca. Data are the means of 6-8 samples, standard errors are between 2 and 4×10^{-9} moles/mg protein.

showed at a faster time than that on the normal FSR, but both FSR showed similar capacities. On the other hand, as shown in Fig. 3b the amounts of bound Ca on the procaine treated FSR were much greater than that on the normal FSR. The amount of bound Ca on caffeine treated FSR was less than that on the normal FSR. Therefore, from these results, it was suggested that the Ca uptake rate on FSR in the inactivated phase depends on the amount of bound Ca on the outer surface of the FSR membrane.

As shown in Fig. 4a the Ca uptake reaction

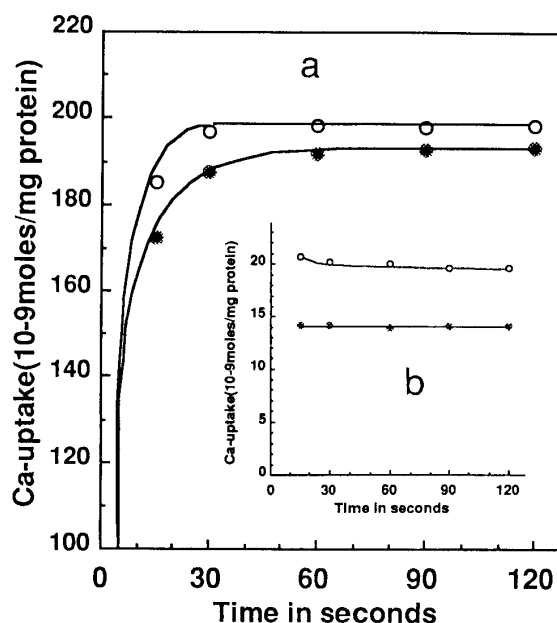


Fig 4 Changes of Ca uptake in EGTA. treated FSR.

Figure a shows the changes of Ca uptake in FSR measured as described in Figure 1 (○), FSR was separated by the millipore filter membrane from the reaction mixture within 10 sec after the addition of 0.5 mM EGTA. (●). The maximum Ca uptake capacity was $198 \pm 3.1 \times 10^{-9}$ moles/mg protein. Data are the means of 6-8 samples, standard errors are between 3 and 5×10^{-9} moles/mg protein. Figure b shows the changes of bound Ca in FSR. Data are the means of 6-8 samples, standard errors are between 3 and 4×10^{-9} moles/mg protein.

in the FSR was stopped by the addition of 0.5 mM EGTA. at definite time intervals after the start of reaction by the addition of ATP. FSR was separated from the reaction mixture within 10 sec after the addition of 0.5 mM EGTA using a millipore filter membrane. The amounts of Ca^{2+} taken up in these EGTA treated FSR were less than that in the FSR separated from the reaction mixture with the millipore filter membrane. The difference in these amounts of Ca taken up was $8-10 \times 10^{-9}$ moles/mg protein, and has statistically a significant value. However, when the amounts of bound Ca were examined by both method, the difference was $8-10 \times 10^{-9}$ moles/mg protein as shown in Fig. 4b. The amounts of bound Ca in the FSR were not suppressed by washing with 5 ml of the reaction solution. Thus, it was suggested that the difference found using the two methods was caused by the bound Ca on the outer surface membrane of the FSR.

Fig. 5 shows that Ca release by caffeine was markedly inhibited in procaine (0.5 mM) treated FSR when compared with untreated FSR. Procaine is known to inhibit CICR (13). It has been reported that procaine binds to the site which influences Ca sensitivity to the Ca regulatory site, and that the inhibitory effects of procaine on Ca release from the FSR were dependent on the extravesicular Ca concentration (17). In this experiment, as shown in Fig. 4b, the bound Ca on the outer surface membrane of the FSR was augmented more than that of normal FSR. However, when FSR fully charged with Ca^{2+} were treated with 0.83 mM caffeine after the addition of 0.5 mM EGTA., there was no Ca release from the FSR whereas the amounts of bound Ca on 0.5 mM EGTA. treated FSR was depressed more than that of normal FSR. The bound Ca in the procaine treated FSR was not depressed by the addition of caffeine (0.83

mM) and EGTA. (0.5 mM). These results suggested that the bound Ca on the outer surface of the FSR membrane could not be removed. $[\text{}^3\text{H}]$ -ryanodine binding to the FSR depends on Ca concentration, as shown in previous works (17, 18). But, caffeine potentiates the Ca sensitivity in the CICR channels and increases $[\text{}^3\text{H}]$ -ryanodine binding to FSR.

The inhibitory effect on Ca release by caffeine in the procaine treated FSR showed that the activity of the ryanodine receptor/Ca release channels are modulated by procaine.

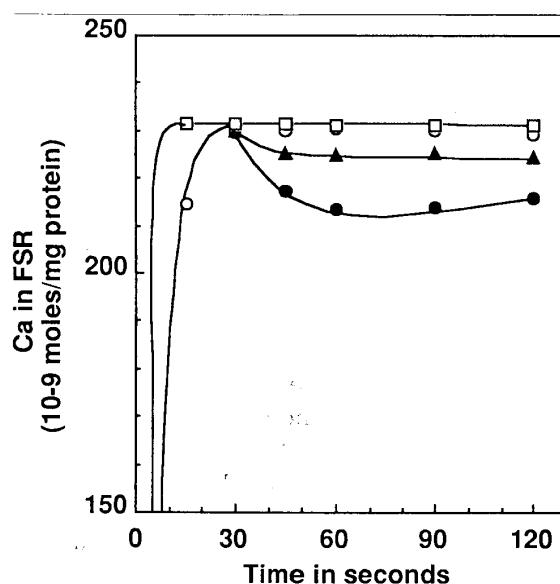


Fig 5 Ca release by caffeine from procaine treated FSR.

The maximum Ca uptake capacity on FSR in this experiment was $232 \pm 4.1 \times 10^{-9}$ moles/mg protein. The Ca release from each treated FSR were examined by 0.833 mM caffeine. Figure shows the changes of Ca uptake in non treated (○) and the Ca release from non treated FSR (●) with 0.83 mM caffeine. The Ca taken up in the 0.95 mM procaine treated FSR (□) was not released by 0.83 mM caffeine, and after the addition of 0.5 mM EGTA. (▲). Data are the means of 6-8 samples, standard errors are between 3 and 6×10^{-9} moles/mg protein

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