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On the Possible Interaction of Caffeine with the Fragmented Sarcoplasmic Reticulum of Rabbit Skeletal Muscle

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筋小胞體 切片에 미치는 Caffeine의 작용에 관한 연구

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要 約

筋小胞體의 Ca能動輸送은 caffeine에 의하여 阻害되므로 筋小胞體 切片에 대한 caffeine의 작용을 조사한 결과는 다음과 같다.

caffeine은 근소포체 표면에 結合하지 않거나 결합하여도 그 결합은 극히 약한 결합으로 생각된다.

caffeine은 筋小胞體 표면의 遊離 SH基를 증가시킨다. 따라서 caffeine에 의하여 근소포체 단백질의 표면변화가 일어나서 그 결과 Ca에 대한 輸送能이 低下되는 것으로 생각된다.

INTRODUCTION

Caffeine has long been known for its pharmacolcgical effects on nerve and muscle tissues and as a diuretic (Bianchi, 1961), but its mechanism of action on the subcellular level in muscle contraction is not fully understood.

Herz and Weber (1965) reported that caffeine (8-10 mM) inhibited the Ca uptake of frog sarcoplasmic reticulum (650-2,000 × G fraction) and that if caffeine was added after a maximal amount of Ca had been taken up, 20-40% of the bound Ca was released. These results suggested that the increased Ca release brought about by caffeine would reflect an increased intracellular level of ionized Ca which in turn affects the contractile mechanism in accordance with the current concepts regarding the essential role of Ca in muscle contraction.

Isaacson and Sandow (1967) reported that 1 mM caffeine, a concentration that produces only twitch potentiation and not contracture in frog sartorius muscle, increased both the uptake and release of Ca in this muscle by about 50%, and

suggested that these results indicate that a basic effect of caffeine on muscle is to directly release Ca from the sarcoplasmic reticulum in proportion to the drug concentration. They considered it unlikely that the drug might also increase the permeability of the membrane to Ca, since it was reported (Feinstein, 1963) that there was no change in Ca content of frog sartorii subjected to 5 mM caffeine for 10 minutes and then washed for 2 hours.

Weber and Herz (1968) have shown that the amount of Ca released from the heavy fractions (2,000-8,000 × G) of the frog and rabbit reticulum by 8-10 mM caffeine could account for all of the caffeine-induced tension, suggesting that the caffeine-induced release of Ca from the reticulum is the direct cause of the contracture.

Fuchs (1969) also reported evidences that caffeine produces contracture through an inhibition of Ca transport of the rabbit sarcotubular membrane fraction (1,500-10,000 × G) and suggested that caffeine might affect both uptake and release of Ca. Fairhurst and Hasselbach (1970) showed that caffeine stimulated the efflux from the heavy sarcotubular fraction (2,000-8,000 × G) whereas the efflux from a light fraction (12,000-35,000 × G) was much less sensitive to this drug. From these observations, they concluded that the action of caffeine is the stimulation of efflux and not influx of Ca from the sarcoplasmic reticulum. Batra and Daniel (1971) also obtained similar results that caffeine (8 mM) had no effect on the influx of Ca into the sarcoplasmic reticulum of rat myometrial muscle.

Ha (1972) studied the nature of caffeine action on the ATPase activity and Ca uptake of fragmented sarcoplasmic reticulum of rabbit skeletal muscle and revealed somewhat detailed properties of the action, suggesting a possibility of an interaction of the alkaloid with sarcoplasmic reticulum, probably with the protein moiety. The following experiments were therefore attempted to test the possibility that caffeine interact with the protein moiety of the sarcoplasmic reticulum.

The experiments were carried out in two approaches; one a direct measurement of the caffeine binding to the membrane fragments using ^{14}C-caffeine, and the other the determination of the change in the sulfhydryl content of the membrane protein in the presence of caffeine employing the method of DTNB reaction.

MATERIALS AND METHODS

1. Preparation of Sarcoplasmic Reticulum Fragments

Adult male albino rabbits were decapitated and back muscle was immediately taken out. Fatty matter and nerve fibers were carefully removed. The muscle was then homogenized with 0.01 N NaOH (400 ml per 100g of muscle) in a Waring blender according to the method of Ebashi and Yamanouchi (1964). During the homogenation, pH was kept at 6.8 by addition of 1 N NaOH. The homogen-

ate was centrifuged twice at 2,000 × G for 20 minutes and the supernatant was successively centrifuged at 8,000, 10,000 and 20,000 × G. The fraction which sedimented at the centrifugal force between 10,000 and 20,000 × G was washed by centrifugation in 20 mM tris (hydroxymethyl) aminomethane-maleate buffer (tris buffer), pH 6.8, containing 50 mM KCl and finally suspended in this solution. All procedures were carried out at below 4°C.

2. Protein Determination

The protein concentration was determined by the Lowry's Cu-folin method (Lowry *et al.*, 1951) standardized by the micro-Kjeldahl analysis of nitrogen using rabbit or bovine crystalline serum albumin as standard.

3. Measurement of Caffeine Binding

The binding of caffeine to the sarcoplasmic reticulum was measured using Millipore filter. Since the specific activity of ^{14}C-caffeine in the reaction mixture was very low (1.2×10^{4} cpm/mM) owing to the relatively high concentration of caffeine employed, counting of radioactivity was always made on both filters and filtrates. Blank filtration was done by filtering the incubation medium containing the labeled compound but no microsomes.

4. Assay of Radioactivity

Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer (Model 33101). The scintillator used was a mixture containing 4 g of 2,5-diphenyloxazole (DPO), 0.2g of 1,4-bis (2-(5-phenyloxazolyl))-benzene (POPOP), 60g of naphthalene, 100 ml of methanol, and 200 ml of ethylene glycol, all dissolved in p-dioxane to 1 liter (Bray, 1960). The Millipore filtrate (0.5 ml) was added to 10 ml of the scintillator and counted for 5 or 10 minutes each at room temperature. When the radioactivity of the filter was counted, the filter was directly dissolved in the scintillator fluid and counted as above.

5. Determination of Endogenous Calcium

The amount of endogenous Ca present in preparations of the sarcoplasmic reticulum was determined with an atomic absorption spectrophotometer (Hitachi Model 207) by reading at the wavelength of 422 nm. Solutions of $LaCl_3$ and CsCl were added to the suspension to a final concentration of 10 mM and 3.6 mM, respectively, to obviate the interference of phosphate. Contamination of Ca in the reagents of the reaction mixture was also determined similarly.

6. Determination of Sulfhydryl Content

To determine the change in the sulfhydryl content of the membrane protein, 0.02 ml of 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) dissolved in tris buffer (pH 7.2) was added to 3.0 ml of the incubation mixture in a photocell and the color developed was read in a Hitachi Model 101 spectrophotometer at 412 nm, according to the method of Ellman (1959).

Two photocells were always used in parallel, one containing control solution and the other containing test solution. The absorbancy was read at one minute intervals for 20 minutes, the control reading preceding by 30 seconds the test sample.

7. Reagents

All reagents used were of reagent grade. ATP was obtained as disodium salt from Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan. Caffeine, as the free base, was a product of Wako Pure Chemical Industries, Ltd., Tokyo, Japan. Tris (hydroxymethyl) aminomethane was obtained from Sigma Chemical Co., St. Louis, U.S.A. DTNB was a product of Aldrich Chemical Co., Milwaukee, U.S.A. Radioactive $^{45}CaCl_2$ (6 mCi/mg) was purchased from International Chemical and Nuclear Corp., U.S.A., and caffeine 1-methyl-^{14}C (4.4 mCi/mM) from New England Nuclear, Boston, U.S.A. All other reagents were supplied from Wako Pure Chemical Industries, Ltd., Tokyo, Japan. All solutions were prepared with deionized, glass-distilled water.

RESULTS

1. Caffeine Binding to the Microsome

The possibility that caffeine may bind to sarcoplasmic membrane and modify the conformational state of the membrane protein, thereby causing an alteration of the Ca-transport system, was tested by the use of ^{14}C-labeled caffeine. The incubation mixture containing the labeled caffeine (specific activity approximately 7×10^4 cpm/ml), 0.1 mg protein/ml, 2 mM ATP, 4 mM $MgCl_2$, 50 mM each KCl and NaCl, and 0.02 mM $CaCl_2$, all dissolved in 20 mM tris-maleate buffer (pH 6.8) was rapidly filtered on a Millipore filter device after 1 minute of incubation at 25°C. The filters were counted for their content of radioactivity with a Packard liquid scintillation counter. Blank measurement on the incubation mixtures containing trichloroacetic acid-denatured microsomes or no microsomes at all were simultan-

Table 1. The binding of radioactive caffeine to microsomes.

Additions*			Radioactivity (Mean CPM/0.1mg protein)
ATP (2 mM)	Ca (0.02 mM)	Microsomes (0.1 mg protein/ml)	
+	+	+	13820
+	−	+	21378
−	+	+	18655
−	−	+	19707
−	−	−	11556

*Basic incubation medium: 50 mM KCl, 50 mM NaCl, 4 mM $MgCl_2$, 5 mM caffeine-^{14}C, dissolved in 20 mM tris-maleate buffer, pH 6.8.

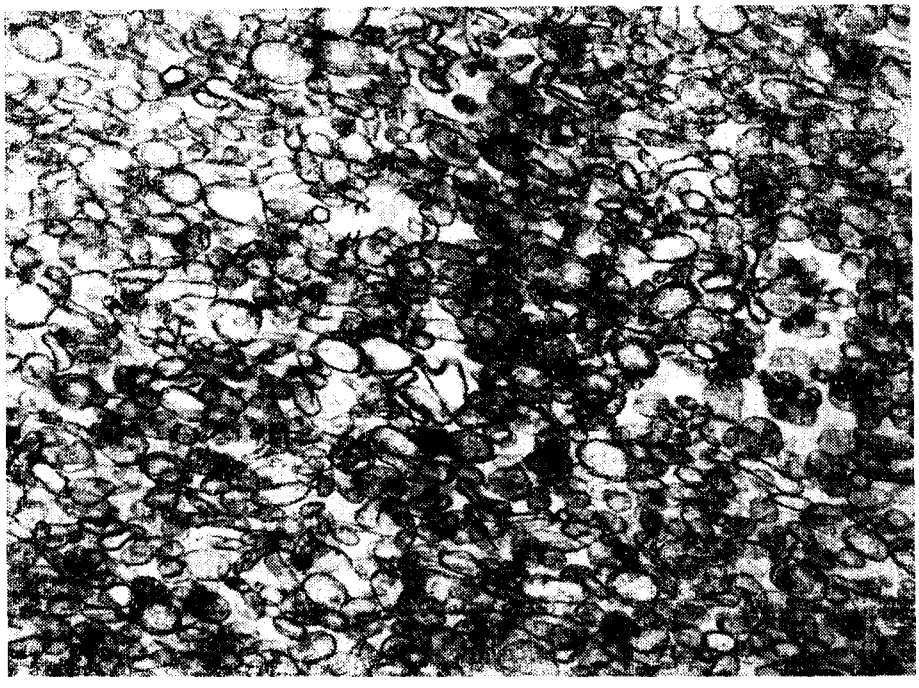

Fig. 1. Electron micrograph of the microsomal preparation of sarcoplasmic reticulum of rabbit skeletal muscle. The microsomal pellets obtained by centrifugation at 20,000× G were fixed first in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.5) and next in 1% OsO_4 in the same buffer. After dehydration, the preparation was embedded in Epon 812, stained with 2% uranyl acetate and lead citrate and observed in a Japan Electron Optics Laboratory Model T-6S electron microscope. × 60,000.

eously run to check the adsorption of the radioactivity to the Millipore filter. The uptake of ^{45}Ca was also measured concomitantly to confirm the vividity of the microsome. The results are shown in Table 1.

The results obtained, however, gave a negative impression as to the binding of the compound to the sarcoplasmic membranes. No statistically significant difference was noted in the radioactivities between test and blank measurements. The measurements were also done in various compositions of the incubation medium, e.g., with or without ATP and Ca. Washing the filter with 5 ml of "cold" tris buffer containing only inorganic ions removed almost all of the radioactivity deposited on the filter. It seemed to be difficult, therefore, to detect directly any binding of the caffeine molecules to the membrane.

2. Change in Microsomal Sulfhydryl Content by Caffeine

Ellman (1959) synthesized a water-soluble (at pH 8.0) aromatic disulfide (5, 5'-dithiobis (2-nitrobenzoic acid)) and showed it to be useful for determination of

sulfhydryl groups. This compound (DTNB) was employed in the present study for the determination of sulfhydryl content of the microsomal protein in the presence of 7.5 mM caffeine. A pair of photocells, one containing a specified incubation mixture but no caffeine (control mixture) and the other containing the same mixture plus 7.5 mM caffeine, were placed in a photometer and the optical densities were read after the addition of the coloring-agent, as described in "Experimental Materials and Methods."

Fig. 2 shows the change in the optical densities with and without caffeine in the presence of ATP. The net differences in the absorbancies between these two mixtures were small but obvious. The presence of ATP (2 mM) seemed to have no influence on the caffeine-induced change in the absorbancy, since practically the same patterns of the change could be observed whether the nucleotide was present or absent (Fig. 3). ATP itself, however, functions as a "protector" against the reaction of DTNB with the sulfhydryl groups of the protein. In Fig. 4 are shown the changes in the absorbancy of the control sample (with no caffeine) in

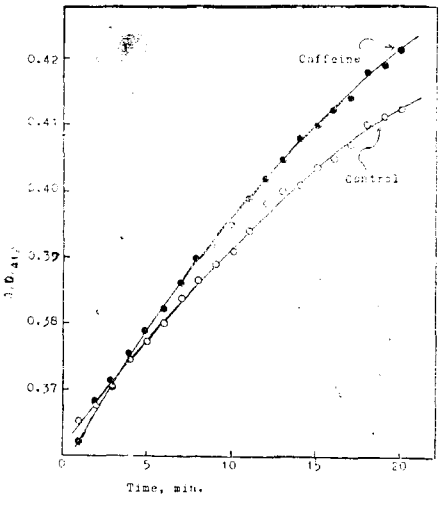

Fig. 2. The caffeine effect on the sulfhydryl content of the fragmented sarcoplasmic reticulum as measured by the DTNB method. The reaction mixture contained in 20 mM tris buffer (pH 7.2) 50 mM NaCl, 50 mM KCl, 4 mM $MgCl_2$, 0.02 mM $CaCl_2$, 2 mM ATP, 0.33 mg protein/ml, and 0.06 mM DTNB. Caffeine concentration was 7.5 mM. The reaction was measured at room temperature.

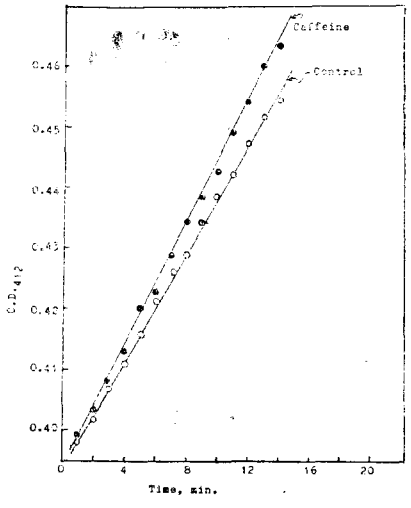

Fig. 3. The caffeine effect on the sulfhydryl content of the fragmented sarcoplasmic reticulum in the absence of ATP. All conditions were the same with those of Fig. 2, except the absence of ATP.

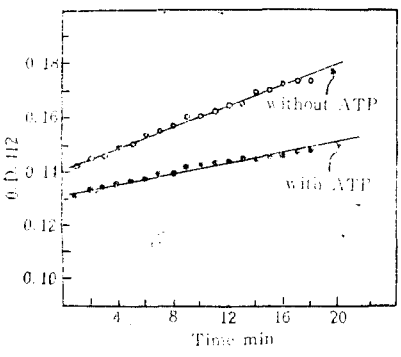

Fig. 4. The effect of ATP on the sulfhydryl content of the fragmented sarcoplasmic reticulum in the absence of caffeine. All conditions were the same with those of Fig. 2, except that the concentration of the microsomal protein was 0.1 mg/ml.

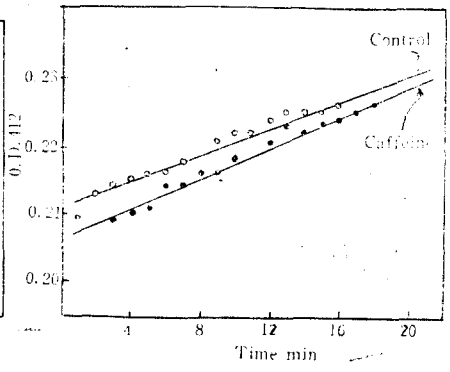

Fig. 5. The caffeine effect on the sulfhydryl content of the fragmented sarcoplasmic reticulum in the absence of Ca. All conditions were the same with those of Fig. 2, except that Ca was absent and the microsome concentration was 0.2mg protein/ml.

the presence and absence of ATP. When ATP was present, the color development was much slower than that in the absence of ATP.

The presence of Ca in the concentration of 0.02 mM either did not seem to interfere the reaction of caffeine with protein sulfhydryl groups (Fig. 5).

DISCUSSION

Electron micrographs of the present preparation of rabbit skeletal microsomes revealed that the microsomal fraction was not heavily contaminated with mitochondria as far as the morphological aspects are concerned (Fig. 1). This is consistent with the lack of detectable succinic dehydrogenase activity in this fraction (Ha, 1967). Furthermore, sodium azide which has been known to inhibit the Ca uptake and the ATPase activity in mitochondria but not the ATPase in microsomes (Carsten, 1969) did not inhibit the ATPase activity of this fraction (Ha, 1972). The activity of $(Na^+ + K^+)$ ATPase was also negligible in the fraction employed in the present experiment (Ha, 1972). Therefore, it is considered that the microsomal preparation of the present study is relatively pure, uncontaminated fragments of sarcoplasmic reticulum.

The inhibitory action of caffeine on the Ca uptake of the sarcoplasmic reticulum has been now well studied and seems to be due to a weak interaction of the drug with the membrane, probably with the protein moiety of the membrane (Ha, 1972).

The attempt to measure directly the binding of caffeine to the membrane component in the present experiment gave no obvious result as shown in Table 1. The concentration of caffeine required for its action on the Ca transport is relatively high. A 5 mM concentration of caffeine causes an inhibition of only 20-30% at most. Consequently, when the labeled caffeine is to be employed, the specific radioactivity expressed as microcuries per unit millimolar cannot be but very low, there must be a very small difference of the radioactivity between the blank and test samples even a significant amount of caffeine is bound, and the existing binding might be overlooked. This may be the case of the present measurement. The possibility of the binding, therefore, still exists and this was tested again by measuring the change in sulfhydryl content of the membrane protein.

When the content of sulfhydryl groups of the sarcoplasmic membrane fragments was measured in the presence of caffeine (7.5 mM) by the use of DTNB, it was observed that the content increased, although to a very small extent, with time (Figs. 2-5). Because the observed difference in absorbancy between the control and caffeine-treated sample was very small, and because the change is very slow whereas the Ca uptake of the sarcoplasmic reticulum fragments is a rather rapid process saturating usually within a minute or less, it cannot be decisively concluded that the caffeine alters the protein molecules that associated with the Ca uptake. The change in the content of sulfhydryl groups of protein by the addition of caffeine, however, would possibly be a reflection of a structural change of the protein molecule which resulted from an interaction between the two molecules. Such an interaction, if really exists, might cause the active Ca-sequestering sites less affinitive for Ca. This lowering of the affinity (or binding constant) would cause a decrease in the uptake of Ca and the enhancement of the Ca release and hence brings about an increase in the ionized Ca level in the sarcoplasm. The increased Ca influx and outflux would also be a consequence of change in permeability to Ca, as suggested by Bianchi (1961); i.e., membrane changes resulting in a greater mobility of Ca through the membrane. The result of the measurements reported by Fairhurst and Hasselbach (1970) of the caffeine effect on Ca efflux under conditions where no ATP was present and hence Ca influx could not occur suggested that an altered membrane permeability might be associated with the drug action, though the measurements were made on the heavy fraction (2,000-8,000 $\times$ G) of rabbit skeletal muscle homogenate.

The DTNB method can best be used at pH 8 or more alkaline side since the reagent is more water-soluble at this pH than at more acidic side. The present determination was carried out at pH 7.2, because the Ca uptake and the ATPase activity were very low at pH 8.0 (Ha, 1972). This may be the cause of the slow change in the coloring observed in the present experiment.

SUMMARY

Since caffeine inhibits the active uptake of Ca by the sarcoplasmic reticulum, the action of caffeine on the fragmented sarcoplasmic reticulum of rabbit skeletal muscle was studied.

Caffeine seemed not to bind tightly to the sarcoplasmic reticulum. The determination of sulfhydryl content of the fragmented sarcoplasmic reticulum, however, suggested that caffeine in some unknown manner influences the protein moiety and thereby increases the sulfhydryl content. The inhibition of Ca uptake by caffeine therefore might be considered as due to the result of this change in protein sulfhydryl content.

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