The Effects of Caffeine on the ATPase Activity and the Calcium Uptake of the Fragmented Sarcoplasmic Reticulum of Rabbit Skeletal Muscle*

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筋小胞體의 ATPase 活性과 칼슘吸收能에 미치는 Caffeine 의 영향

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要　約

토끼의 骨骼筋 小胞體의 ATPase 活性과 Ca 吸收能에 미치는 caffeine 의 영향을 조사하였다.

遠心分離로 分劃된 小胞體에서 2,000~8,000×G 分劃의 ATPase 活性은 caffeine 에 의하여 增大되지만 8,000×G 以上의 分劃에서는 아무 영향도 받지 않았다. Caffeine 에 의한 이 活性增大는 이 分劃에 混在하는 mitochondria 의 ATPase 活性이 增大된 結果라고 해석된다.

小胞體의 2,000~10,000×G 分劃과 10,000~20,000×G 分劃의 Ca吸收能은 反應液內 Ca 의 농도가 200 nmoles/mg protein 정도 이상일 때는 caffeine 에 의하여 현저히 阻害되지만, Ca 의 농도가 이 以下일 때는 2,000~10,000 分劃에서만 이 阻害現象을 볼 수 있다. 低濃度 Ca 에서의 이 阻害現象은 caffeine 에 의하여 mitochondria 의 Ca吸收도 阻害되기 때문에 나타나는 것으로 해석된다.

Caffeine 에 의한 筋收縮의 誘發 및 痙縮現象은 筋小胞體의 Ca 吸收가 이 物質에 의하여 阻害되고 또 蓄積된 Ca 이 放出되기 때문에 일어나는 것으로 해석된다.

INTRODUCTION

There are now considerable experimental supports for the hypothesis that the muscle relaxation involves the removal of Ca from the myofibrils (Weber, 1966). One of the most remarkable findings in the study of this muscle relaxation is probably the fact that fragments of sarcoplasmic reticulum(microsomes) are able, in the presence of ATP and Mg^{++}, to take up Ca against a large concent-

ration gradient (Ebashi, 1960; Ebashi and Lipmann, 1962; Hasselbach and Makinose, 1962). These fragments are also capable of hydrolyzing ATP in the presence of Mg (Ebashi and Lipmann, 1962).

The state of Ca accumulated by the sarcoplasmic reticulum is still unknown, though it is of fundamental importance to know whether this Ca is accumulated in the reticular vesicles by an active transport process or accumulated merely by binding at the active sites of the lipoprotein membrane. There have been appeared numerous indirect evidences which support either one of the two possible mechanisms (Makinose and Hasselbach, 1965; Ha, 1967; Ebashi and Endo, 1968; Carvalho and Leo, 1967; Carvalho, 1968; Martonosi, 1969; Chevallier and Butow, 1971; MacLennan and Wong, 1971).

In the meantime, there have been carried out intensive studies on the effects and the mechanism of various potentiators of muscle contraction, like caffeine. Axelsson and Thesleff(1958) showed that caffeine initiated a process in the muscle membrane leading to contraction and that it did not reduce the transverse membrane resistance significantly. Bianchi(1961) observed that caffeine(2.5-5.0 mM) increased resting Ca influx approximately threefold in normally polarized fibers of frog sartorius muscle. He also found that the efflux of Ca is also markedly increased by caffeine, and suggested that the increased Ca efflux brought about by caffeine would reflect an increased intracellular level of ionized Ca which in turn affects the contractile mechanism in accord with the current concepts regarding with 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 the basic effect of caffeine on muscle is to directly release Ca from the sarcoplasmic reticulum in proportion to the drug concentration.

On the other hand, Carsten and Mommaerts (1964) failed to show any significant effect of caffeine (5 mM) on the accumulation of Ca by sarcotubular vesicles of rabbit skeletal muscle prepared by centrifugation between 10,000-40,000 xG, and concluded that the pharmacological properties of caffeine were not explainable by its small influence on Ca accumulation. Similar results were reported later by Weber(1968). Carvalho(1967, 1968) described that the fragmented sarcoplasmic reticulum of rabbit skeletal muscle binds Ca^{++}, Mg^{++}, K^+, and H^+ to the same binding sites and that caffeine (10 mM) has no effect on the binding of Ca, but releases the labile fraction of Ca, which presumably is accumulated in excess of the bound Ca. He concluded that the effect of caffeine observed *in vivo* studies on the fluxes of Ca cannot explain in terms of their direct effects on the Ca bound by the sarcoplasmic reticulum. Similar results were later showed also by Bondani and Karler(1970).

Recently, Fuchs(1969) reported evidences that caffeine produces contracture through an inhibition of Ca transport of the rabbit sarcotubular membrane fraction (1,500-10,000 xG) and suggested that caffeine might affect both the uptake and release of Ca. More recently, Fairhurst and Hasselbach (1970) measured the Ca efflux in systems in which the sarcotubular vesicles were first

partially loaded with Ca-oxalate in the presence of a limiting supply of ATP, under which conditions the energy-dependent inward transport of Ca ceased after the rapid exhaustion of the ATP, and showed that caffeine stimulated the efflux from the heavy sarcotubular fraction(2,000-8,000xG), whereas the efflux from a light fraction(12,000-35,000 xG) was much less sensitive to this drug. From these observations, they concluded that the action of caffeine is the stimulation of efflux and not the inhibition of influx of Ca from the sarcoplasmic reticulum. Batra and Daniel(1971 a) also obtained similar results.

As described above, attempts to demonstrate a direct effect of caffeine on the sarcotubular Ca transport in order to link this effect to the well-known caffeine contracture have not met with uniform success. The mechanism of the interaction of caffeine with the sarcotubular membrane is also still entirely unknown. The present investigation was therefore undertaken to provide more details of the action of caffeine on Ca transport of the fragmented sarcoplasmic reticulum of rabbit skeletal muscle, and to clarify the mechanism of the action. The caffeine effects hitherto reported were those on the heavy sarcoplasmic fractions(below 10,000xG) which are obviously not the typical microsomal fraction. In the present investigation, the effect of the alkaloid on the lighter fractions of the fragmented sarcoplasmic reticulum (above 10,000xG) was studied under various conditions. General properties of the ATPase activity and Ca transport of the membrane fragments were also studied simultaneously as much as there are disagreements among investigators as to the state of Ca in the sarcoplasmic reticulum and the mechanism of its Ca uptake, as already described.

MATERIALS AND METHODS

Sarcoplasmic reticulum fragments were prepared from male adult rabbits as described previously (Ha, 1971). Fractions of the fragments sedimented at the centrifugal forces of 2,000-10,000, 10,000-20,000, and 20,000-34,000 xG were used for the experiment. Preparations not aged older than 72 hours were always used.

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

The inorganic phosphate (Pi) liberated from the hydrolysis of ATP was measured by Nakamura's method (Nakamura, 1950). The presence of high concentration of caffeine interferred the photometric readings by forming a precipitated complex with molybdenum phosphate. Charcoal filtration of the reaction mixture could remove the caffeine and eliminated the interference. The filtration, however, also removed a part of the liberated Pi from the mixture and hence underestimation of the Pi was inevitable. The formation of the precipitate, however, was negligible for the photometric quantitization of Pi when the concentration of Pi or caffeine was lower than 0.35 mM or 7.5 mM, respectively. The subsequent measurements were, therefore, always done under these conditions.

The ATPase activity and the Ca uptake were measured with essentially the same methods as described elsewhere (Ha, 1971).

The amount of accumulated Ca was calculated from the difference between the radioactivity of the filtrate and initial activity of the reaction mixture.

Radioactivity was assayed in a Packard Tri-Carb liquid scintillation spectrometer. The scintillator used was a mixture described by Bray(1960).

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 10mM 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.

The change in the sulfhydryl content of the membrane protein was measured 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 absorbance at 412 nm was read at one minute intervals for 20 minutes, the control reading preceding by 30 seconds the test sample.

All reagents used were of reagent grade and all solutions were prepared with deionized, glass-distilled water.

RESULTS

1. General Properties of the ATPase Activity

The homogenate of the muscle is composed of fragments of various sizes of the sarcoplasmic reticulum resulted from the mechanical disintegration of the muscle (Peachey, 1965).

In addition, a large amount of mitochondria, intact or fragmented, should also be contained. Most of these mitochondrial fragments may be fractionated off by successive centrifugations; however, a part of them may escape from the typical mitochondrial fraction and may be contained in lighter fractions. Weber et al.(1966) have reported that 8,000 to 36,000 xG fraction was not entirely free of mitochondrial fragments since they found a rate of respiration of 6 nmoles O_2/minute/mg protein. This contamination should be taken into consideration when the ATPase activity and Ca uptake of the microsomal fraction are to be measured since these two activities are also seen in the mitochondrion (Penefsky et al., 1960; Pullman et al., 1960; Wadkins, 1960).

In the present experiment, the muscle homogenate was fractionated into three fractions by centrifugation and each fraction was measured for the ATPase activity. Fig. 1 represents a typical result of the measurement. The specific activity of the enzyme was the highest in the light fraction (L) which sedimented between 20,000 and 34,000 xG, followed by the middle fraction (M: 10,000–20,000 xG). The heavy fraction (H: 2,000–10,000 xG) had the lowest enzymatic activity. The addition of sodium azide(0.05 mM) to the H and M fractions reduced the ATPase activity but a little(Fig. 2), revealing that there was practically no significant contribution of the mitochondrial part to the liberation of Pi from ATP in these fractions. Sodium azide has been reported to inhibit the mitochondrial ATPase activity to about 15% of its control when the concentration is greater than 0.05mM (Pullman et al., 1960). That the mitochondria contribute only insi-

gnificantly to the ATPase activity of the H fraction of skeletal muscle microsomes was also found by Fairhurst and Hasselbach (1970). Therefore, in the determination of the ATPase activity which followed, the contamination of mitochondria or their fragments were neglected.

Beginning with the Skou's observation (1957)of the presence of a monovalent cation-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) in fractionated crab nerve, the microsomal fraction from a variety of tissues, such as brain, kidney and erythrocytes, was found to have this enzyme,$(Na^+ + K^+)$-ATP-ase, activity (Skou, 1962; Post et al., 1960; Lee and Yu, 1963). There are contradictory reports as to the existence of this enzyme activity in the sarcoplasmic reticulum of striated muscle(Fratantoni and Askari, 1965; Rubin and Katz, 1967; Samaha and Gergely,
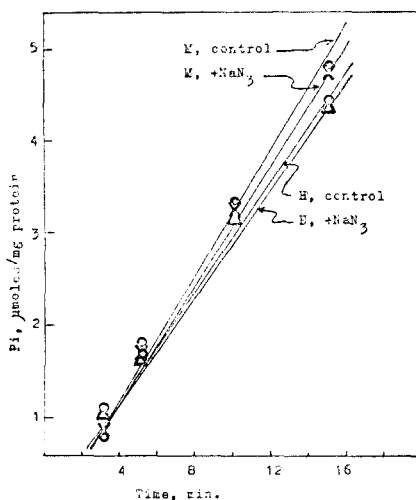

Fig. 2. The effect of NaN_3 on the ATPase activity of H and M fractions. The reaction mixture was the same with that of Fig. 1, except the addition of 0.05 mM NaN_3.

Table 1. ATPase activity measured in the presence of Ca and/or G-strophanthin.

Addition \\ Fraction	H	M	L
Control	160.3	177.0	
0.2 mM Ca	266.0	321.3	344.9
0.2 mM Ca+0.25 mM G-str.	284.4	328.0	353.3
0.2 mM Ca+2.5 mM G-str.	269.5	319.8	344.1
2.5 mM G-str.	162.2	170.8	

Values are expressed in nmoles Pi/mg protein/min. Control medium consisted of 50 mM NaCl, 50 mM KCl, 4 mM $MgCl_2$, 2 mM ATP, 20 mM tris buffer(pH 6.8), 0.1-0.2 mg protein/ml. Ca-free media contained 0.5 mM GEDTA to chelate the trace of the contaminated Ca. The measurements were done at 20° for 10 minutes.
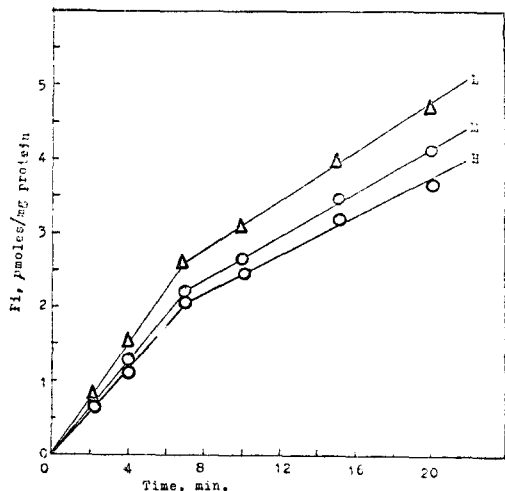

Fig.1. The APTase activity of the heavy(H), middle(M), and light(L) fractions. The reaction mixture contained in 20mM tris buffer(pH 6.8) 50 mM NaCl, 50mM KCl, 4mM $MgCl_2$, 0.2mM $CaCl_2$, 2mM ATP. The microsomal concentration was 0.2mg protein/ml. The reaction was carried out at 22°.

1966; Martonosi, 1968). In the present experiment, the ATPase activity was measured in the presence of G-strophanthin which is known to inhibit the $(Na^+ + K^+)$-ATPase (Charnock and Potter, 1969), and Table 1 shows the results. It is obvious from the table that our preparation has only two kinds of ATPase; one which is activated by Ca (extra

ATPase) and the other which does not require Ca (basic ATPase) for the activity. No $(Na^+ + K^+)$-ATPase was detected, though the conditions of the measurement were those under which a maximal activity of the enzyme would appear (Skou, 1957); the addition of G-strophanthin in the concentration of 0.25 or 2.5 mM did not cause any significant decrease or increase in the activity. ATPase activity revealed in the control medium (Table 1), therefore, could be considered as Mg^{++}-ATPase, and the increment caused by the addition of Ca would represent the $(Ca^{++}+Mg^{++})$-ATPase. There was no difference in the distribution of these ATPases between the H and M fractions. All the following measurements, except for the effect of temperature, were done on the total ATPase, not distinguishing the two types.

The ATPase activities of the three fractions were measured at various temperatures between 10° to 37°. Like most enzymatic reactions, the total ATPase activity increased as the temperature increased (Fig. 3). This
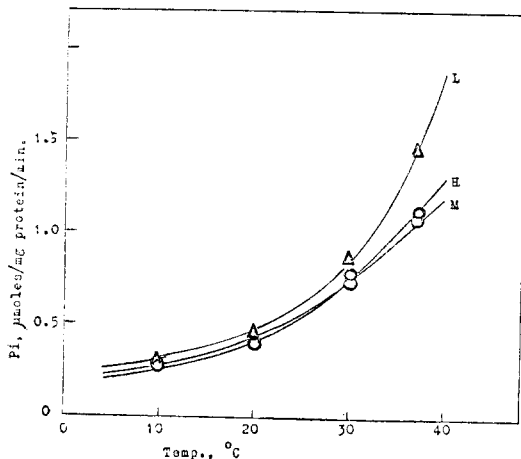
temperature-dependency was commonly seen in all of the three fractions with the same tendency. The mean data shown in Fig. 3 were used to construct Arrhenius plots for the calculation of apparent activation energy of the ATPase. These plots are given in Fig. 4, where it is seen that the apparent activation energy of the total ATPase is approximately 15 to 12 kcal/mole. The activation energies of the total, $(Ca^{++} + Mg^{++})$-, and Mg^{++}-ATPases of the H and M fractions were similarly calculated and are shown in Table 2.
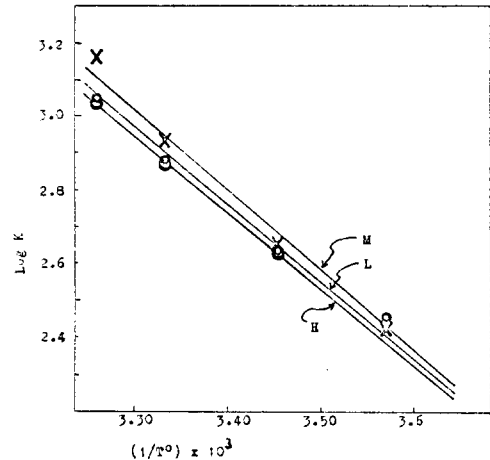

Fig. 4. Arrhenius plots of the effect of temperature on the reaction rate of the total ATPase of the H, M and L fractions. (Refer to Fig. 3.)

Table 2. Activation energies of the ATPases (kcal/mole).

Fraction	Total ATPase	$(Ca^{++}+Mg^{++})$-ATPase	Mg^{++}-ATPase
H	16.5	22.7	10.3
M	15.0	25.5	9.2

Fig. 3. The effect of temperature on the total ATPase activity of the H,M and L fractions. Reaction conditions were the same with those of Fig. 1.

2. The Effect of Caffeine on the ATPase Activity

In the present study, the effect of caffeine on the total ATPase of the three fractions

was investigated in detail under various conditions and the effect was compared with that on the Ca uptake of the microsomes. The II fraction was further divided into H_1 (2,000-8,000xG) and H_2(8,000-10,000xG) fractions. Of the four fractions, only the H_1 fraction was affected by caffeine. All other fractions did not respond to caffeine with regard to their ATPase activity (Fig. 5). The ATPase activity in the H_1 fraction was significantly increased by caffeine of the concentration higher than 5 mM.

Since the existence of some amount of mitochondria is expected in the H_1 fraction, the enhancement of the ATPase activity in this fraction by caffeine was examined to see whether the increment was caused by the mitochondrial ATPase or not. Fig. 6 shows the effect of caffeine on the ATPase activity of the H_1 fraction in the presence of sodium azide. It can be seen from the figure that the presence of azide does not decrease the ATPase activity either in the
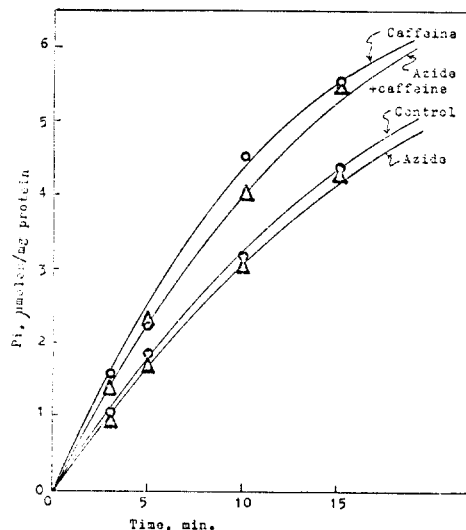

Fig. 6. The effect of sodium azide on the caffeine inhibition of the ATPase activity of the H_1 fraction. The reaction mixture was the same with that of Fig. 1, except the addition of caffeine(7.5 mM) and/or sodium azide(0.05 mM). The microsome concentration was 0.08 mg protein/ml. The reaction was carried out at 30°.
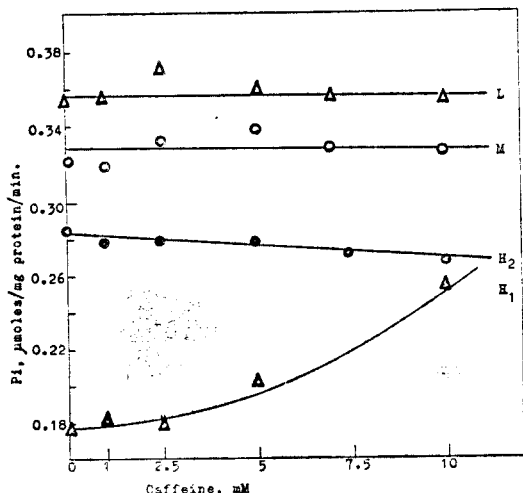

Fig. 5. Effect of caffeine concentration on the ATPase activity of the four fractions. The reaction mixture was the same with that of Fig. 1, except the addition of caffeine as indicated in the abscissa.

presence or absence of caffeine. It is, therefore, suggested that the ATPase activity contributed by the mitochondria is negligible in our preparation. Though the contribution of the mitochondrial ATPase was thus little, the presence of the organelle was not deniable as will be described later. There was no temperature-dependency or pH-dependency of the caffeine effect.

3. General Properties of the Calcium Uptake

The Ca uptake of the sarcoplasmic reticulum fragments was measured on the H and M fractions with $^{45}CaCl_2$. Our preparation usually contained an intrinsic Ca of an average of 35 nmoles/mg protein. This value was always taken into consideration in the subsequent calculation of the amount of Ca taken up by the microsomes.

The H fraction (H_1 and H_2) has essentially

the same capacity of Ca uptake with the M
fraction which can be considered to consist
mainly of microsomal vesicles. Although the
mitochondria which may be contained in the
H fraction contributed little to the ATPase
activity of that fraction (Fig. 6), its contribu-
tion to the overall Ca accumulation of the
fraction was not negligible as can be seen
from Fig. 7, where the effect of 50 μM
dicumarol on the Ca uptake was shown for
the H and M fractions. This compound
decreased the Ca uptake of the H fraction by
20-30%. Dicumarol (50 μM) was known to
inhibit selectively the Ca uptake of the
mitochondrion when the uptake is supported
by the oxidative phosphorylation (Weber,
1968). The effect of dicumarol was not sig-
nificant in the M and L fractions.

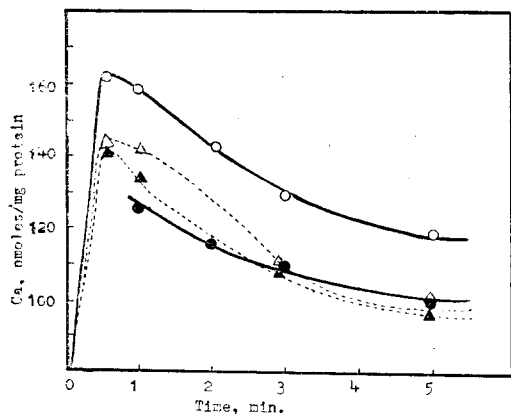The rate of Ca uptake was very rapid

Fig. 7. The Ca uptake of the H and M fractions
and the effect of dicumarol on the uptake.
The incubation medium contained in 20 mM
tris buffer(pH6.8) 50 mM NaCl, 50 mM KCl,
4mM $MgCl_2$, 2 mM ATP, 0.02 mM $^{45}CaCl_2$ and
0.1 mg protein/ml. The incubation was carried
out at 30°. Dicumarol was added in the conc-
entration of 0.05 mM. Open circles, control of
the H fraction; filled circles, dicumarol-treated
H fraction; open triangles, control of the M
fraction; and filled triangles, dicumarol-treated
M fraction.

and the maximum uptake was observed at
30 seconds in the H and M fractions (Fig. 7).
The accumulated Ca was released slowly
into the medium after 1 minute of the incu-
bation. Sometimes the release was not obser-
ved until 5 minutes after the start of the
incubation. The cause of this release is not
yet known; however, it is obvious that the
cause of the release is neither the age of the
microsome nor the ATP concentration, since
the release was frequently seen in prepara-
tions not older than 12 hours and was also
observed to occur in the medium of very
high concentration of ATP (5 mM).

Although the maximum amount of Ca in-
corporated into the microsomes (M fraction)
varied with one preparation to another,
usually values around 150 nmoles/mg protein
were obtained at 20° when the incubation
medium contained 20 μM $CaCl_2$, 2 mM ATP
and 0.05-0.1 mg protein/ml.

The temperature-dependency of the micro-
somal Ca uptake was measured for the H
and M fractions from 0° to 37°. The uptake
was very small at 0° and increased sharply
as the temperature increased up to 25°.
There was no significant change in the Ca
uptake in the temperature range between 20°
and 30°. At higher temperatures than 30°,
the activity dropped suddenly. The Q_{10} app-
roximated from the figure gave a value of
about 2 in the range between 0° and 25° and
the optimum temperature usually obtained
was around 25° for both fractions. The acti-
vation energy for the Ca uptake of the H
and M fractions was 5.5 kcal/mole for the H
fraction and 4.7 kcal/mole for the M fraction.

The Ca uptake of the M fraction was
measured at various pH at 25°. The pH of
the medium was adjusted with tris-maleate-

HCl buffer. The uptake dropped suddenly at either side of pH 6.0-6.8. The optimum pH was thus relatively broad ranging from 6.0 to 6.8. The optimal pH was all the same for the H, M and L fractions; it was also the same regardless of the temperature.

The effect of the ATP concentration on the Ca uptake of the M fraction was measured at 25° and the results are shown in Fig. 8. At low concentration of ATP, the Ca accumulation was small, but as the concentration of ATP increased up to 0.1 mM, the uptake of Ca increased greatly. Higher concentrations of ATP than 0.1 mM no longer exerted any significant increase in the uptake. The so-called "passive binding" of Ca was very small ranging from 5 to 10 nmoles/mg protein. This passive binding was observed to the same extent at various temperatures (0°-37°) and pH's (5.0−8.0) studied.
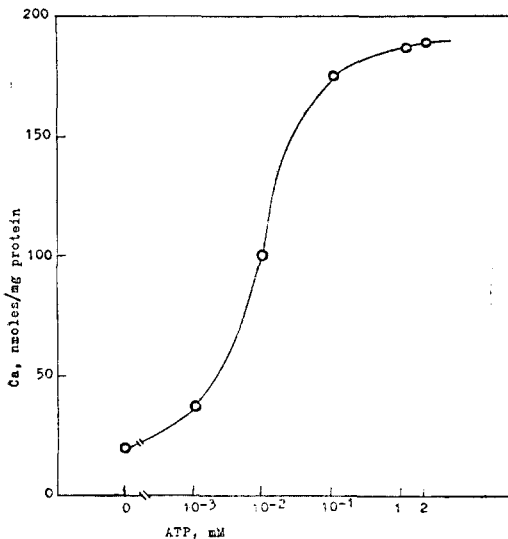
The effect of the medium Ca concentration

Fig. 8. The Ca uptake of the M fraction in various concentration of ATP. The incubation medium was the same with that of Fig. 7, except the concentration of ATP. The incubation was carried out at 25° for 1 minute.

on the Ca uptake of the M fraction was measured in two different manners with regard to the Ca concentration; one in which the total Ca concentration was varied from 1×10^{-6} to 2×10^{-4}M (total system), and the other (free system) in which the total Ca was kept constant to 2×10^{-4}M and the free Ca concentration was varied from 3×10^{-7} to 2×10^{-4}M by the addition of a calculated amount of glycoletherdiamine tetraacetic acid (GEDTA) assuming the binding constant as 3×10^5 M^{-1} at pH 6.8. In the total system, the concentration of Ca in the medium was a limiting factor of the Ca accumulation until the concentration reached about 2×10^{-4} M (1 μmole/mg protein). The amount of Ca taken up was almost directly proportional to that of Ca present in the medium (Fig. 9).

The concentration of free Ca also plays an important role in the uptake of Ca. Although the total Ca present in the free system(0.2 mM) is in excess of that required for the saturation, it is the free ionic form that is available for the microsome to take up. From Fig. 9, it is seen that the concentration of free Ca, not GEDTA-bound form, is the limiting factor of the accumulation. The Ca uptake system was insensitive to a Ca concentration of 3×10^{-7} M but took up when the concentration exceeded this value. Considering the reaction, Ca+GEDTA⇌GEDTA−Ca, it may be that, as the microsome takes up free Ca^{++}, more and more free ions would be liberated from GEDTA−Ca chelate and there must be always free Ca^{++} available for uptake. Nevertheless, at very low concentrations of the free ion, the uptake was significantly low. This is considered as due to the lack of enough free Ca^{++} that are required to activate the transport system of
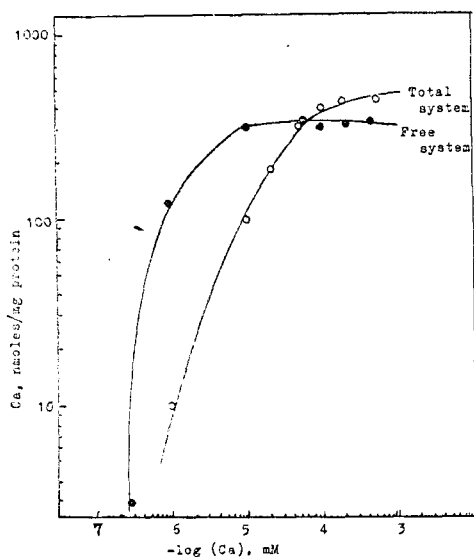
Fig. 9. The effect of total Ca and free Ca ion concentrations on the Ca uptake of the M fraction. Incubation conditions were the same with those of Fig. 7, except the Ca concentration. Incubation was done at 25° for 1 min.

In the total system, the abscissa indicates directly the concentration of the total Ca present in the medium; and in the free system, the Ca concentrations indicated in the abscissa are those of the free Ca concentration, the total Ca reservior being 0.2 mM.

the membrane. When, however, the free concentration is higher than 1×10^{-5} M, no further increase in the Ca uptake was seen. At and above 1×10^{-5} M, the system may be considered as to be fully activated and as the Ca incorporation proceeds, the Ca-buffer system supplies the free ions until the saturation is reached.

4. The Effect of Caffeine on the Calcium
   Uptake

The effects of caffeine (7.5 mM) on the Ca accumulation were measured on the H, M and L fractions and the results are shown in Fig. 10 for the H fraction and in Fig. 11 for the M fraction. The L fraction responded in much the same manner as the M

fraction and hence the result is not presented. As has been reported by Weber and Herz (1968) and by Fuchs(1969), the H fraction is the most sensitive to caffeine. The M fraction is relatively insensitive to the alkaloid when compared with the H fraction, but the drug effect on this fraction is nevertheless significant. The H fraction seemed to contain an abundant amount of mitochondria, since the addition of 50 μM dicumarol greatly inhibited the Ca uptake. The M fraction was affected little by dicumarol.

When the effect of caffeine on the M fraction was measured in a medium containing low concentration of the total Ca (10μM), no effect was observed at any concentration of the drug (1–10 mM). At this low concentration of Ca which corresponded to 50 nmoles/ mg protein, practically all of the Ca in the medium was incorporated into the microsomes and no Ca was remained in the solution even

Fig. 10. The effects of caffeine and dicumarol on the Ca uptake of the H fraction. All incubation conditions were the same with those of Fig. 7, except the addition of 7.5 mM caffeine and/or 0.05mM dicumarol. The measurement was done from 1 minute of the incubation and, hence, the initial rate of the uptake is not shown (refer to Fig. 7).
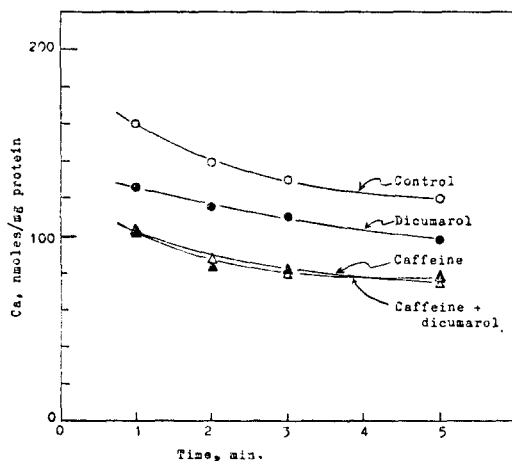
Fig. 11. The effects of caffeine and dicumarol on the Ca uptake of the M fraction. All incubation conditions were the same with those of Fig. 7, except the addition of 7.5 mM caffeine and/or 0.05 mM dicumarol. The measurement was done from 1 minute of the incubation and, hence, the initial rate of the uptake is not shown(refer to Fig. 7).
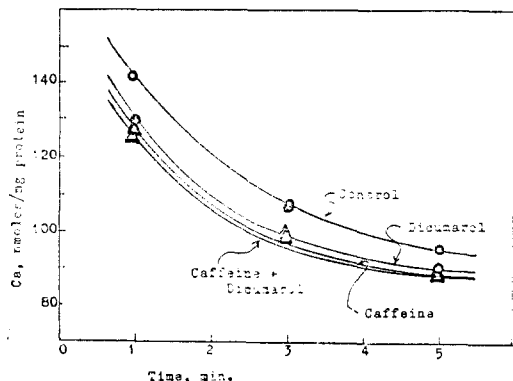
in the presence of 10 mM caffeine. The H fraction also took up almost all of the Ca from its medium in the absence of caffeine; however, caffeine did inhibit the uptake of Ca in this fraction. At Ca concentrations higher than 200 nmoles/mg, both fractions were affected by the drug, the H fraction being inhibited more remarkably than the M fraction. Dicumarol (50 μM) inhibited the Ca uptake of the H fraction by about 25% of the control, while caffeine (7.5 mM) inhibited it by about 45%. When dicumarol and caffeine were present together, the degree of overall inhibition did not exceed that of caffeine alone. These observations suggest that caffeine inhibits both mitochondrial and microsomal Ca uptake.

The inhibitory action of caffeine on the Ca uptake of the microsomal vesicles was measured at various caffeine concentrations for the H and M fractions at Ca concentration of 0.02 mM (200 nmoles/mg protein) at

which a significant inhibition by caffeine was observable in both fractions as described above. As presented in Fig 12, the Ca uptake of the H fraction was inhibited by about 20% when 1 mM caffeine was added. With 2.5 mM caffeine added, the inhibition was almost maximum and no further inhibition was observed when the caffeine concentration was increased up to 10 mM. On the other hand, the response of the M fraction increased gradually as the concentration of caffeine increased until 5 mM, above which no further significant inhibition was observable.

There are contradictory reports as to the most effective temperature for the caffeine action on the Ca transport of the microsome (Weber and Herz, 1968; Ogawa, 1970; Fuchs, 1969). Furthermore, all of the reports are concerned with heavier fractions which were the most caffeine-sensitive. In addition, most of the studies were conducted at temperatures (generally at 20°—25°) which are not physiological for the mammalian muscle. In the
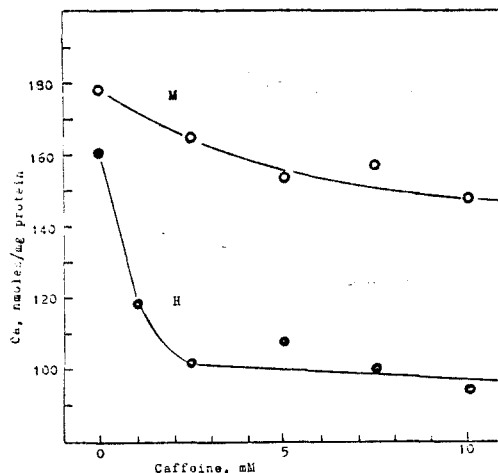

Fig. 12. The Ca uptake of the H and M fractions in various concentrations of caffeine. Incubation conditions were the same with those of Fig. 7, except the addition of caffeine. The uptake was measured at 30° for 1 minute.

present experiment, caffeine was tested for its inhibitory effect on the Ca transport at various temperatures. The rate of inhibition caused by caffeine was the highest(21%) at the physiological temperature of the experimental animal (37°). At 0°, caffeine still inhibited the Ca uptake by about 10% of the control. It seemed, however, to be safe to conclude that the inhibitory action of caffeine is not temperature-dependent; at all temperatures studied caffeine acted practically to the same degree.

As was described already, the Ca uptake has a rather wide range of optimum pH (6.0 -6.8). The same range was also observed in the caffeine-treated microsome for the H and M fractions. At all pH examined, the caffeine inhibited the Ca incorporation practically to the same extent, suggesting no pH-specificity for the action.

The concentration of ATP in the medium either did not change the pattern of the caffeine effect. At every concentrations of ATP studied, no particularity of caffeine action was observable in either fraction. At low concentration of ATP (0.001-0.1 mM) the effect of this drug was obscure presumably because of the low uptake of Ca (Fig. 8). Caffeine did not affect the passive binding.

The presence of oxalate (1 mM) in the assay medium which contained 0.1 mM Ca greatly increased the amount of Ca incorporated into the vesicle of the M fraction. The presence of this Ca-trapping agent seemed to prevent completely the inhibition of Ca uptake by caffeine. The amount and rate of Ca uptake was essentially the same whether caffeine was present or absent.

Although it seemed unlikely that caffeine competes in any sense with Ca for the bind-

ing site of sarcoplasmic vesicles, as a number of cations do, from the view point of the neutral charge of caffeine at physiological pH, its effect was tested at various total or free Ca concentrations that are shown in Fig. 9. Caffeine manifested no observable inhibition at lower concentrations of total Ca (1-10 μM), while it caused a significant inhibition at these concentration of free Ca. These results are consistent with those described in the effect of Ca concentration on the Ca uptake in the absence of caffeine.

5. The Interaction of Caffeine with the Microsome

The present investigation on the nature of caffeine action on the ATPase activity and Ca uptake of microsomes suggested a possibility of an interaction of the alkaloid
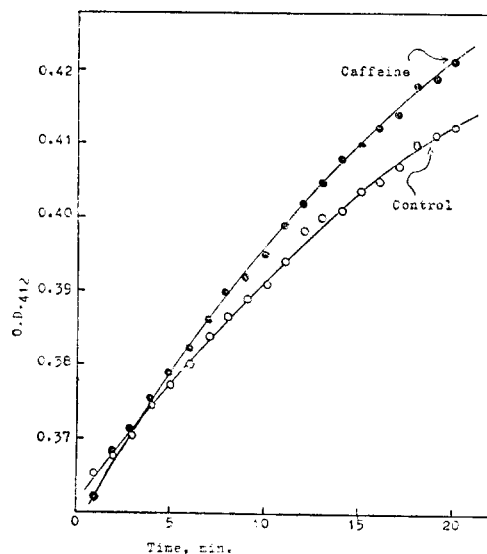

Fig. 13. The caffeine effect on the sulfhydryl content of the M fraction as measured by the DTNB method. The reaction mixture contained in 20 mM tris buffer (pH 7.2) 50mM NaCl, 50 mM KCl, 4 mM MgCl$_2$, 2 mM ATP, 0.33 mg protein/ml, and 0.06mM DTNB. Caffeine concentration was 7.5mM. The reaction was measured at room temperature.

with sarcoplasmic reticulum, probably with the protein moiety. In the present experiment, therefore, the change in the sulfhydryl content of the microsomal protein in the presence of caffeine was measured.

Fig. 13 shows the change in the optical densities with and without caffeine in the presence of ATP. The net difference 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 absorbance, since practically the same patterns of the change could be observed whether the nucleotide was present or absent. The presence of Ca in the concentration of 0.02 mM either did not seem to interfer the reaction of caffeine with protein sulfhydryl groups.

DISCUSSION

1. The ATPase Activity and the Caffeine Effect

The microsomal preparation used in the present investigation was fractionated into 4 fractions to discriminate the activity of ATPase and Ca uptake of the sarcoplasmic membrane from those activities contributed by the mitochondria. The H fraction (H_1 $+H_2$) seemed to contain a substantial amount of mitochondria as evidenced by the measurement of Ca uptake of the fraction in the medium containing dicumarol, though the ATPase activity of mitochondria in this fraction was negligible compared to that of sarcoplasmic membrane fragments. On the other hand, there was no significant contribution of these activities from mitochondria in the M and L fractions. The contamination of mitochondria and their fragments in the

H fraction, therefore, should always be taken into consideration when the ATPase activity and Ca uptake of the sarcoplasmic reticulum of the H fraction are to be measured.

Electron micrographs revealed that our microsomal fraction (M fraction) was not heavily contaminated with mitochondria as far as the morphological aspects are concerned. This is consistent with the lack of detectable succinic dehydrogenase activity in this fration (Ha, 1967). Sodium azide which has been known to inhibit the Ca uptake and the ATPase activity in mitochondria, but not the ATPase in microsomes (Fanburg and Gergely, 1965), did not inhibit the ATPase activity of the M fraction and inhibited only slightly that activity of the H fraction.

In our preparation, there was negligible ($Na^+ + K^+$)-ATPase in all fractions and only the basic and extra ATPases were present in an approximately equal activities. These results are essentially identical with those reported by Hasselbach and Makinose(1963) and by Scales and McIntosh(1968). On the other hand, Carvalho(1968) reported that a considerable ($Na^+ + K^+$)-ATPase was found in the rabbit microsomes. The monovalent cation-activated ATPase is generally known to be present in the plasma membrane, which has a relatively low activity of the other two enzymes, and has been known to be intimately linked to Na^+-K^+ exchange at the cellular membrane (Post et al., 1960; Dunham and Glynn, 1961). Since the microsomal fraction is mainly consisted of sarcoplasmic membranes whose function is, as far as presently known, the transport of Ca (Martonosi and Feretos, 1964; Weber et al., 1966), the function of the fraction is markedly different from that of the plasma mem-

brane. The difference in the distribution of the enzyme seems to reflect this functional aspect. The microsomal fraction, especially the H fraction, may of course contain fragments of plasma membrane; however, these fragments should occupy only a little portion of the fraction. The activity of $(Na^+ + K^+)$-ATPase manifested by these fragments therefore may not contribute significantly to the overall ATPase activity of the fraction.

The apparent activation energies calculated for the total ATPase was approximately 16 kcal/mole. The Mg^{++}-ATPase has an activation energy of 9-10 kcal/mole, which is somewhat higher than that reported by Shamoo et al.(1971) for the microsomal fraction isolated from mucosal epithelial cells of urinary bladders of a fresh-water turtle (3.7-11.6 kcal/mole, with an average of 8 kcal/mole). The value for $(Ca^{++} + Mg^{++})$-ATPase was found to be 22-26 kcal/mole. This is very close to that found in $(Na^+ + K^+)$-ATPase of rat brain microsomes (23 kcal/mole, Hexum et al., 1970), but is more than twice that of $(Na^+ + K^+)$-ATPase obtained from rabbit renal cortex membranes (10.2 kcal/mole, Charnock et al., 1971). The activation energies of the two kinds of enzymes are practically the same for both H and M fractions. This is considered also to mean that both fractions are equipped with the enzymes equally. This view is further supported from the results that the pH-dependency is the same for the H, M and L fractions.

The microsomal ATPase activity was practically not affected by caffeine, as was previously observed by the author (Ha, 1971). The ATPase activity of the H_1 fraction, however, was significantly enhanced by caffeine while other lighter ones responded

little to this compound. As much as the H fraction of our preparation had only a little contribution of ATPase from mitochondria, as revealed from the results obtained in the presence of azide (Fig.2), the increment might be considered to be solely due to the action of caffeine on the sarcotubular fragments in the H_1 fraction. However, if the sarcoplasmic membrane fragments are equally equipped with the enzyme as already described, the caffeine effect should be observed in all fractions equally, which was not the case of the present experiment. Therefore, the possibility that the enhancement is due to the stimulation of the mitochondrial ATPase by the drug cannot be excluded. It may be that the contribution of the mitochondrial ATPase is negligibly small and inhibition by azide on this small amount of the ATPase activity therefore could not be detected. On the other hand, when the mitochondrial ATPase activity is stimulated by caffeine, the increased activity may be apparent now so as to be detectable as the increment of the overall activity. This view will be discussed later in connection with the effect of caffeine on the Ca uptake.

2. Calcium Uptake and the Caffeine Effect

While the ATPase activity of mitochondria in the H fraction was negligibly low, the Ca uptake by the mitochondria was significant, suggesting the presence of substantial amount of mitochondria, as revealed by the measurement in which the overall Ca uptake of the H fraction was determined in the presence of dicumarol (Fig. 10). If we assume that the mitochondrial Ca uptake in the H fraction is completely inhibited by dicumarol, the remaining Ca uptake activity which was 70-80% of that measured in the absence of

dicumarol would be the activity revealed by the fragmented sarcoplasmic reticulum.

The Ca concentration in the surrounding medium is an important controlling factor of the uptake of Ca. The uptake increases lineally as the total Ca increases up to about 0.1 mM. This linearity has also been reported by Martonosi and Feretos (1964). With regard to this relationship, a question arises whether the increase of Ca uptake by increasing Ca in the medium is wholly an ATP-dependent uptake, or if it is in part due to a passive binding of Ca when Ca concentrations increased (Batra and Daniel. 1971 b). However, it seems unlikely that the increase of Ca uptake by high Ca concentration is due to the greater passive binding of Ca, since the passive binding was almost the same at different Ca concentrations.

The concentration of free Ca^{++}, not the total Ca, also influences the Ca uptake of the microsomes. Negligible amount of Ca uptake occurred when the free Ca^{++} concentration was made as low as 3×10^{-7}M by the addition of GEDTA. When the concentration was raised above 3×10^{-7}M, there was an uptake of sharp increase with the increasing concentration of Ca^{++}. Batra and Daniel (1971 b) have obtained the same relationship between Ca uptake and free Ca^{++} concentration for rat uterine smooth muscle. The Ca uptake system seems not to function at the free Ca^{++} concentration below 3×10^{-7}M in the medium. This concentration of ionized Ca is near the threshold of Ca for contractions in glycerinated muscle (Weber et al.. 1964).

It has been reported that the caffeine effect was very little or negligible on the M fraction while it was significant on the H fraction of the skeletal muscle homogenate.

(See "Introduction.") The ineffectiveness of caffeine on the M fraction was confirmed in the present study only when the effect was measured in a medium of low Ca concentration. When the amount of Ca present in the medium was 50-100 nmoles/mg protein, the caffeine effect was not observable in the M fraction. When, however, the Ca concentration was raised to above 200 nmoles/mg protein, an average of usually 20% of inhibition of the Ca uptake could be measured in the presence of caffeine. Weber and Herz (1968) determined the caffeine action on fractions of 650-2,000xG, 2,000-8,000xG, and 8,000-20,000 xG, and reported that the effect was significant only in the two heavier fractions. They measured the effect in the Ca concentration of 100-250 nmoles/mg protein. At this range of Ca concentration, we either could not observe any effect of the drug in the M fraction. However, the inhibition in the M fraction was apparent in the present experiment at the Ca concentrations of above 200 nmoles/mg protein. In the low Ca concentration, where the caffeine effect was not apparent, the microsomes took up nearly all the Ca present in the medium and caffeine seemed not to be able to inhibit this uptake. From our results, it may be concluded that caffeine only influences when the microsomes are partly saturated; its inhibition does not tide over the strong affinity of the microsomes for Ca when the microsomes are extremely depleted of Ca. In fact, Herz and Weber (1965) found that if the sarcoplasmic reticulum (650−2,000xG) contains only a half maximal amount of Ca or less, very little or no Ca is released by caffeine. The Ca uptake of the H fraction was inhibited by caffeine even in the low con-

centration of Ca where the M fraction did not respond to the drug. At this low Ca concentration the H fraction, like the M, sequestered almost all of the Ca available in the medium, practically no Ca remaining in the bulk phase. This higher sensitivity of the H fraction is also revealed from the measurement in which the inhibition of caffeine was determined as a function of caffeine concentration (Fig. 12).

As much as the caffeine effect was apparent in the M fraction when the effect was measured in a relatively high concentration of Ca, the author considers that the drug effect observed in the H fraction at the low Ca concentration might be due to the effect of the drug on mitochondria present in that fraction. In this low concentration of Ca, the sarcoplasmic fragments in the H fraction like those of the M fraction overcome the inhibition by caffeine and take up Ca, while the contaminated mitochondria in this fraction are inhibited by the drug, reducing consequently the overall Ca uptake. That caffeine might affect the mitochondrial Ca uptake is evidenced by the finding that nearly the same extent of inhibition was observed even when dicumarol was present along with caffeine. Caffeine decreased the Ca uptake of the H fraction and the extent of the decrease was practically the same whether or not dicumarol was present in combination with caffeine. Dicumarol also reduced the Ca uptake but to a lesser extent than caffeine did. The sensitivity of mitochondria to caffeine is also suggested by the finding of Nayler(1967), who reported that mitochondrial and microsomal fractions that was isolated from cardiac muscle which had been immersed in caffeine-enriched physio-

logic saline solution contained less Ca than did the mitochondrial and microsomal fractions isolated from control preparations.

The ATPase of the H_1 fraction of our preparation was increased by caffeine, while that of other fractions was insensitive. Two possible explanations were proposed for this increment as already discussed; one attributing the effect to the mitochondrial ATPase and the other considering the microsomal ATPase as the site of the drug action. It seems now more probable to consider that the increase in the ATPase activity observed was due to the enhancement of the mitochondrial ATPase by caffeine.

Since caffeine induced more decrease in the Ca uptake than dicumarol did, and since the coexistence of caffeine and dicumarol had essentially the same effect as the presence of caffeine only, our conclusion is that caffeine inhibits the Ca uptake of the sarcoplasmic reticulum of the M fraction as well as that of the H fraction and that the alkaloid also reduces the Ca uptake of mitochondria as well to the nearly same extent with that of dicumarol.

3. Caffeine Interaction with the Membrane

The inhibitory action of caffeine on the Ca uptake of the sarcoplasmic reticulum seems to be due to a weak interaction of the drug with the membrane, probably with the protein moiety of the membrane, because 1) the drug effect appears only when the drug concentration is rather high (1-10 mM), 2) it only causes about 50% of the uptake inhibition at most, 3) the effect does not come out at low Ca concentration where the Ca capacity of the microsome is hardly saturated, and finally 4) the effect disappears when oxalate is present in the medium

causing continued inward-flux of Ca.

When the content of sulfhydryl groups of the sarcoplasmic membrane fragments was measured in the presence of caffeine (7.5 mM), it was observed that the content increased, although to a very small extent, with time (Fig. 13). The change in the content of sulfhydryl groups of protein by the addition of caffeine would possibly be a reflection of a structural change of the protein molecule. Such an interaction 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, which would be reflected by the Ca outflux observed by Bianchi(1961) in the absence of extracellular Ca and the increased outflux and influx in the presence of Ca. The increased Ca influx and outflux would also be a consequence of change in permeability to Ca, as suggested by Bianchi(1961). 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×G) of rabbit skeletal muscle homogenate. The slight change in membrane resistance produced by caffeine and lack of membrane depolarization as noted by Axelsson and Thesleff(1958), however, suggest that caffeine does not cause an increase in membrane permeability.

SUMMARY

The effects of caffeine on the ATPase activity and Ca uptake of the fragmented sarcoplasmic reticulum isolated from rabbit skeletal muscle were studied.

The ATPase activity of the heavy fraction (2,000-8,000xG) was stimulated by caffeine while that of other lighter fractions was not. It is suggested that the enhancement of the ATPase activity in the heavy fraction is due to the increment in the mitochondrial ATPase by the caffeine treatment.

The Ca uptake of the heavy and middle (10,000-20,000xG) fractions was inhibited by caffeine when measured at the medium Ca concentration higher than 200 nmoles/mg protein, while only that of the heavy fraction was inhibited when measured at the Ca concentration below 200 nmoles/mg protein. Experiments with dicumarol suggested that caffeine inhibits the Ca uptake of the mitochondria as well as that of the sarcoplasmic reticulum and that the inhibition of the Ca uptake by caffeine in the low Ca concentration in the heavy fraction is due to the inhibition of the mitochondrial Ca uptake by caffeine.

It appeared highly probable that the potentiation of muscle contraction caused by caffeine is solely due to the inhibition of the Ca uptake by and to the release of the accumulated Ca from the sarcoplasmic reticulum.

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