BLOCKERS OF THE UNCOUPLING ACTION OF CAFFEINE ON Ca^{++} TRANSPORT FUNCTION IN SARCOPLASMIC RETICULUM MEMBRANES

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Release of Ca^{++} ions from cisterns of the sarcoplasmic reticulum (SPR) is known to be the cause of caffeine contracture and of the potentiating action of caffeine on a single contraction of skeletal muscle. Addition of caffeine to isolated SPR vesicles filled with Ca^{++} ions as a result of active transport leads to rapid release of 20-30% of the stored Ca^{++} into the surrounding medium [6, 10]. Caffeine not only releases Ca^{++}, but it also acts on active transport of Ca^{++} ions by vesicles of the reticulum. Caffeine has been shown to inhibit uptake of Ca^{++} ions without any inhibitory effect under these circumstances on Ca-dependent ATPase activity [2]. This effect of caffeine may be linked with uncoupling of transport and hydrolysis in energy metabolism and may be due to direct binding of caffeine with Ca-dependent ATPase [1, 10]. It may be accepted that both effects of caffeine are manifestations of its action on the same center. However, a different opinion also is held: Ca^{++} release under the influence of caffeine is connected, not with ATPase, but with some unidentified channel-forming protein [8].

To shed light on this problem an investigation was carried out using an approach based on the use of known blockers of caffeine-induced Ca^{++} release: ruthenium red and local anesthetics [9]. If the uncoupling effect of caffeine and Ca^{++} release through its action are manifestations of caffeine-induced structural changes in Ca-dependent ATPase, blockers of caffeine-induced Ca^{++} release ought also to block the uncoupling effect of caffeine.

EXPERIMENTAL METHOD

The caffeine-sensitive fraction of SPR membranes was isolated from white muscles of rabbit hind limbs. The tissue was homogenized as described previously [4]. To increase the yield of membranes 10 mM caffeine was added to the homogenization medium. The total membrane fraction sedimented by centrifugation from 10,000 to 36,000g was extracted in the cold in medium containing 0.6 M KCl, 0.1 mM EDTA, 0.2 mM $CaCl_2$, human serum albumin (0.6 mg/ml), and 5 mM histidine, pH 7.2. The membrane suspension was centrifuged at 11,000g (20 min) to sediment the caffeine-sensitive fraction, and then again at 40,000g (60 min) to sediment caffeine-insensitive membranes (light fraction). The residue thus obtained were suspended in medium containing 25% glycerol (vol. %), 0.1 mM EDTA, 0.2 mM $CaCl_2$, and 10 mM histidine, pH 7.2 (at 4°C). For further purification the fractions were layered in a centrifuge tube above 4 ml of the same medium and centrifuged at 36,000g (60 min). The caffeine-sensitive fraction was obtained as the residue and the light fraction as a thick suspension in the bottom part of the tube. ATPase activity and the average efficiency of Ca^{++} transport by SPR membranes were determined by pH-metry [2]. The incubation medium contained 100 mM NaCl, 4 mM $MgCl_2$, 5-20 mM sodium oxalate, 25 μM $CaCl_2$, 2 mM ATP, 20-30 μg/ml SPR protein, and 2.5 mM imidazole, pH 7.05 (at 37°C). The protein concentration was measured by the biuret reaction. Electrophoresis was carried out by the method in [6].

EXPERIMENTAL RESULTS

An important distinguishing feature in the action of caffeine on the Ca-transport function of SPR membranes is dependence of the effect of caffeine on the sedimentation characteristics of the SPR vesicles. Caffeine has virtually no effect on Ca^{++} transport by the light fraction, but strongly inhibits the transport function of the heavy fraction of SPR mem-

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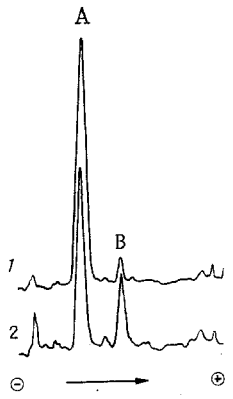


Fig. 1

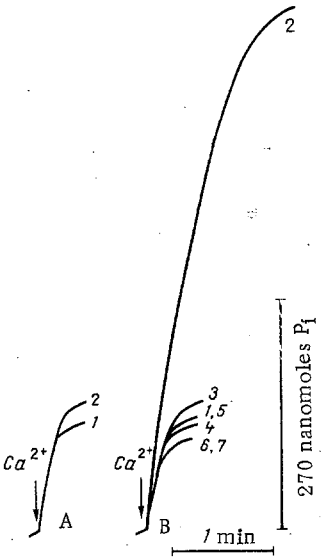


Fig. 2

Fig. 1. Densitograms of gels after electrophoretic separation of light (1) and caffeine-sensitive (2) fractions of SPR membranes. A) Ca-ATPase; C) calsequestrin.

Fig. 2. Effect of caffeine on ATP hydrolysis during active transport of Ca^{++} by light (A) and caffeine-sensitive (B) fractions of SPR membranes. 1) Control, 2) caffeine (5 mM); 3) caffeine (5 mM) + tetracaine (amethocaine) (0.2 mM); 4) caffeine (5 mM) + ruthenium red (1.5 μM); 5) caffeine (5 mM) + procaine (10 mM); 6) control + tetracaine (0.2 mM); 7) control + ruthenium red (1.5 μM). P_i) Inorganic phosphorus.

branes. Previously the writer described a method of obtaining a purified fraction of SPR membranes highly sensitive to caffeine, but the relatively low yield and insufficient stability of this fraction during keeping necessitated further research in this direction. It was found that the yield of the caffeine-sensitive fraction can be increased three- to fourfold (to 0.5-0.6 mg protein/g tissue) if homogenization is carried out in medium containing 10 mM caffeine. The yield of the light fraction was thereby increased by 1.5-2 times (to 1 mg protein/g tissue). The stability of the caffeine-sensitive fraction increased if medium containing 25% of glycerol was used to purify and preserve it. Gel-electrophoresis showed that the caffeine-sensitive fraction differs from the light fraction of SPR membranes in its lower content of Ca-dependent ATPase (55 ± 5 and 80 ± 5% respectively), and the presence of a high content of protein corresponding in electrophoretic mobility to calsequestrin (Fig. 1). The high content of calsequestrin in the caffeine-sensitive fraction, together with the writer's recent electron-microscopic observations [3], show that the caffeine-sensitive fraction is in fact a fraction of fragments of terminal cisterns of SPR, from which Ca^{++} ions are released in response to excitation. The results of determination of the content of Ca-dependent ATPase in the caffeine-sensitive fraction and light fraction with the aid of alamethicin [5] agree with the data of electrophoresis (Table 1). The caffeine-sensitive fraction did not differ from the light fraction in efficiency of Ca^{++} transport (Ca/ATP ratio). A pH-metric trace of ATP hydrolysis during active transport of Ca^{++} by different fractions of SPR membranes is illustrated in Fig. 2. It showed that addition of Ca^{++} to the incubation medium containing Mg-ATP, oxalate, and SPR membranes, led to a sharp rise in the rate of acidification of the medium. After a short time the rate of acidification fell to its initial level, indicating that the vesicles of the reticulum had taken up all the added calcium. The rate of acidification of the medium in the absence of Ca^{++} is determined mainly by hydrolysis of ATP under the influence of nonspecific ATPase. Efficiency of the transport process (the mean value of Ca/ATP) can be calculated from the increase in H^+ concentration and, correspondingly, of inorganic phosphate during Ca^{++} transport. The more phosphate that accumulates during transport of added Ca^{++}, the lower the values of Ca/ATP. It will also be clear from Fig. 2 that addition of 5 mM caffeine to the incubation mixture containing the caffeine-sensitive fraction of the

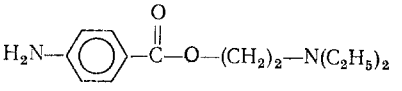
TABLE 1. Comparison of Ca-ATPase Activity in Caffeine-Sensitive and Light Fractions of SPR (M ± m)

SPR fraction	ATPase, %	ATPase activity	Ca/ATP
Caffeine-sensitive	46	13,7±1,2	1,3±0,1
Light	67	20,5±0,5	1,3±0,1

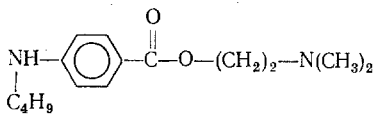
Legend. Calculation of ATPase in percent was based on specific activity of purified Ca-ATPase (30 μmoles P_i/min/mg protein). ATPase activity was measured in the presence of alamethicin.

SPR membranes leads to a sharp decrease in the efficiency of Ca^{++} transport. Caffeine had virtually no action, even in a concentration of 10 mM, on Ca^{++} transport by vesicles of the light fraction. Addition of ruthenium red in a concentration of 3 μM to the incubation mixture completely abolished the effect of caffeine. Dependence of the effect of caffeine on the ruthenium red concentration in the incubation medium in the presence of different concentrations of caffeine is illustrated in Fig. 3. Irrespective of the caffeine concentration, its effect was completely blocked by ruthenium red in a concentration of 3 μM. The action of ruthenium red is reversible.

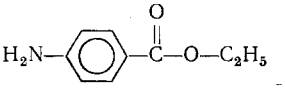
It was shown previously that Ca^{++} release from vesicles of SPR under the influence of caffeine is blocked by certain local anesthetics and, in particular, by procaine and tetracaine (amethocaine) [9, 10].



Procaine (pK_a – 8.95)



Tetracaine (pK_a – 8.24)



Benzocaine (pK_a – 3.19).

It will be clear from Fig. 2 that procaine and tetracaine also blocked the uncoupling effect of caffeine. Tetracaine blocked this effect by 50% in a concentration of 2×10^4 M (I_{50}), against the background of 5 mM caffeine (Fig. 3). The value of I_{50} for procaine was about 10 times greater. Considering the ability of the ruthenium red cation to block the effect of caffeine, it might be supposed that the blocking action of procaine and tetracaine was due to the presence of a positive charge on the dialkylethanolamine fragment. However, this is not so. We found that uncharged ethyl p-aminobenzoate (benzocaine) also abolished the effect of caffeine. It must be pointed out that tetracaine and ruthenium red cannot only block the effect of caffeine, but they can also increase the efficiency of Ca^{++} transport by the caffeine-sensitive fraction of SPR a little in the absence of caffeine (Fig. 3). Tetracaine and ruthenium red do not affect the efficiency of Ca^{++} transport by the light fraction. This suggests that the caffeine-sensitive fraction contains a certain amount of an endogenous factor acting like caffeine.

The results thus show clearly that Ca^{++} release from SPR vesicles under the influence of caffeine and inhibition of the transport function of ATPase by caffeine are closely interconnected: Both effects are blocked by ruthenium red and by local anesthetics. The uncoupling effect of caffeine on the transport function is the result of its action on a special form of Ca-dependent ATPase located in the terminal cisterns of SPR. Considering also that tetracaine blocks the potentiating effect of caffeine on whole muscle, it is logical to suggest that Ca^{++} release from cisterns of SPR under the influence of caffeine and the potentiating

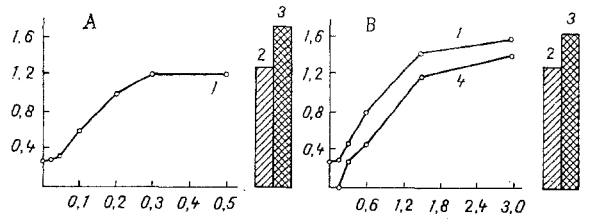


Fig. 3. Blocking uncoupling effect of caffeine by tetracaine (A) and ruthenium red (B). Abscissa, concentration: A) mM, B) μM; ordinate, value of Ca/ATP. 1) Caffeine (5 mM); 2) control; 3A) control + tetracaine (0.2 mM); 3B) control + ruthenium red (1.5 μM); 4) caffeine (10 mM).

effect of caffeine also are connected with the action of caffeine on Ca-dependent ATPase of the terminal cisterns.

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