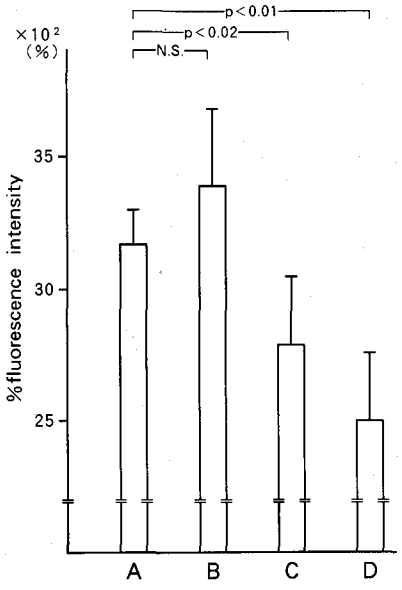

Figure 4. Fluorescence intensity of lysozyme incubated during 14 days after 2-h preincubation with or without α-ketoaldehyde dehydrogenase. Fluorescence intensity is expressed as percent increase of fluorescence compared to that of the corresponding sample stored at −40 °C after the same preincubation. A, B, C and D represent groups A, B, C and D as described in 'Materials and methods'. The data are the means ± SD of 4 experiments.

other AGEs in macrophages, and that there is a mechanism for the elimination of AGEs by macrophages. Brownlee et al.[18] have suggested that the aminoguanidine might inhibit the production of AGEs. We have assumed that α-ketoaldehyde dehydrogenase plays a role in the regulation of the accumulation of products of the Maillard reaction in the body. α-Ketoaldehyde dehydrogenase has been shown to act on carbonyl compounds, such as methylglyoxal and 3-DG. 3-DG is produced as an intermediate in the Maillard reaction and may act as a potent cross-linking agent[9, 10].

The results of the present study suggested that α-ketoaldehyde dehydrogenase acted on 3-DG and inhibited a 3-DG-stimulated increase in the fluorescence of lysozyme. It is thus possible that α-ketoaldehyde dehydrogenase might play a role in controlling pathogenesis attributed to aging, such as arteriosclerosis, both by preventing the progression of the Maillard reaction, and by inhibiting the formation of cross-links; both these reactions involve 3-DG.

Abbreviations: 3-deoxyglucosone: 3-DG; sodium dodecyl sulfate: SDS; diethyl-aminoethanol: DEAE; advanced glycosylation end-product: AGE; para-chloromercuribenzoate: *p*-CMB; 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole: FFI.

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Solubilization and characterization of a ouabain-sensitive protein from transverse tubule membrane-junctional sarcoplasmic reticulum complexes (TTM-JSR) in cat cardiac muscle

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Summary. A new glycoprotein of 31,500 dalton, which has a high affinity for ouabain, and is independent of (Na^+-K^+)-ATPase, was solubilized from transverse tubule membrane and junctional sarcoplasmic reticulum complexes (TTM-JSR) of cat cardiac muscle. This protein could be extracted only in small amounts from sarcolemma-plasma membrane (SLM-PL) fragments, suggesting that it indeed originates from the TTM-JSR.
Key words. Ouabain-sensitive protein; solubilization; characterization; TTM-JSR; cat heart.

There have been many studies[1-16] of the positive inotropic effect (PIE) of cardiac glycosides on cardiac muscles. A plurality of ouabain-binding sites is well known, and we wish to describe the isolation and behavior of regulatory proteins from the transverse tubular membrane-junctional sarcoplasmic reticulum system (TTM-JSR), which could be regarded as a regulator of mechanical contraction or force-generation of muscle cells[17,18]. In the present report, we describe a protein which has been isolated from cat cardiac muscle and has a high binding-affinity for ouabain.

Materials and methods. 1) Isolation of TTM-JSR and sarcolemma-plasma membrane complexes (SLM-PL): Cardiac ventricle muscle was removed from anesthetized cats. TTM-JSR and SLM-PL were isolated using a slight modification of the previous method[4,19] to improve the purity of TTM-JSR. 2) Photoaffinity labeling by ^{3}H-ouabain: 200 µg of TTM-JSR or SLM-PL (measured as protein) were incubated at 20 °C for 30 min in 1 ml of Tris maleate buffer (20 mM, pH 6.8) containing 120 mM NaCl, 2 mM $MgCl_2$, 0.4 mM phenylmethylsulfonylfluoride and 0.5 µM of radioactive ouabain. The mixture with ^{3}H-ouabain was maintained at 0 °C (ice-water) and photolyzed with a wave length of 342 nm (1000 W) for 30 min. 3) Elution: The band with a protein of 31,500 dalton was cut out of the gel and crushed in elution buffers (pH: 5.3, 7.4, and 11.0), of which constituents were (in mM, except for SDS): 0.1% SDS, 0.1 EDTA, 5 dithiothreitol, 150 NaCl, and various substances for buffering (in acidic, 100 citric acid, 200 Na_2HPO_4; in physiological, 50 Tris-HCl; in alkaline, 100 NaOH and 50 Na_2HPO_4). After 4 h, the suspension was centrifuged briefly at 15,000 × g. The supernatant was then mixed with four volumes of cold aceton (− 20 °C, and centrifuged for 10 min at 10,000 rpm. The precipitate was used. 4) Protein determination of bands in SDS-PAGE: The gel stained with Coomassie brilliant blue (CBB) was scanned with a densitometer (Densitorol, DMU-33C, TOYO, Tokyo). The stained bands were compared with a standard, obtained by carrying out the same analysis, of starting with a known amount of the standard protein, bovine carbonic anhydrase (Bio-Rad Laboratories), on the gel.

Results and discussion. Figure 1 shows a typical micrograph of the TTM-JSR (A) and SLM-PL (B) used in the present experiments. The TTM-JSR fraction prepared possesses both ATP dependent Ca^{2+}-uptake and Ca^{2+}-release activities. The release was potentiated markedly by a relatively low concentration (1×10^{-6} M) of ouabain, as reported previously[4]. The mean values of (Na^+-K^+)- and Ca^{2+}-ATPase activities represented by released inorganic phosphate (Pi) of TTM-JSR were 0.022 µmoles Pi/s/g protein and 0.15 µmoles Pi/s/g protein, respectively. After photoaffinity labeling with ^{3}H-ouabain, SDS-PAGE[20] of TTM-JSR was carried out, and radioactivities were determined in each protein band incorporated. As shown in figure 2, ^{3}H-ouabain

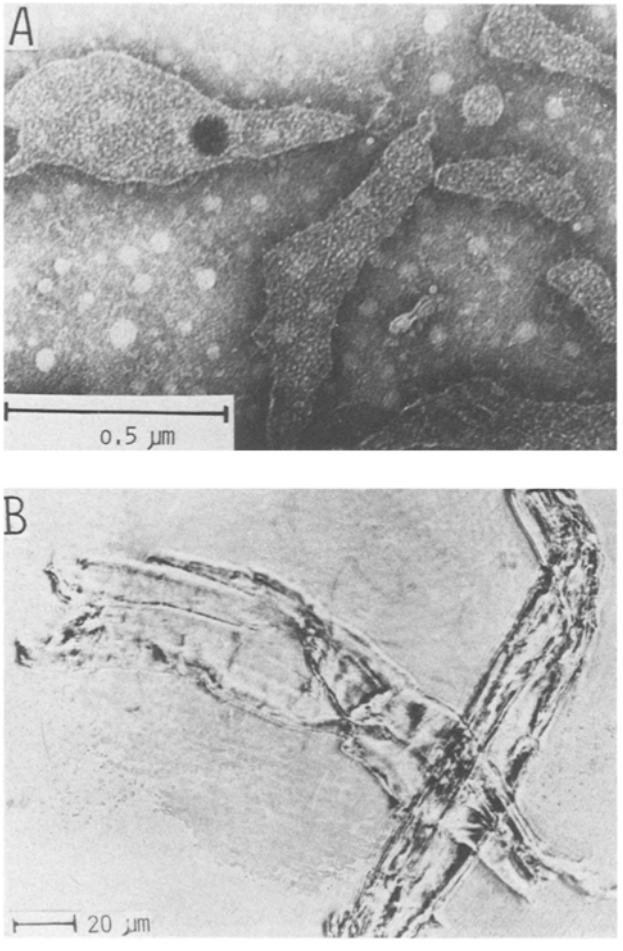

Figure 1. Micrographs of cat cardiac ventricle muscles. *A* Electron micrograph of transverse tubule membrane-junctional sarcoplasmic reticulum complexes (TTM-JSR); negatively stained. *B* Phase-contrast micrograph of sarcolemma-plasma membrane complexes (SLM-PL).

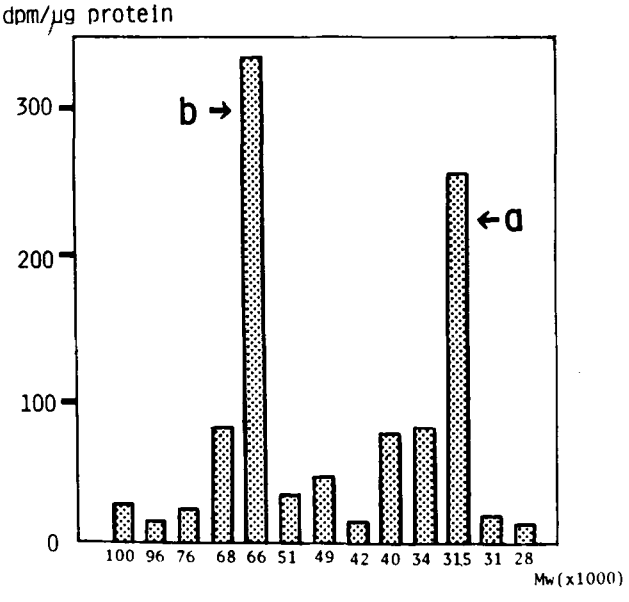

Figure 2. Radioactive profile of SDS-gel electrophoresis of TTM-JSR after photoaffinity labeling with ^{3}H-ouabain (ouabain concentration: 0.5 µM).

was predominantly incorporated into both proteins with molecular weights of 31,500 (a) and 66,000 (b). Incubation in the buffer containing cold ouabain (1 mM) after completion of the ^{3}H-ouabain-incubation reduced greatly the amount of ^{3}H-ouabain incorporated into both proteins. Photoactive proteins corresponding to (a) and (b) in the figure were scarcely found in SLM-PL (this fraction showed: $(Na^+\text{-}K^+)$-ATPase activity, 0.275 µmoles Pi/s/g protein; Ca^{2+}-ATPase activity, 0 µmoles Pi/s/g protein). Efficiency of the photoactivity with ^{3}H-ouabain was 1.45%, which bears comparison with other reports[21]. Both proteins were proved to be glycoproteins by the concanavalin A (Con A)-peroxidase method[22] (data will be published elsewhere). This glycoprotein-nature of the proteins in question was significant not only for characterization but also for isolation of the proteins, because Con A affinity chromatography[23] could be used effectively for the analytical isolation.

To investigate the characteristics of the 31,500 dalton protein, it was isolated from TTM-JSR by Con A affinity chromatography[23], and from the 31,500 dalton band of the gel an extraction was then made under three different pH conditions, acidic (pH 5.3), physiological (pH 7.4) and highly alkaline (pH 11). The SDS-PAGE profile of the material extracted under acidic conditions showed two protein bands (a and b in lane 2 of fig. 3 B). The ratio (mean protein yield) of a and b bands was 90:10. That of the material extracted under physiological conditions also showed two bands of protein (a and b in lane 3 of fig. 3 B) with the ratio, a:b = 40:60. That of the material extracted under alkaline conditions showed three bands (a, b and c in lane 4 of fig. 3 B) with the ratio, a:b:c = 5:85:10. These results demonstrate that both 66,000 dalton and 55,000 dalton proteins originated from the 31,500 dalton protein and would suggest that the b- and even c-band proteins are kinds of polymer of the 31,500 dalton protein.

The most common suggestion about the mechanism of PIE is that it is produced by the influence of cardiac glycosides on the Na-K-pump[2,7,8,10–16]. However, some controversy remains; one view[14–16] emphasizes the dependence of PIE on inhibition of the pump, while others[2,7,8,10–13,15] claim that it is independent of this inhibition. Irrespective of such controversy, a binding of the glycosides to the high affinity-site of $(Na^+\text{-}K^+)$-ATPase has been considered commonly as a fundamental step leading to PIE. $(Na^+\text{-}K^+)$-ATPase is known to be mainly composed of an α-chain[25] (mol. wt approximately 100,000), which is known as the catalytic subunit, and a glycosylated β-chain (mol. wt 35,000–70,000), the function of which is unknown[21,26,27]. Several investigators have reported that only the α-chain is photolabeled with ^{3}H-ouabain but the glycosylated β-chain is not[21,26,27]. In the present experiment, the 31,500 dalton protein and its dimer are probably all glycosylated proteins, and they are photolabeled with a low concentration of ^{3}H-ouabain (fig. 2). Therefore, the 31,500 dalton protein seems to be different not only from the α-chain but also from the β-chain of the enzyme. This characterization would coincide with the fact that the 31,500 dalton protein is extractable mainly from TTM-JSR and not from SLM-PL.

We have already reported that ouabain occupies a considerable part of the cellular space of cardiac muscle, when a positive inotropic action is monitored[24]. Also, the ouabain-binding capacities in isolated SR-JSR and SLM-PL of cat cardiac muscles were found to be 5.71 ± 0.56 pmoles/mg protein of SR-JSR and 0.66 ± 0.04 pmoles/mg protein of SLM-PL, respectively[19]. Present results are similar, and show that the capacity to band ouabain in the SLM-PL is approximately one-tenth of that found in the TTM-JSR. As shown in the table, calculations of the specific ^{3}H-ouabain activity incorporated into the proteins demonstrate that the protein yield for the 31,500 dalton protein and its dimer (mol. wt 66,000) is 0.5% of the total TTM-JSR protein,

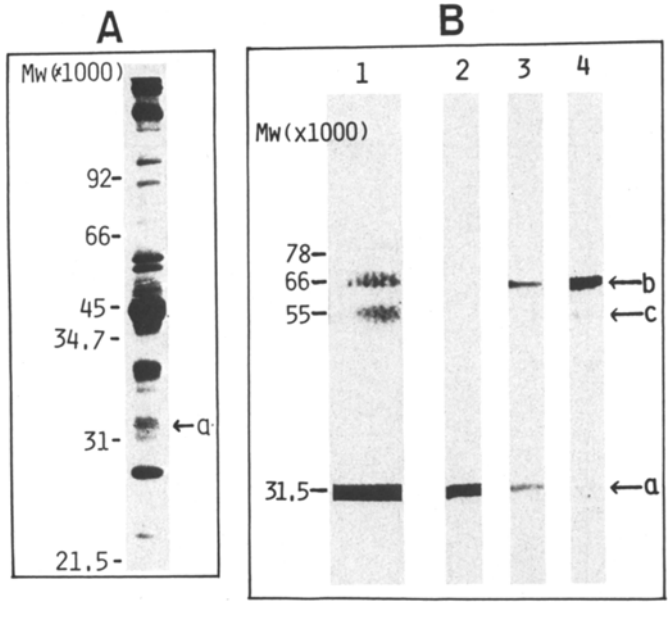

Figure 3. Purification and characterization of the 31,500 dalton protein from TTM-JSR of cat cardiac ventricle muscles. All lanes are Coomassie blue-stained profiles of gels obtained by SDS-gel electrophoresis. *A* SDS-PAGE of TTM-JSR. *B*1 SDS-PAGE of Con A sensitive protein from TTM-JSR. *B*2, 3, 4 SDS-PAGE of proteins obtained by elution from the band A of 31,500 dalton of lane B1 at pH of 5.3, 7.4 and 11.9, respectively.

Ouabain concentration in transverse tubule membrane and junctional SR complexes (TTM-JSR) in the 31,500 dalton protein and its dimer

	Protein yield µg/g muscle	Ouabain incorporated pmoles/mg protein
TTM-JSR (M ± SE)	420 ± 38	5.10 ± 0.13*
31,500 protein and its dimer	2.0 ± 0.17	974**

* ^{3}H-ouabain uptake by TTM-JSR was measured just before the photolabeling with a UV-lamp. ** This value was calculated by photolabeling experiment with ^{3}H-ouabain. Efficiency of photolabeling with ^{3}H-ouabain to the TTM-JSR protein was 1.45%.

and the amount of ouabain incorporated in both proteins was approximately 200-fold (974/5.1) that incorporated in the total TTM-JSR protein. This indicates that almost all the ouabain molecules in the TTM-JSR, which could be a regulator for contractile force, are bound to the newly-found protein and none other.

Noticeable points in the results obtained are that the newly-isolated protein is distinguished by its high binding capacity for ouabain even when the concentration of the drug is low, and that the protein is probably of TTM-JSR origin. We have as yet no decisive and direct evidence to show that the binding of ouabain to this 31,500 dalton protein is responsible for the inotropic effect on cardiac muscle, but the afore-mentioned points are interesting, since the low concentration of ouabain capable of binding to the protein is within the concentration-range which causes PIE. Thus, when ouabain is administered in the low concentration necessary to cause PIE, it seems probable that in the heart ouabain is bound first of all mostly to the newly-described protein selectively, rather than to the α-chain or β-chain of the membrane, and causes PIE. It follows, then, that the new protein should not be neglected in discussions of the mechanism of the PIE.

The study of the 31,500 dalton protein in skeletal[28, 29] and cardiac muscles[30] is still continuing, and its function has not yet been decisively demonstrated. However, we consider that the 31,500 dalton protein, which is extractable mainly from TTM-JSR of muscles in various species of animals, is not only interesting because of the present findings, but also because 1) it is extractable from TTM-JSR, which is regarded as a system of structures specially differentiated for the excitation-contraction (E-C) coupling mechanism; 2) our recent studies on rapid cooling contracture[6], K-contracture, and Ca-release from sarcoplasmic reticulum[4] of cat and frog heart muscles have shown that the PIE of cardiac glycosides on heart takes place through the influence of the glycosides on the E-C coupling process; and 3) in skeletal and heart muscles, the E-C coupling process has been reported to be inhibited by a substance[28–30], which has recently been shown to bind the 31,500 dalton protein (data will be published elsewhere).

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