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ASSOCIATION OF BASAL ATPase ACTIVITY AND CHOLESTEROL WITH A DISTINCT GROUP OF RABBIT SKELETAL MUSCLE MICROSOMAL PARTICLES

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SUMMARY

Basal ATPase is readily separated from the Ca^{2+}-ATPase of the sarcoplasmic reticulum. The median density distributions of cholesterol and basal ATPase activities are almost identical. Digitonin has been successfully employed in determining the association of cholesterol with specific vesicles in rat liver microsomal preparations. Treatment of rabbit skeletal muscle microsomal preparations with digitonin alters the density distribution patterns of basal ATPase activity and cholesterol in an identical fashion. Protein distribution displays a less marked change in median density. Enzymic activity associated with calcium transport, measured under differing conditions, is largely unaffected.

It is concluded that cholesterol and basal ATPase activity are associated with a distinct group of rabbit skeletal muscle microsomal particles.

INTRODUCTION

Rabbit skeletal muscle microsomal fractions isolated by differential pelleting procedures rapidly transport calcium ions in the presence of oxalate [1–3]. Microsomal fractions prepared from rabbit skeletal muscle by density gradient centrifugation techniques also transport calcium ions. However, these preparations display a higher specific activity than those prepared by differential pelleting [4, 5]. Microsomal fractions prepared by both procedures contain cholesterol [7–9] and enzymic activities in addition to their calcium transporting activity [5, 10]. The cholesterol to protein ratio in microsomal preparations prepared by density gradient centrifugation is 50–75 % of the ratio obtained in preparations obtained by differential pelleting [9]. Some workers have postulated that cholesterol plays a role in the calcium transporting process. Data supporting such an interpretation has been obtained from experiments involving diethyl ether extractions of muscle microsomal vesicles [8, 11, 12] and by the use of digitonin, a detergent which binds specifically to 3β-OH steroids such as cholesterol.

More recent experiments involving the use of ether and heptane [13] have indicated that cholesterol, while present in muscle microsomal preparations, does not play a role in calcium transport or storage. In addition, skeletal muscle microsomes

isolated from rats with 20, 25 diazocholesterol-induced myotonia contained a dehydrogenated and esterified product of the drug [14]. However, the calcium-transporting activity of the microsomes isolated from such rats demonstrated essentially normal calcium-transporting activities [15, 16]. Previous results from this laboratory have indicated the total absence of cholesterol from the membranes involved in calcium transport [17].

Thus, because of the presence of cholesterol in muscle microsomal preparations and its noninvolvement in calcium transport together with the known heterogeneity of rabbit skeletal muscle microsomal vesicles [9, 18] it was of interest to determine which of the microsomal particles contain cholesterol. To this end digitonin, which has been successfully used in the identification of cholesterol-containing vesicles in liver microsomal preparations [19, 20], was employed. In addition to this analysis with respect to cholesterol, the calcium-dependent transport ATPase [21] and the calcium-independent or basal ATPase [22, 23] activities were determined in the experiments described here.

MATERIALS AND METHODS

Young adult rabbits weighing approx. 2.5 kg were used in this study. The white muscle from the hind limbs was removed immediately after death and placed in ice-cold 0.25 M sucrose buffered with 3 mM imidazole · HCl to pH 7.4. The tissue was then minced and the mince homogenised in 3 vols of 0.25 M sucrose/3 mM imidazole · HCl, pH 7.4, forming a 25 % homogenate. After preliminary centrifugations at 600 × *g* for 10 min and 15 000 × *g* for 15 min in an MSE 8 × 25 ml angle rotor (cat. no. 59594) operating in an MSE Superspeed 40 centrifuge the 100 000 × *g*, 60 min pellets were resuspended in 0.25 M sucrose/3 mM imidazole · HCl, pH 7.4. Such microsomal preparations are essentially free of mitochondrial contamination as indicated by succinate dehydrogenase analysis [24].

The microsomal suspensions were treated following the procedure of Thines-Sempoux et al. [19]. A 2-ml portion of the treated and untreated microsomal suspensions was layered on exponential sucrose density gradients. These gradients were prepared according to the procedure of Birnie and Harvey [25]. The volume of the gradients, which were buffered with 3 mM imidazole · HCl, pH 7.4, was 15 ml and ranged in density from 1.04 to 1.21 g/ml. The underlay consisted of 2 ml of 2.0 M sucrose. The ratio of heavy to light sucrose used in the production of the gradients was 1.5 : 1. Density gradient equilibration was achieved by centrifugation at 30 000 rev./min for 600 min in an MSE 3 × 25 ml swinging bucket rotor (cat. no. 59590) using an MSE Superspeed 40 centrifuge (MSE Ltd., Crawley, U. K.). The temperature of centrifugation was 5 °C. Twenty fractions (volume 1 ml) were collected manually from each gradient after centrifugation. Refractive indices were determined using an ABBE 60 refractometer (Bellingham & Stanley Ltd., London). Sucrose concentrations were obtained from the refractive indices by consulting standard tables.

Protein concentrations were determined by an automated Lowry procedure [26]. ATPase activities were assayed according to the method of Duggan [23, 27] using the semi-automated procedure of Marzban and Hinton [27]. Lipid analysis was carried out as previously reported [29]. All calculations were carried out using an IBM 370/135 computer.

The results are presented in the form of normalized histograms. The diagrams are plotted on a density scale (ρ) and the ordinate gives the frequency within the corresponding span of density [20].

Digitonin (grade 1) was obtained from the Sigma Chemical Company, St. Louis, No. 63178, U.S.A.

RESULTS AND DISCUSSION

The median density distributions of basal ATPase activity ($\rho = 1.149$) and of cholesterol ($\rho = 1.151$) in the control preparation indicate a possible association of cholesterol with vesicles displaying basal ATPase (Figs 1a and 1c). In addition, it is evident that basal ATPase activity and the calcium-transport ATPase are readily separable by sucrose density gradient centrifugation (Figs 1c and 1d) confirming previous results from this laboratory involving rate separations of muscle microsomal constituents [5, 30]. Digitonin forms an insoluble equimolar complex with cholesterol and thus brings about a change in the physical characteristics of those microsomal vesicles which contain cholesterol, namely an increase in the density of these particles [20, 31]. The distribution of skeletal muscle microsomal vesicles following treatment with digitonin and equilibration on a sucrose density gradient are shown in Figs 1a–1e. The density distributions of cholesterol of untreated and digitonin-treated microsomes after equilibration on a sucrose density gradient are shown in Fig. 1a. As shown, the median density is shifted in a significant manner by 0.028 density units.

In contrast, the distribution of protein (Fig. 1b) is modified to a much lesser extent after digitonin treatment. The shift in the median density in this case is only 0.013 density units. This indicates that cholesterol is associated with a relatively small amount of the protein in this preparation.

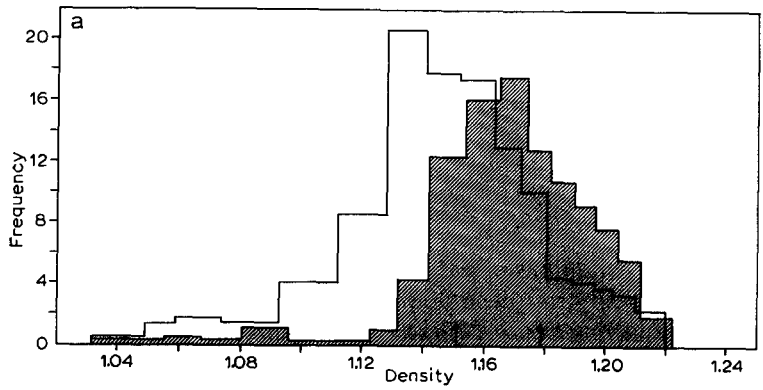
The distribution of basal ATPase activity after digitonin treatment and density gradient equilibration is shown in Fig. 1c. Here an increase in the median density of 0.030 density units greater than the control value is evident. Taken together, this increase (0.030) and the increase observed for cholesterol (0.028) indicate that cholesterol is associated with those vesicles of rabbit skeletal muscle microsomal preparations which contain basal ATPase activity.

Fig. 1e shows the distribution of K^+-stimulated ATPase [23] of microsomal vesicles. The median density of the treated material is not significantly affected when compared to the control and in fact shows a slight negative shift (-0.002), possibly due to the detergent properties of digitonin [20].

Similarly, with the Ca^{2+}-dependent ATPase activity, the median density is again changed, when compared to the control value, in a negative direction (-0.002) for the same reason as before (Fig. 1d).

Table I shows the median densities in both treated and untreated microsomes after equilibration in sucrose density gradients and the changes observed in these values for the chemical constituents and enzymic activities named above. Since the Ca^{2+}-dependent ATPase shows no density shift it can be inferred that cholesterol is not associated with those microsomes involved in calcium transport, supporting previous results from this laboratory [17].

These results suggest that in the intact muscle cell those areas of the sarcoplasmic reticulum involved in Ca^{2+}-uptake, i.e. the longitudinal elements of the sarco-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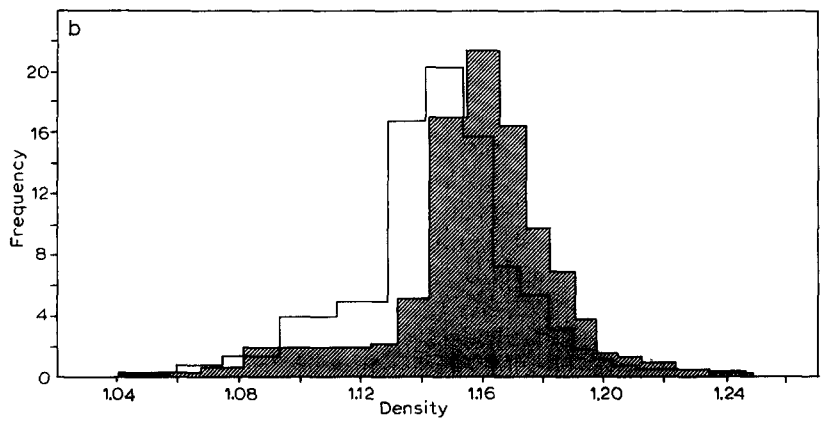
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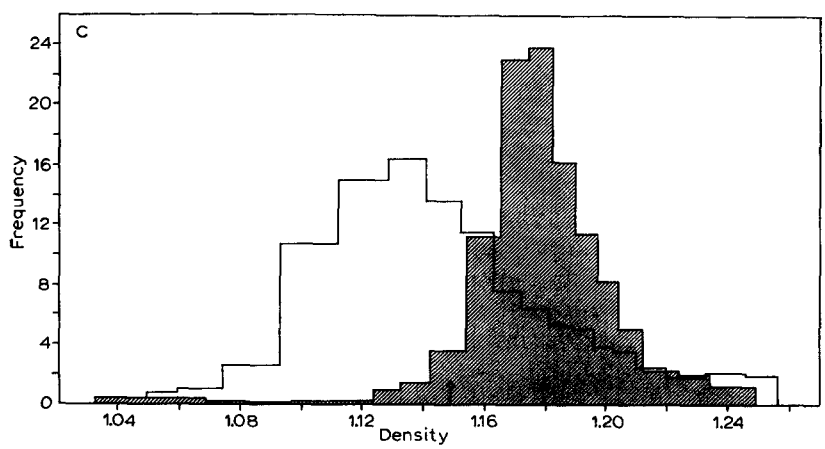
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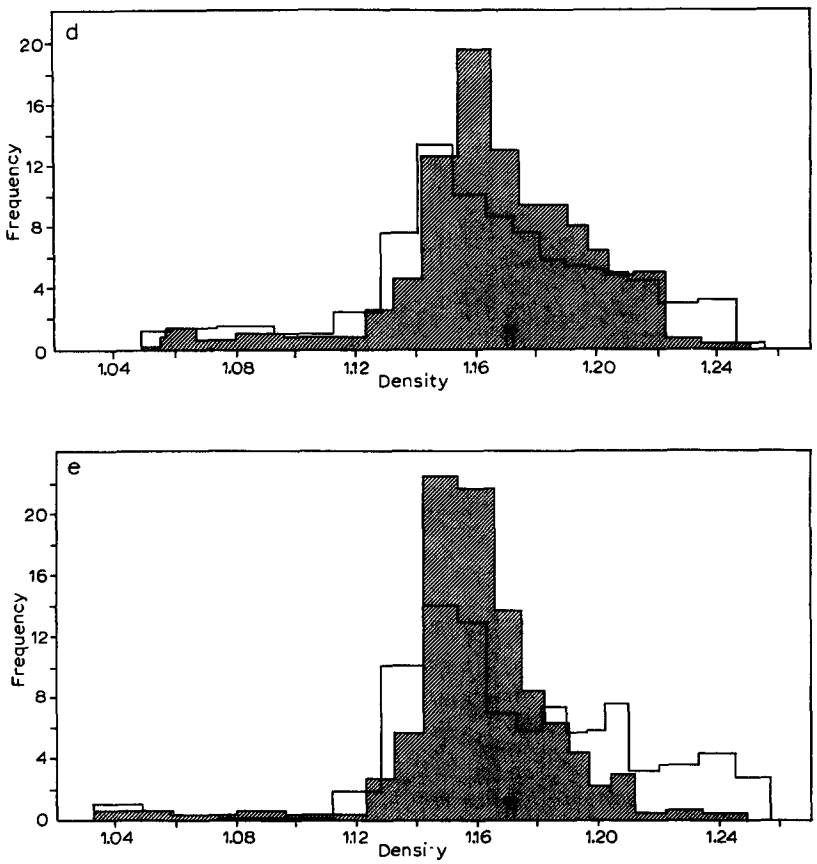

Fig. 1. Influence of the treatment with digitonin on the density distribution of rabbit skeletal muscle microsomal constituents in sucrose density gradients. Treatment of microsomes and analysis for constituents were carried out as described in Materials and Methods. The shaded area represents the distribution after treatment with digitonin, with the median density indicated by a black arrow. The median density value for control microsomes is indicated by the white arrow. (a) cholesterol, (b) protein, (c) basal ATPase, (d) Ca^{2+}-dependent ATPase and (e) K^{+}-stimulated ATPase.

plasmic reticulum [32], do not contain cholesterol. The cholesterol-associated microsomal particles may possibly arise from the sarcolemma or plasma membrane of skeletal muscle during the homogenisation process.

Papahadjopoulos et al. [33] have suggested that, while cholesterol is needed in order to provide a generally stable membrane framework, it is excluded from highly functional areas of membranes, e.g. those involved in transport. The absence of cholesterol from membranes involved in the transport of calcium lends weight to this theory.

Work is in progress to subfractionate further the vesicles present in rabbit skeletal muscle microsomal preparations.

TABLE I

MEDIAN DENSITIES OF CONSTITUENTS IN CONTROL AND DIGITONIN-TREATED MICROSOMAL VESICLES EQUILIBRATED IN SUCROSE DENSITY GRADIENTS

Constituent	Median density		Variation of treated from control ($\times 10^4$)
	Control	Digitonin-treated	
Protein	1.150	1.163	130
Cholesterol	1.151	1.179	280
Basal ATPase	1.149	1.179	300
K^+-stimulated ATPase	1.171	1.169	−20
Ca^{2+}-dependent ATPase	1.172	1.170	−20

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