The Differential Release of Basal ATPase, Ca^{2+} -Dependent ATPase, 5'-Nucleotidase and Cholesterol During Homogenization of Skeletal Muscle

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The influence of homogenization times on the presence of constituents in the microsomal fraction of skeletal muscle was investigated. Membranes having Ca^{2+} -activated ATPase activity have a fragmentation pattern distinct from that of membranes displaying Ca^{2+} -independent or basal ATPase activity. These latter membranes were found in highest specific concentration in the microsomal fraction prepared from homogenates subjected to short periods of homogenization. 5'-Nucleotidase (EC 3.1.3.5) activity paralleled that of basal ATPase on short periods of homogenization, as also did the specific concentration of cholesterol. Longer periods of homogenization led to a decrease in the specific activity of basal ATPase, which reached its lowest value at 120 s of homogenization, whereas the specific activity of 5'-nucleotidase and the specific concentration of cholesterol decreased initially in a similar manner to basal ATPase, but both increased substantially after the longest period of homogenization.

The skeletal-muscle cell contains a number of highly organized membrane systems (Franzini-Armstrong, 1973), namely, the sarcolemma, which incorporates the plasma-membrane, the transverse tubules or T-system and finally the sarcoplasmic reticulum, which is the site of Ca^{2+} storage and release.

The microsomal fraction obtained from skeletal-muscle homogenates contains a number of enzymic activities, including at least two distinct ATPase activities (Hasselbach & Makinose, 1963; Weber *et al.*, 1966), one of which is responsible for the active accumulation of Ca^{2+} bringing about relaxation and is termed the Ca^{2+} -dependent ATPase (Hasselbach & Makinose, 1961; Martonosi, 1972). The other ATPase activity is apparent in the presence of EGTA and has been termed the Ca^{2+} -independent or basal ATPase (Weber *et al.*, 1966; Duggan, 1968*b*). Microsomal preparations may also contain ATPase activities arising from contaminating mitochondrial membranes, and, less commonly, from contractile proteins.

We have shown that muscle microsomal fractions may be divided into two groups depending on

their cholesterol content (Headon *et al.*, 1977). Group 1 microsomal fraction comprises vesicles having cholesterol as a membrane constituent. These membranes have a median density of 1.15 g/cm^3 after equilibrium centrifugation in sucrose density gradients at 4°C . Basal ATPase activity has been shown to be associated with this group of microsomal components (O'Flaherty *et al.*, 1975; Joyce & Headon, 1975). Vesicles from the Ca^{2+} -transporting regions of the sarcoplasmic reticulum are found in group 2 microsomal fraction, which are characterized by the absence of cholesterol as a detectable membrane constituent (Headon & Barrett, 1974). The latter also differ from group 1 membranes in that they have a median density of 1.17 g/cm^3 after equilibrium centrifugation in sucrose and they sediment more slowly in a sucrose density gradient.

The present study was designed to examine the susceptibility of the various membrane systems to homogenization and to provide information regarding the membrane composition of skeletal-muscle microsomal preparations.

Experimental

Tissue preparation and fractionation

Equal weights of minced muscle were homogenized in 3 vol. of 0.25 M -sucrose/ 5 mM -imidazole/HCl (pH 7.4) at 4°C by using a Waring blender for 30, 60, 90 and 120 s. The microsomal fraction and other subcellular fractions were prepared from these

Abbreviation used: INT, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride.

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homogenates by the method of Barrett & Headon (1975).

Analyses

The fractions were assayed for protein (Leighton *et al.*, 1968), phospholipid (Bartlett, 1959) and cholesterol (O'Flaherty *et al.*, 1975). Succinate dehydrogenase activity was measured as succinate-INT reductase activity by a modification (Porteous & Clark, 1965) of the method of Pennington (1961). Ca^{2+} -dependent ATPase and basal ATPase were assayed by the method of Duggan (1968*a,b*) using the semi-automated procedure of Marzban & Hinton (1970). The $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase was assayed in the presence of 100mM- Na^+ and 10mM- K^+ . Before assay, fractions were diluted to the same protein concentration. The ouabain concentration and the ouabain/protein ratio in the assay mixture were similar to that reported by Sulakhe *et al.* (1973). 5'-Nucleotidase activity was determined by the method of Hinton *et al.* (1970). At these concentrations the activity was maximally sensitive to ouabain. One-dimensional electrophoresis of membrane proteins was carried out in gradient-pore polyacrylamide gels (Barrett & Headon, 1975).

Results and Discussion

The distributions of a number of constituents and enzymes between the microsomal fractions were studied for homogenization times of 30, 60, 90 and 120s. With the exception of ATPase activities, the amount of each constituent in a fraction is expressed as a percentage of that in the original homogenate. Recoveries in the various subcellular fractions for protein, phospholipid, cholesterol, and succinate-INT reductase were 95, 95, 97 and 101% respectively of the amount in the homogenate. In an interpretation of the effect of increasing homogenization time, consideration must be given to the possible denaturation of the enzymes being assayed. The activity of succinate-INT reductase in the homogenate was unaffected over the range of homogenization times chosen. The activities of Ca^{2+} -dependent ATPase, basal ATPase, 5'-nucleotidase and $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase cannot be accurately determined in the homogenate due to interference by a variety of phosphatase activities. The effect of homogenization on the stability of these enzymes was stimulated by treating a microsomal suspension with a Waring blender. The results indicated that the ATPase activities were stable under the homogenization conditions used. Homogenization times of 120s have been used by other workers (e.g. Martonosi, 1968; Louis & Shooter, 1972) in the routine preparation of muscle microsomal material.

The activities of basal ATPase, 5'-nucleotidase and Ca^{2+} -dependent ATPase together with the cholesterol/phospholipid (w/w) ratio in the microsomal fractions plotted against homogenization time are shown in Fig. 1. To prevent interference by mitochondrial ATPase the ATPase activities were assayed in the presence of 50mM-azide (Fanburg & Gergely, 1965). The specific activities of basal ATPase and Ca^{2+} -dependent ATPase, expressed in terms of phospholipid content, indicate an appreciably different pattern of fragmentation during homogenization. The specific activity of Ca^{2+} -dependent ATPase reaches a peak after a homogenization time of 60s. This peak in specific activity coincides with the lowest cholesterol/phospholipid ratio for the four homogenization times studied. This correlates well with the previous finding that membranes displaying this enzyme activity do not contain cholesterol (Headon & Barrett, 1974; O'Flaherty *et al.*, 1975).

The specific activity of basal ATPase follows a different trend, being highest at 30s and decreasing thereafter. The cholesterol/phospholipid ratio closely follows the specific activity of basal ATPase up to and including a homogenization time of 60s. Since basal ATPase has been shown to be located in a cholesterol-containing membrane (O'Flaherty *et al.*, 1975), this close correlation is to be expected. However, between 90 and 120s the cholesterol/phospholipid ratio increases, whereas the specific activity of basal ATPase continues to decrease.

Fig. 1. Cholesterol/phospholipid (w/w) ratio (■) and the specific activities of Ca^{2+} -dependent ATPase (▲), basal ATPase (●) and 5'-nucleotidase (○) in the microsomal fraction prepared from 30, 60, 90 and 120s homogenates

5'-Nucleotidase activity more closely parallels the cholesterol/phospholipid ratio. The specific activity of this enzyme increases on longer periods of homogenization, which treatment leads to a decrease in the specific activity of the basal ATPase. This finding is interpreted as indicating the presence of two types of cholesterol-containing membranes, one containing Ca^{2+} -independent ATPase and 5'-nucleotidase and appearing in the microsomal fraction after short periods of homogenization. The other membrane type displays 5'-nucleotidase activity and is ruptured into vesicles that are present in the microsomal fraction in high concentration after longer periods (90 and 120s) of homogenization. The results thus indicate that these membranes and the membranes displaying Ca^{2+} -dependent ATPase activity are differentially fragmented during homogenization.

The possibility that the cholesterol-containing membranes not associated with basal ATPase might contain the ouabain-sensitive ($\text{Na}^{+} + \text{K}^{+}$)-dependent ATPase in addition to 5'-nucleotidase was investigated. The ouabain sensitivity of this enzyme activity was assayed in the microsomal fractions from 30 and 120s homogenates. The results are presented in Table 1. The activity of this enzyme increases with increasing homogenization time. However, its contribution to the overall ATPase activity decreases from 15.6% in the fraction from a 30s homogenate to 11.6% in that from a 120s homogenate. In terms of specific activity relative to phospholipid content the activity displayed by the microsomal fraction prepared from the 120s homogenate is approx. 60% of that obtained after homogenization for 30s.

The membranes appearing in high concentration after 120s of homogenization may therefore originate from the transverse tubules and/or from the regions of the sarcoplasmic reticulum, such as the terminal cisternae or central fenestrated collar, which are thought not to transport Ca^{2+} (Winegrad, 1968). Thines-Sempoux *et al.* (1969) and Amar-Costesec *et al.* (1974) have shown that, with the exception of Golgi apparatus, endocytotic vesicles and secondary lysosomes, the intracellular membranes of liver do not contain cholesterol. These

reports, along with the already proven absence of cholesterol from the Ca^{2+} -transporting regions of the sarcoplasmic reticulum, would suggest that the entire sarcoplasmic reticulum does not contain cholesterol as a constituent and would not be a source of cholesterol-containing microsomal fraction. The most probable source of these additional membranes would be the transverse tubule system that arises by invaginations of the plasma membrane and thus separates the intracellular contents from the extracellular medium, a function similar to that of plasma membrane, and for this reason is likely to be rich in cholesterol.

To further characterize the various vesicles the protein components of the microsomal fraction for each homogenization time were analysed to determine compositional differences in membrane proteins between the various membrane types. The

Fig. 2. The electrophoretic separation of protein components in the microsomal fraction and (a) 30s, (b) 60s, (c) 90s and (d) 120s homogenates
Electrophoresis was carried out in 6–27% polyacrylamide-gel gradients containing 0.1% (w/w) sodium dodecyl sulphate. Migration was towards the anode, i.e. from top to bottom in the Figure.

Table 1. Activity of ($\text{Na}^{+} + \text{K}^{+}$)-dependent ATPase in the microsomal fraction from 30 and 120s homogenates
Values are given as means $\pm$ s.d. The number of determinations is given in parentheses. Ouabain inhibition is expressed as a percentage of the activity in the control.

Homogenization time(s)	Activity ($\mu\text{mol}/\text{fraction per min}$)		Inhibition (%)
	Control	Oubain-treated	
30	1.390 $\pm$ 0.053 (8)	1.174 $\pm$ 0.062 (8)	15.6
120	2.375 $\pm$ 0.120 (7)	2.100 $\pm$ 0.164 (7)	11.6

protein components of membranes for each of the four homogenization times were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in gradient-pore polyacrylamide gels. The separations obtained are shown in Fig. 2. The major protein band evident in Fig. 2 after short periods of homogenization is generally considered to be the principal protein component in skeletal-muscle microsomal fraction, having a molecular weight in the region of 100 000 and containing the Ca^{2+} -dependent ATPase (Martonosi, 1969, 1972; MacLennan *et al.*, 1974; Barrett & Headon, 1975). Proteins having molecular weights of less than 90 000 constitute approx. 41, 27, 26 and 37% of the total protein in the fraction from 30, 60, 90 and 120 s homogenates respectively. The 100 000-mol.wt. band increases in concentration up to a homogenization time of 90 s. Homogenization for 90 and 120 s leads to heterogeneity in the region of this band. In the microsomal fraction from a 120 s homogenate a band with a molecular weight somewhat greater than 100 000 and marked with an arrow in Fig. 2 is present in greater concentration than the 100 000-mol.wt. band. These results lend support to the fact that variations in the membrane content of the microsomal fraction occur on various periods of homogenization as evidenced by the enzymic and constituent data.

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