LOCALIZATION OF ADENYLATE CYCLASE IN SKELETAL MUSCLE SARCOPLASMIC RETICULUM AND ITS RELATION TO CALCIUM ACCUMULATION

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1. Introduction

Histochemical and biochemical studies have shown that adenylate cyclase is present in the SR of cardiac and skeletal muscle [1,2]. There is considerable evidence for an association between cAMP and SR function, especially in heart, where cAMP stimulation of calcium accumulation was proposed to be responsible for the inotropic effect of catecholamines [3,4]. However, in fast skeletal muscle this relationship is less firmly established, especially since catecholamines reduce the rate of relaxation [5]. To further study the potential role of the microsomal adenylate cyclase in fast skeletal muscle, we examined the localization of adenylate cyclase relative to calcium accumulation in chicken pectoralis. Our results indicate that the adenylate cyclase activity is found primarily in the terminal cisternae of the SR, and is not associated with the calcium accumulating function.

2. Methods

SR microsomes were prepared from the pectoralis muscle of 3–4 month old White Leghorn chickens as in [6]. The microsomal pellet was suspended in 10 mM imidazole, 1 mM DTT, pH 7.4. Loading of fresh microsomes with [^{45}Ca]oxalate and the separation of loaded vesicles on a discontinuous sucrose gradient was done as in [7]. The gradient fractions were suspended in imidazole–DTT and quick frozen in liquid nitrogen.

The amount of calcium loaded was determined by counting 50 μl of each fraction in 10 ml Bray's scintillation fluid. Adenylate cyclase activity was measured as in [8] after solubilization (30 min at 4°C) in 0.5% Lubrol-PX, 0.2 mM EGTA. The assay medium contained 25 mM Tris–HCl, pH 7.5, 5 mM magnesium acetate, 1 mM DTT, 1 mM cAMP, 50 units/ml creatine kinase, 5 mM phosphocreatine and 1–3 × 10^6 cpm [α-^{32}P]ATP, in final vol. 0.1 ml. Basal ATPase activity was measured in 20 mM Tris maleate, pH 6.8, 100 mM KCl and 4 mM $MgCl_2$. Ca^{2+}–ATPase is defined as the increase in enzyme activity upon addition of 0.3 mM Ca^{2+}. Na^+–K^+–ATPase was the difference between basal activity and the activity measured in 10 mM Tris, pH 7.4, 5 mM $MgCl_2$, 10 mM NaCl and 10 mM KCl. All samples contained 1.0 mM Na_2ATP, 5 mM NaN_3 and 0.2 mM EGTA. The final protein concentration was 10–20 μg/ml. The reaction was stopped after 5 min at 30°C with 150 μl 30% trichloroacetic acid (TCA). The samples were centrifuged at 10 000 × g for 15 min and phosphate was measured as in [9], with the modification of 18–20 h color development at 4°C. Protein was measured as in [10].

For cytochemical localization of adenylate cyclase the following procedure was used. A piece of chicken pectoralis was quickly removed, minced in cold 1.25% glutaraldehyde, 0.1 M cacodylate buffer (pH 7.4), fixed for 15 min in cold fixative, and washed 4 times (15 min each) in cold cacodylate buffer. 100 μm strips were cut and incubated for 30 min at 37°C in 80 mM Tris–maleate buffer (pH 7.4) with 8%

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sucrose (w/v), 2 mM lead nitrate, 20 mM NaF, 10 mM $MgCl_2$, 10 mM theophylline, and 1 mM AMP–P(NH)P, purified on a Dowex AG-50 W-X4 cation exchange resin [11,12]. Following incubation, the tissue was stained en bloc with 0.25% aqueous uranyl acetate and then dehydrated through a graded series of acetones and embedded in Spurr low viscosity resin [13]. Sections were examined unstained to confirm the specificity of the reaction, and then stained with lead citrate [14] to enhance contrast.

3. Results and discussion

Separation of calcium loaded microsomes on a discontinuous sucrose gradient showed an inverse relationship between adenylate cyclase activity and calcium uptake in the four fractions obtained (table 1). The adenylate cyclase activity was highest in the lighter band, and was barely detectable in the pellet, which contained the greatest amount of calcium. Control experiments showed that under the conditions of the assay (in the presence of 0.5% Lubrol-PX and 0.2 mM EGTA), the lower adenylate cyclase activity was not due to calcium inhibition of the enzyme (table 2). Na^+–K^+–ATPase paralleled the distribution of the adenylate cyclase and, as reported [15], was not inhibited by ouabain. Ca^{2+}–ATPase was present in significant amounts in all fractions.

Local specialization in SR function has been shown [16]: calcium release at the terminal cisternae and calcium accumulation primarily in the longitudinal tubules. By these criteria the light fraction of our separation rich in adenylate cyclase and Na^+–K^+–ATPase and lacking calcium accumulating ability could correspond to the terminal cisternae. This assumption was confirmed by cytochemical examination of the distribution of adenylate cyclase in non-disrupted muscle (fig.1). The majority of the reaction product was found at the Z-lines in the terminal cisternae of the longitudinal tubules.

If we assume an association between structure and function, our findings could suggest a role for adenylate cyclase and Na^+–K^+–ATPase in the calcium release step of excitation–contraction (E–C) coupling. It is presumed that, in fast muscle, depolarization of the terminal cisternae may play a role in calcium release [17]. A relationship between cAMP and K^+ or Na^+ fluxes across biological membranes was reported in several systems [18–20]. It is interesting that in heart tissue the inotropic effect of ouabain, which is

Table 2
Effect of Lubrol and EGTA on adenylate cyclase activity of loaded microsomes

EGTA	Lubrol	Specific activity (pmol cAMP/mg protein/min)
–	–	11.0 (0.3)
1.0 mM	–	11.9 (0.1)
–	0.5%	5.6 (0.6)
0.2 mM	0.5%	12.8 (1.2)
1.0 mM	0.5%	12.9 (1.2)

A microsome subfraction, loaded with 79.2 nmol calcium/mg protein, was preincubated at 4°C for 30 min in the presence of EGTA and/or Lubrol-PX as shown. Adenylate cyclase was measured as in Methods. Results are the mean and range of duplicate measurements

Table 1
Adenylate cyclase and ATPase activity of SR microsome subfractions

Fraction	Calcium loading (nmol calcium/mg protein)	Adenylate cyclase (pmol cAMP/mg protein/min)	Na^+–K^+ ATPase (nmol P_i/mg protein/min)	Ca^{2+}–ATPase (nmol P_i/mg protein/min)
B1	28.8	545.5 ± 23.9	168.6 ± 24.5	106.6 ± 19.1
28S	54.0	101.1 ± 5.0	129.3 ± 11.4	196.3 ± 6.3
B2	106.9	35.5 ± 4.7	87.5 ± 7.2	147.2 ± 8.6
Pel	6395.0	3.7 ± 3.0	35.5 ± 13.7	210.9 ± 18.9

Fractions were prepared and assayed for enzymatic activity as described in Methods. Results are expressed as mean ± SD of three determinations

Abbreviations: B1, float on 28% sucrose; B2, float on 40% sucrose; 28S, suspended in 28% sucrose; Pel, pellet

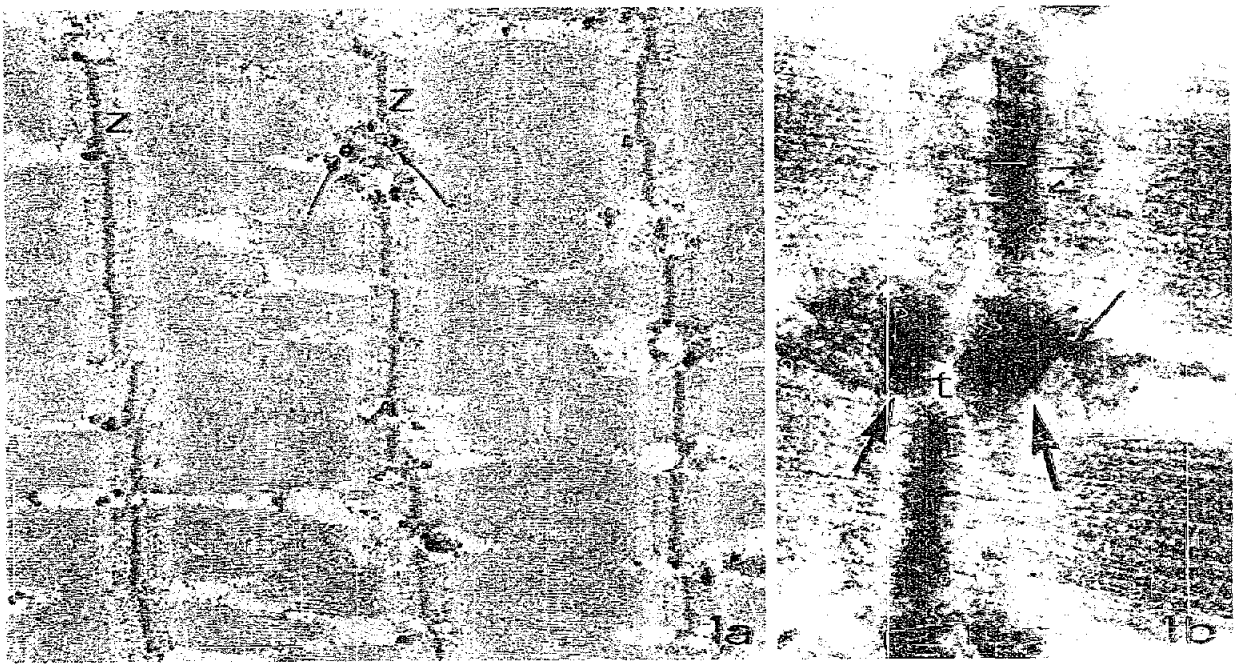

Fig.1. Cytochemical localization of adenylate cyclase in chicken pectoralis muscle. 1a: Electron micrograph showing reaction product indicative of adenylate cyclase activity in the terminal cisternae of the ST (arrows) which are situated at the 'Z' bands (z). Virtually no reaction product can be seen in the longitudinal SR (× 15 750); 1b: Higher magnification electron micrograph showing reaction product in the terminal cisternae of SR (large arrow) below the 'Z' band (Z). Some reaction product can be seen at junction of the longitudinal SR and the terminal cisternae (small arrow). The 'T' tubule (t) between the terminal cisternae of the triad is free of reaction product (× 100 000).

known to interact with the Na^+—K^+—ATPase, was abolished by an adenylate cyclase inhibitor [21].

The fast muscle microsomal adenylate cyclase was not stimulated by isoproterenol at concentrations up to 10^{-4} M or PGE_1 (data not shown), but was susceptible to calcium inhibition (fig.2). Calcium reduced the velocity of the enzyme over 10-fold with an apparent K_i of 0.2 mM at 5 mM Mg^{2+}. This effect may play a role in the regulatory function of the enzyme. The data presented in table 2 suggest that the calcium sensitive site faces the sarcoplasm. If cAMP is essential

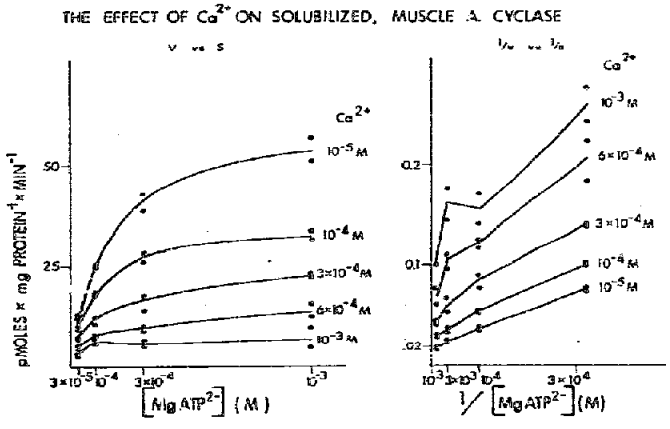

Fig.2. Effect of Ca^{2+} on solubilized muscle adenylate cyclase. SR microsomes were incubated for 15 min at 4°C after being diluted 1:1 with 1% Lubrol-PX. 0.125 M sucrose, 1 mM DTT, 1 mM EGTA, 10 mM Tris, pH 7.6 and 10 mM NaF. They were then centrifuged at 148 000 × *g* for 30 min. Adenylate cyclase activity was measured in the supernatant as described in Methods. The free calcium concentrations were calculated from multiple equilibria equations describing the interactions of Ca^{2+}, ATP and EGTA [24].

for excitation–contraction coupling the inhibition of adenylate cyclase by calcium released from the terminal cisternae may initiate the refractory period described [22] and increased by calcium [23].

In summary, we have shown that in fast skeletal muscle the microsomal adenylate cyclase is associated with the non-calcium-accumulating terminal cisternae of the SR. Based on the relationship of cAMP to membrane depolarization and calcium fluxes in other systems, we envisage as a working hypothesis that cAMP, probably via phosphorylation of a membrane component, enhances calcium release from the terminal cisternae of the SR.

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