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SHORT COMMUNICATIONS

Labeling of sarcoplasmic reticulum peptides with ^{32}P-phosphate and fluorescein 5′-isothiocyanate

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PIP* is formed in sarcoplasmic reticulum, which indicates the presence of a PI kinase in these membranes. As a result of this phospholipid phosphorylation the SR Ca^{2+} transport ATPase activity increases [1, 2]. Furthermore it can be demonstrated that phosphorylase kinase enhances the rate of PIP formation which suggests an involvement of this kinase in this phospholipid phosphorylation. Evidence is presented for an association of phosphorylase kinase with SR vesicles [3–5]. Immunofluorescence studies indicate that an antigen identical or related to phosphorylase kinase can be located within the sarcolema and the sarcoplasmic reticulum [6–9]. In agreement with these observations, it is demonstrated that isolated rabbit skeletal muscle sarcoplasmic reticulum contains membrane associated phosphorylase kinase which sediments in sucrose gradients with the light and heavy SR fractions. Recently Dombradi *et al.* [5] confirmed that phosphorylase kinase activity is also present in T-tubules.

I-strain mice muscle genetically deficient in cytosolic phosphorylase kinase, contains membrane associated phosphorylase kinase activity [9]. This membrane associated phosphorylase kinase activity certainly does not function as a glycogen phosphorylase phosphorylating enzyme since no phosphorylase *b* to *a* conversion occurs in these murine muscles [10]. An alternate function for this membrane associated phosphorylase kinase could be the above mentioned phosphorylation of phosphatidylinositol. In agreement with this conclusion, indeed the pure acidic phospholipid, phosphatidylinositol, can be phosphorylated by phosphorylase kinase. During standard purification of phosphorylase kinase, both phosphorylase kinase and phosphatidylinositol kinase are enriched in parallel approximately to the same degree. These two activities cannot be separated employing several chromatographic techniques [11]. Polyclonal as well as monoclonal antibodies directed specifically against the α subunit of phosphorylase kinase immunoprecipitate in parallel both phosphorylase kinase and phosphatidylinositol kinase. Therefore, it was interesting to find out if polypeptides identical or related to phosphorylase kinase which might belong also to phosphatidylinositol kinase are demonstrable in SR membranes. The present study will show that polypeptides which can be phosphorylated and which react with FITC like the α subunit of phosphorylase kinase are present in isolated vesicles of the rabbit skeletal muscle sarcoplasmic reticulum.

Materials and methods

For preparation of SR membranes New Zealand white rabbits were anesthetised by injection of Nembutal into the ear vein and, following bleeding, hind and back muscles were excised. Homogenization and differential centrifugation was carried out according to ref. 12. Phosphorylation of these membranes was carried out in a total volume of 300 μl containing 10 mM γ-^{32}P ATP, 10 mM EDTA, 1 mM EGTA, 100 mM KCl, 200 mM Tris–HCl pH 7.6, 250 μM Vanadate and SR membranes (10 mg/ml) at 27°. Aliquots were removed at 0.5, 1, 1.5 min to determine the radioactivity bound unspecifically to the membranes. The reaction was started by addition of 16 μl, 400 mM $MgCl_2$, chelation with EGTA and EDTA yield free concentrations of 1.6 nM Ca^{2+} and 5.1 mM Mg^{2+}. At appropriate time intervals aliquots are applied to Whatman GF/C filter paper discs which were washed twice with 10% trichloroacetic acid, 1% K_2HPO_4, 1% $Na_4P_2O_7$ followed by a third wash with 10% trichloroacetic acid. The filter discs were further washed with water and acetone and dried at 100°. Finally the discs were extracted with chloroform : methanol : conc. HCl (100 : 100 : 0.6 v/v) to remove all the lipid bound radioactivity. The filter discs were washed with acetone, water and acetone. The remaining radioactivity, representing protein phosphorylation, was measured.

For SDS gel and autoradiography purposes an aliquot of 150 μl of the incubation mixture was removed to a separate tube at the 15th minute and was precipitated with trichloroacetic acid. The phosphorylated pellet was washed twice with 1 ml chloroform : methanol : HCl as previously mentioned to remove the radioactivity extractable into the organic phase. SDS-PAGE was performed using 1.5-mm-thick slab gels as described by [13] which after drying were applied on Kodak films X-OMAT-S for autoradiography.

FITC labeled SR was prepared by incubating for 3 min isolated SR vesicles (2 mg/ml) with FITC (10 μM) in 25 mM Tris–HCl, 0.1 mM EGTA pH 8.6 at 25°. The incubation was terminated by addition of 400 μl ice-cold solution containing 200 mM β-glycerophosphate, 50 mM mercaptoethanol and 10 mM ATP pH 6.5. Finally the solutions were dialyzed overnight against 200 mM sucrose, 20 mM Tris and 1 mg/ml albumin pH 7.0. FITC labeling of phosphorylase kinase was carried out according to ref. 14.

Results and discussion

Figure 1 shows the time course of phosphate incorporation into SR membrane proteins (10 mg/ml) upon incubation with γ^{32}P ATP/Mg^{2+} at 1.6 nM free Ca^{2+}. When this phosphorylation is catalysed by the endogenous kinase 0.03 moles phosphate per 100,000 g SR protein are incorporated. Addition of 300 μg/ml phosphorylase kinase increases the initial velocity as well as the total amount of incorporated phosphate approximately 2-fold. The phosphorylated proteins were identified by SDS gel electrophoresis and autoradiography. Figure 2 shows that a phosphorylated band corresponding in molecular weight to that of the α subunit of phosphorylase kinase is easily visible. On the other hand only a trace amount is detectable at the position of the β subunit. The similarity of this phosphoprotein to the α subunit of phosphorylase kinase was further extended by phosphorylation experiments with exogenously added protein kinases.

Addition of phosphorylase kinase (Fig. 2) enhances the level of incorporation into the α (Mr 145,000) and β (Mr 128,000) subunits of phosphorylase kinase. A band of Mr 94,000 corresponding to phosphorylase *a* also appears. Phosphorylase *a* is not visible when the SR membranes are phosphorylated in the presence of the endogenous kinases or the cAMP-dependent protein kinase. This observation

* Abbreviations used: PIP, phosphatidylinositol-4-phosphate; PI, phosphatidylinositol; SR, sarcoplasmic reticulum; FITC, fluorescein 5′-isothiocyanate; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

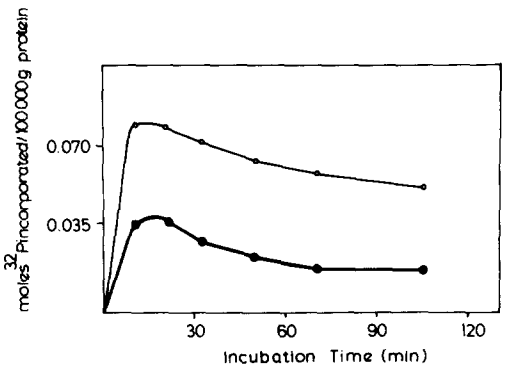

Fig. 1. SR membranes (10 ml/mg) were phosphorylated in the presence of 1.6 nM free Ca^{2+}. The phosphorylation was catalyzed by endogenously (●) or exogenously added phosphorylase kinase (300 μg/ml) (○). Samples were removed for determination of protein bound radioactivity as described in Materials and Methods.

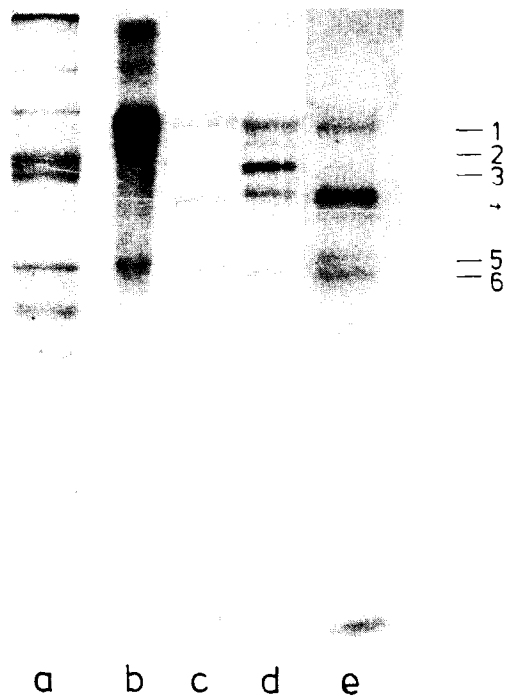

Fig. 2. ^{32}P incorporation into SR membranes by endogenously or exogenously added protein kinases. Isolated SR membranes were incubated with γ-^{32}P-ATP as described in Materials and Methods and then subjected to SDS-PAGE. After staining with Coomassie blue (lane a) the gels were dried and subjected to autoradiography (lanes b–c). Lane (b) represents autophosphorylated phosphorylase kinase; (c) phosphorylation of SR membranes proteins by endogenous protein kinases; (d) phosphorylation of SR membranes in the presence of exogenously added phosphorylase kinase (300 μg/ml) and (e) phosphorylation of SR membranes in presence of 70 μg/ml cyclic AMP dependent protein kinase. Marks at the right show the migration distanced of the following proteins: (1) α subunit of phosphorylase kinase, (2) β subunit of phosphorylase kinase, (3) phosphorylase *a*, (4) a protein of MW 88,000 corresponding to glycogen synthase, (5) possible proteolytic degradation product of α subunit of phosphorylase kinase (6) calsequestin (MW 53,000).

confirms that indeed endogenous phosphorylase kinase activity does not function as a phosphorylase *b* to *a* converting enzyme. Phosphorylase *a* formation is visible only after exogenous addition of phosphorylase kinase (compare Fig. 2, lane c and d). Bands of *ca* Mr 88,000 and Mr 53,000 corresponding to glycogen synthase and calsequestrin, respectively, are also phosphorylated both in absence and presence of exogenously added phosphorylase kinase. In these experiments the Ca^{2+}-transport ATPase was not phosphorylated since the reaction was carried out in the presence of vanadate which inhibits the aspartyl phosphate formation.

cAMP-dependent protein kinase (Fig. 2, lane e) increases the incorporation into the α subunit of phosphorylase kinase. An additional band corresponding to Mr 80,000 appears when the SR membranes are phosphorylated in presence of cAMP-dependent protein kinase, which might represent a proteolysis fragment of the α subunit of phosphorylase kinase.

FITC selectively labels the α subunit of phosphorylase kinase [14]. SR membranes labeled with the same concentration of FITC showed a number of labeled peptides (Fig. 3); the most intensively labeled polypeptide represents the major protein of SR membranes, the Ca^{2+} transport ATPase (Fig. 3, lane a, broadest band, middle). Among the other labeled peptides one protein shows approximately the same Mr as the α subunit of phosphorylase kinase. FITC reacts specifically with an ATP-binding site on the α subunit of phosphorylase kinase [15]. Therefore we conclude that the FITC labelled polypeptide of the same Mr as the α subunit represents this subunit itself.

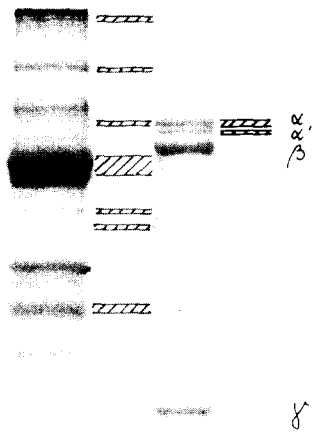

Fig. 3. SDS PAGE of FITC labeled SR membranes (150 μg). FITC labeling was carried out as described in Methods. The labeled bands were visualized under u.v. light and the pattern is schematically drawn on the side (dashed lanes). Lane a represents the FITC labeled SR-membrane proteins following Coomassie blue staining, lane b labeled phosphorylase kinase.

Immunological studies using monoclonal antibodies against the α and β subunit of phosphorylase kinase showed also the presence of both these subunits in SR membranes [16]. The data presented in this report confirm and extend these observations. It has been shown here chemically that the α and β subunits of phosphorylase kinase are associated with SR membranes. Since phosphorylase kinase exhibits phosphatidylinositol kinase activity, it might be possible that the phosphatidylinositol kinase present in these membranes is also associated with these polypeptides identified here.

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Inactivation of the carcinogen, 5-hydroxymethylchrysene, by glutathione conjugation *via* a sulphate ester in hepatic cytosol

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5-Hydroxymethylchrysene (5-HCR), a potent carcinogen [1] and a major metabolite of 5-methylchrysene in rat liver [2], has been reported by Okuda *et al.* [3] to be activated to the highly mutagenic sulphate conjugate, 5-HCR sulphate, by rat liver sulphotransferase in the presence of a 3′-phosphoadenosine 5′-phosphosulphate (PAPS)-generating system. The mutagenicity of 5-HCR towards *Salmonella typhimurium* TA98, exerted by a dialysed soluble supernatant fraction (S105) of a liver homogenate from the untreated rat in the presence of the PAPS-generating system, was much higher than that exerted by the hepatic post-mitochondrial fraction in the presence of an NADPH-generating system [3]. This strongly suggests that, so far as examined by the mutagenicity test, sulphate conjugation plays a more important role than monooxygenation in the metabolic activation of 5-HCR in untreated rat liver. 5-HCR sulphate, isolated from the incubation mixture and identified with the synthetic specimen, has been demonstrated to have potent intrinsic mutagenicity towards TA98 and also demonstrated by a fluorophotometric study of calf thymus DNA incubated with the sulphate to bind covalently to the nucleic acid through its 5-methylene carbon with loss of a sulphate anion [3].

During the course of our investigation on the metabolic activation of 5-HCR in the presence of rat liver S105 fortified with PAPS, the authors found addition of glutathione (GSH) to the S105-PAPS system retard the mutagenicity of 5-HCR towards TA98. The present communication deals with (1) metabolic inactivation of the intrinsic mutagenicity of 5-HCR sulphate by S105 in the presence of GSH, (2) isolation and characterization of a GSH conjugate formed from 5-HCR by the rat liver S105-PAPS system fortified with GSH as well as from 5-HCR sulphate by S105 in the presence of GSH, and (3) inhibition of covalent binding of 5-HCR sulphate to calf thymus DNA by S105 in the presence of GSH.

5-HCR dissolved in dimethyl sulphoxide was incubated at 37° for 20 min with S105 of a liver homogenate from male Wistar rats, weighing 160–180 g, in 0.1 M phosphate buffer, pH 7.4, containing TA98 and PAPS in the presence and in the absence of GSH. According to the method of Ames *et al.* [4], the bacterial suspension was then diluted with soft agar and placed on hard agar plates to count the number of His^+ revertant colonies appearing after 48 hr at 37°. The mutagenicity of 5-HCR exerted by the rat liver S105-PAPS system was completely retarded in the presence of GSH (Fig. 1A).

After incubation of 5-HCR under these conditions without the bacterial suspension, methanol was added to the incubation mixture (1 ml) in order to remove cytosolic proteins and most of inorganic salts. The aqueous methanolic solution was subjected to high pressure liquid chromatography (HPLC) carried out on an octadecylsilica column (Nucleosil $7C_{18}$, 7 μm in particle size, 4 × 300 mm)