Characterization of Endogenous Phosphorylation in Isolated Cardiac Sarcolemma

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Abstract. The cardiac sarcolemma contains kinases which catalyze the incorporation of ^{32}P-phosphate into acid stable and acid precipitable membrane components of low molecular weight. The phosphorylation is not influenced by cyclic AMP or calmodulin. Analysis of phosphorylation products using proteolytic digestion, organic solvent extraction, thin layer chromatography and gel filtration reveals both polypeptides and lipids as kinase substrates. Polypeptides are phosphorylated at their serine and threonine residues, while lipid phosphorylation gives rise to ^{32}P-labelled phosphatidylinositol phosphates and some nonidentified compounds. Phosphorylated polypeptides and phosphorylated lipids do not separate in SDS polyacrylamide gel electrophoresis. On the basis of the fast time course of ^{32}P-phosphate incorporation, it may be supposed that endogenous phosphorylation may play a role in the short term regulation of the cardiac sarcolemmal function.

Key words: Membrane phosphorylation — Sarcolemma — Proteolipid — Phosphatidylinositol

Introduction

Membrane phosphorylation is an important process in the regulation of cellular activities. In heart muscle interest has been focused on the phosphorylation of the sarcolemma (SL) and sarcoplasmic reticulum (SR). Cyclic AMP-dependent and Ca^{2+}/calmodulin-dependent phosphorylation has been extensively studied in isolated SL and SR, and a number of membrane-bound protein substrates have been identified (Tada and Katz 1982). So far, much less attention has been given to endogenous phosphorylation reactions that are not influenced by the second messengers cyclic AMP and calcium.

Endogenous phosphorylation occurs predominantly at low molecular weight (M_r) membrane components. The amount of ^{32}P-phosphate incorporated into these components in isolated fragments of cardiac SL is comparable to the amount of ^{32}P-phosphate incorporated into various membrane-bound substrates of cyclic

Table 2. Extraction of covalently bound ^{32}P-phosphate from phosphorylated SL membranes and estimation of phosphorylated amino acids in partial acid hydrolyzates of membranes, organic solvent extracts, and non-extractible residue.

	^{32}P-radioactivity [%]	Ser-P [% of total][a]	Thr-P [% of total][a]
Total	100.0	5.63 ± 0.51	2.02 ± 0.22
CM + ACM extract	70.5 ± 1.8	4.80 ± 0.73	1.66 ± 0.31
Residue	29.4 ± 3.3	0.69 ± 0.10	0.31 ± 0.12

[a] Values for serine phosphate (Ser-P) and threonine phosphate (Thr-P) represent experimental data that were not corrected for hydrolysis of the phosphocompounds (cf. Bylund and Huang 1976).

Table 3. Amino acid composition of the low M, phosphocompound(s) of the heart SL [Mol — %].

Asp	8.42	Met	0.87
Thr	5.85	Ile	3.42
Ser	10.48	Leu	6.75
Glu	12.22	Tyr	0.91
Pro	3.68	Phe	2.54
Gly	22.73	His	1.63
Ala	5.64	Lys	5.16
Cys	1.33	Arg	3.67
Val	4.85		

For details concerning the electrophoretic separation, extraction and amino acid determination see Material and Methods.

Endogenous phosphorylation is not confined to the membrane protein alone. The ^{32}P-phosphate label is likely found in the phospholipid species of the SL membrane. When phosphorylated membranes were treated with CM and ACM mixtures large amounts of ^{32}P-radioactivity were extracted into the organic solvents. The ratio of the radioactivity soluble in organic solvents to the residual, insoluble radioactivity was about 7 to 3 (Table 2). The same restult was obtained when CM extraction had been omitted and membranes had been extracted with ACM only.

Thin-layer chromatography of ACM extract revealed phosphatidylinositol--4-phosphate as the most highly labelled phospholipid (Fig. 4). The formation of ^{32}P-labelled phosphatidylinositol-4-phosphate was rapid. It proceeded in parallel

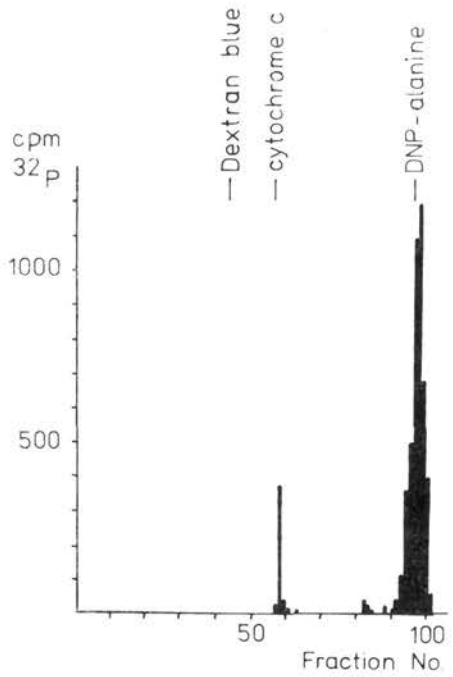

Fig. 3. Gel filtration of solubilized cardiac SL phosphocomponents on a Sephacryl S-200 column (1.5 × 86 cm). Solubilization of phosphorylated membranes and chromatography were accomplished in 4 mol.l^{-1} sodium trichloroacetate, pH 7.0. Fraction volume 1.2 ml, flow rate 2.4 ml/h.

with the overall membrane phosphorylation (Fig. 2). Traces of ^{32}P-labelled phosphatidylinositol-4,5-biphosphate were present in the organic solvent, and ^{32}P-phosphatidic acid was detected only when membrane phosphorylation was carried out in the absence of the calcium chelator EGTA (experiments not shown).

Some ^{32}P-labelled membrane components that were extracted into ACM mixtures exhibited extremly low R_f values in TLC. The mobility of these components was lower than that of any of the reference phospholipids chromatographed in parallel. It is possible that the non-identified components represent lyso-derivatives of phosphatidylinositols, or proteolipids, or even proteins. The extraction of ^{32}P-phosphate labelled proteins into the organic solvent was suggested by the presence of phosphoserine and phosphotreonine in the respective acid hydrolyzates (see Table 2).

Discussion

The present experiments have shown an endogenous kinase activity in isolated cardiac sarcolemma in the absence of external effector substances. The kinase activity appeared to be strongly associated with the membrane, since it could not

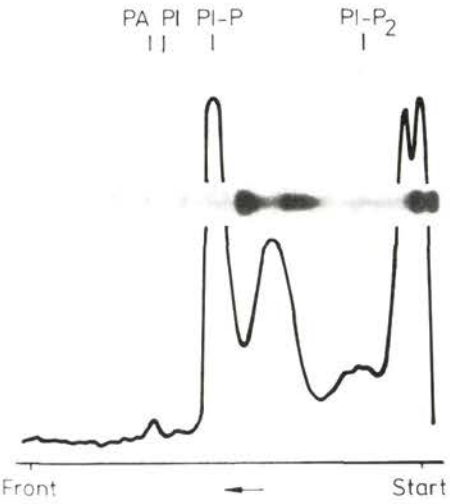

Fig. 4. TLC, autoradiograph (insert) and densitogram of ^{32}P-labelled compounds extracted from phosphorylated cardiac SL with ACM. Reference phospholipids were phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PI-P), and phosphatidylinositol-4,5-biphosphate (PI-P_2). The chromatogram was developed twice in the same solvent.

be removed by various washing and extraction procedures used for membrane preparation. This activity could be distinguished from that of the sarcolemma-bound cyclic AMP-dependent and Ca^{2+}/calmodulin-dependent protein kinases by its insensitivity to cyclic AMP and to calmodulin as well as by the unique substrates that were phosphorylated. An essential property of these phosphorylated substrates is their fast electrophoretic mobility. Although it proved impossible to separate the fast moving compounds into individual components by SDS-PAGE evidence is presented that both proteins and lipids are phosphorylated. It follows that two different classes of kinases are involved in the phosphorylation reactions: protein kinases and lipid kinases.

Phosphorylation of low M_r proteolipids by endogenous kinases has been observed before in erythrocyte membranes (Gaetjens 1976), as well as in membranes of frog skeletal muscle (Barany et al. 1977), or in membranes of frog skeletal muscle (Barany et al. 1977), and in purified Na^+, K^+-ATPase preparations of various origin (cf. Reeves et al. 1980). The phosphoproteolipid extracted by Barany et al. (1977) from skeletal muscle had an amino acid composition similar to that of the electrophoretically purified phosphocomponent(s) of cardiac SL. Its phosphorylation has been demonstrated in living muscle.

Phosphorylation of phosphatidylinositol, degradation of phosphatidylinositol phosphates, and resynthesis of phosphatidylinositol are essential elements of the

phosphatidylinositol cycle. The cycle which has been studied in varios tissues and membrane preparations, plays an important role in the action of hormones and neurotransmitters. It is thought to be associated with movements of Ca^{2+} ions across cellular membranes (Michell (1979). To our knowledge, it is for the first time that a highly active phosphatidylinositol kinase has been described in cardiac SL. The similar mobility characteristics of the phosphorylated protein and the phospholipid in SDS-PAGE suggests a strong association between them although similar electrophoretic mobilities of separate phosphoprotein and phospholipid entities cannot be ruled out.

Attention is called to a report of Feldman and Weinhold (1977). These authors succeeded in solubilizing lipoproteins from the cardiac SL using deoxycholate. Their lipoproteins contained a protein of a high electrophoretic mobility (M_r 12,000) and strongly associated phospholipids, among them phosphatidylcholine, phosphatic acid, cardiolipin, lysophosphatidylethanolamine and phosphatidylinositol. A ratio of 90 moles of phospholipid per mole of protein could be found. This complex contained a high amount of sialic acid residues, and it reacted with PAS.

Lipoproteins with M_r 10—12,000 dalton, that are tightly associated with lipid, are also present in other muscle cell membranes. They have been implicated in cation binding and cation transport. The cardiac SL complex characterized by its multiple phosphorylation sites may well belong to this class of lipoproteins.

Acknowledgement. The authors wish to thank Mr. Dettmer, Zentralinstitut für Molekularbiologie, Berlin, for the determination of the amino acid composition, and the staff of the SSB of the VE Fleischkombinat Eberswalde-Britz for their skillful and quick preparation of pig hearts.

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Received April 19, 1983 / Accepted June 8, 1983