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SOLUBILIZATION OF RAT HEART SARCOLEMMA 5′-NUCLEOTIDASE BY PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C

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Summary

The influence of a phosphatidylinositol-specific phospholipase C treatment on rat heart sarcolemmal 5′-nucleotidase was investigated. Upon complete hydrolysis of all phosphatidylinositol in the sarcolemma, 75% of 5′-nucleotidase activity was found in the solubilized form. The insolubilized enzyme after this treatment has the same K_m for AMP as the untreated, sarcolemmal-bound enzyme (0.04 mM), whereas the solubilized enzyme has a 40-fold increase in K_m for AMP (0.16 mM). Other sarcolemmal-bound enzymes were not affected by the same treatment. Hence, the specific involvement of phosphatidylinositol in the binding of 5′-nucleotidase to the sarcolemma of the rat heart is clearly demonstrated.

Introduction

The role of endogenous adenosine in the physiological regulation of coronary resistance is well-documented [1,2]. In addition, adenosine has been suggested to limit the inotropic and metabolic effects of catecholamines in myocardium [3—6]. Adenosine formation has been shown to occur mainly via dephosphorylation of AMP by 5′-nucleotidase [7]. Although this enzyme was demonstrated to be located mainly on the myocardial cell membrane [8,9], the nature of association of 5′-nucleotidase with sarcolemmal membrane

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Abbreviation: PI, phosphatidylinositol.

remains largely unknown. In this communication, we report the solubilization of the sarcolemmal-bound 5′-nucleotidase from the rat heart by treatment with a phosphatidylinositol (PI)-specific phospholipase C [10].

Materials and Methods

PI-specific phospholipase C (*Staphylococcus aureus*; 8.7 mg/ml in 0.25 M sucrose/10 mM Tris-HCl, pH 7.4, specific activity 0.08 I.U./mg at 37°C) was kindly given to Dr. M.S. Nijjar of this University by Dr. Martin G. Low, Department of Biochemistry, University of Birmingham, U.K. Phospholipase C, *Clostridium welchii* (type I, 4.3 units/mg) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. [U-^{14}C]AMP (specific radioactivity, 479.0 mCi/mmol) was purchased from New England Nuclear, Canada. Other chemicals were of analytical grade.

Cardiac sarcolemmal membrane fraction was prepared as described previously [11]. The sarcolemmal suspension (0.475 ml, 2.1 mg protein/ml) was incubated with 25 μl (0.018 I.U.) of PI-specific phospholipase C for 30 min at 37°C. After the incubation period, the reaction tubes were cooled at 0°C and an aliquot was taken to assay for 5′-nucleotidase activity. Subsequently, the incubation mixture was centrifuged at 25 000 $\times g$ for 10 min and the resultant supernatant was further centrifuged at 100 000 $\times g$ for 60 min. The supernatant was collected and the pellet washed twice with 0.25 M sucrose/10 mM Tris-HCl, pH 7.4, and resuspended in the same medium (1 mg protein/0.5 ml). 5′-Nucleotidase activity was determined in the supernatant and pellet fractions as well as in the reconstituted suspension.

Rat heart sarcolemma suspension (1 mg) was also incubated with *Clo. welchii* phospholipase C (1.33 mg) in 1 mM $CaCl_2$ and 0.2 mg bovine serum albumin (final volume, 0.5 ml) for 10 min at 30°C. The incubation was terminated by the addition of 2 mM EGTA. Fractionation of the mixture after incubation was performed as described above. Control preparations were treated identically without addition of any phospholipase.

The rate of ATP hydrolysis in sarcolemmal fraction was determined as described previously [12]. 5′-Nucleotidase was assayed according to a modified procedure of Avruch and Wallach [13]; [U-^{14}C]AMP (0.2 mM) was used as substrate and dicyclohexylammonium *p*-nitrophenylphosphate (2 mM) was added to the assay mixture to avoid the interference of non-specific phosphatase activities. The determination of K^+-stimulated *p*-nitrophenylphosphatase activity was performed as described by Lamers et al. [14]. Protein was measured by the method of Lowry et al. [15] with bovine serum albumin as standard.

Results

In order to demonstrate unequivocally that a specific association was present between phosphatidylinositol and 5′-nucleotidase, the sarcolemma from rat heart was treated separately with PI-specific phospholipase C and phospholipase C from *Clo. welchii*. As shown in Table I, 80% of 5′-nucleotidase was released from the sarcolemma by the treatment with PI-specific phospholipase

TABLE I

EFFECTS OF PI-SPECIFIC PHOSPHOLIPASE C AND *Clo. WELCHII* PHOSPHOLIPASE C ON RAT HEART SARCOLEMMA ENZYMES

Rat heart sarcolemmal fraction was prepared as described in Methods and was treated with the phospholipase C as indicated. After treatment, the mixture was centrifuged and the pellet fraction was assayed for enzyme activities. Values for control and PI-specific phospholipase C represent the mean ±S.E. of four experiments.

Treatment	Mg^{2+}-ATPase	$(Na^+ + K^+)$-ATPase	K^+-stimulated p-nitrophenylphosphatase	5′-Nucleotidase
	P_i formed ($\mu mol \cdot h^{-1} \cdot mg^{-1}$)		*p*-Nitrophenyl formed ($\mu mol \cdot h^{-1} \cdot mg^{-1}$)	Adenosine formed ($nmol \cdot min^{-1} \cdot mg^{-1}$)
Control	32.5 ± 2.0	8.7 ± 2.0	1.2 ± 0.4	75.7 ± 8.4
PI-specific phospholipase C	30.9 ± 2.4	8.3 ± 2.1	1.2 ± 0.4	14.1 ± 3.4
Phospholipase C from *Clo. Welchii*				
Exp. A	19.1	5.0	1.0	71.7
Exp. B	17.9	5.1	1.1	77.9

C, whereas other sarcolemma-bound enzymes assayed were not affected by this treatment. In contrast, treatment of the sarcolemma with the phospholipase C from *Clo. welchii*, which hydrolyzes a variety of neutral phospholipids [16], caused a general decrease in all the ATPase activities, but did not solubilize any 5′-nucleotidase or alter its activity in the sarcolemma. Phospholipid analysis of the sarcolemma upon PI-specific phospholipase C treatment showed that all PI in the sarcolemma was selectively hydrolyzed. Similar experiments with the *Clo. welchii* phospholipase C did not show any significant change in PI content (5.22 nmol lipid phosphorus/mg protein) before and after incubation, although over 70% of phosphatidylcholine and phosphatidylethanolamine in the sarcolemma was hydrolyzed.

TABLE II

SOLUBILIZATION OF 5′-NUCLEOTIDASE IN RAT HEART SARCOLEMMA BY PI-SPECIFIC PHOSPHOLIPASE C

Rat heart sarcolemma suspension was incubated with PI-specific phospholipase C as described in the text. The suspension was separated into supernatant and pellet fractions by ultracentrifugation. These fractions were reconstituted to the original volume in subsequent experiments. 5′-Nucleotidase activity was monitored in each step of treatment.

	5′-Nucleotidase activity ($nmol \cdot min^{-1} \cdot 0.5\ ml^{-1}$)	
	Control sarcolemma	Sarcolemma treated with PI-specific phospholipase C
Direct after incubation	76.1	112.8
Supernatant fraction	1.2	57.4
Pellet fraction	77.0	12.9
Reconstitution of supernatant and pellet fraction	—	122.7

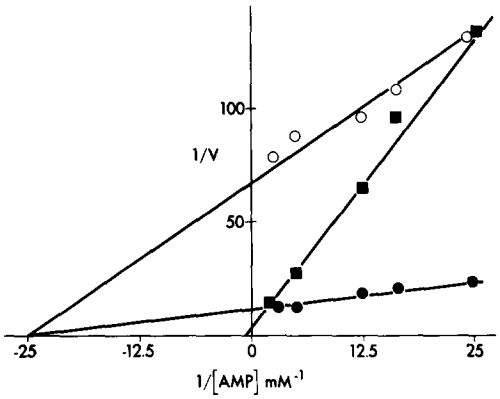

Fig. 1. Double reciprocal plots of adenosine formation at various concentrations of AMP. Rat heart sarcolemmal suspension was incubated with PI-specific phospholipase C and the mixture was subsequently centrifuged to yield a supernatant and a pellet fraction. The pellet fraction was resuspended with buffer to the same volume as the supernatant fraction. 5′-Nucleotidase activities were assayed in the supernatant fraction (■), pellet fraction (○) and in the untreated sarcolemma suspension (●). Enzyme activity is expressed as nmol of adenosine formed · min^{-1} · 0.5 ml^{-1} of each enzyme preparation.

Activation of 5′-nucleotidase activity was also observed in the sarcolemmal suspension after PI-specific phospholipase C treatment (Table II). This activation was abolished after the treated sarcolemmal suspension was separated into the supernatant and pellet fraction by centrifugation. The sum of enzyme activity in these two fractions was almost equal to enzyme activity in the untreated sarcolemmal suspension. An increase in 5′-nucleotidase activity was again observed when the supernatant and pellet fractions of the treated sarcolemmal suspension were recombined.

The K_m of 5′-nucleotidase in the supernatant and pellet fractions after PI-specific phospholipase C treatment was determined. As depicted in Fig. 1, the K_m of the enzyme in the pellet fraction was similar to that of the untreated enzyme in the sarcolemma (0.04 mM), whereas the K_m in the supernatant (solubilized enzyme) is about 40 times higher (1.6 mM).

Discussion

From the results obtained in this study, the specific dependency of rat heart sarcolemma 5′-nucleotidase on PI is clearly demonstrated. Similar results were obtained with rat liver microsomes and pig lymphocytes by PI-specific phospholipase C treatment [17]. However, the nature of enzyme activation immediately after solubilization is not clear. One possible explanation is that the enzyme may undergo conformatory changes to a more active form after solubilization, with concurrent change in V and K_m for 5′-AMP. Since further activation of enzyme activity was observed when the pellet was added to the solubilized enzyme, we postulate that the solubilized enzyme may have a requirement for lipid or other components in the pellet fraction for maximum activity. Hence, full activation was only observed when the pellet fraction was present after solubilization. Further studies on the lipid requirements

of the solubilized 5′-nucleotidase are necessary to establish this point.

The release of 5′-nucleotidase from the sarcolemmal membrane upon treatment with PI-specific phospholipase C appears to be specific, since other membrane-bound enzymes such as Mg^{2+}-ATPase, $(Na^+ + K^+)$-ATPase and K^+-stimulated *p*-nitrophenylphosphatase were not affected by this treatment. Our results indicate that the nature of binding of 5′-nucleotidase with membranes is different from that of $(Na^+ + K^+)$-ATPase. This view is supported further by the fact that the ATPase activities, unlike 5′-nucleotidase activity, were markedly decreased upon treatment of the sarcolemma with phospholipase C from *Clo. welchii*. Since 5′-nucleotidase activity was not altered by this treatment and at the same time PI was not hydrolyzed, it is conceivable for us to conclude the specific involvement of PI in the binding of 5′-nucleotidase in heart cell membrane.

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References

1 Belloni, F.L. (1979) Cardiovasc. Res. 13, 63—85
2 Rubio, R. and Berne, R.M. (1975) Progr. Cardiovasc. Dis. 18, 105—122
3 Schrader, J., Baumann, G. and Gerlach, E. (1977) Pflugers Arch. 372, 29—35
4 Wakade, A.R. and Wakade, T.D. (1978) J. Physiol. 282, 35—49
5 Dobson, J.G., Jr. (1978) Circ. Res. 43, 785—792
6 Hedqvist, P. and Fredholm, B.B. (1979) Acta Physiol. Scand. 105, 120—122
7 Arch, J.R.S. and Newsholme, E.A. (1978) Essays Biochem. 14, 83—113
8 Rostgaard, J. and Behnke, O. (1965) J. Ultrastruct. Res. 12, 579—591
9 Gordon, G.B., Price, H.M. and Blumberg, J.M. (1967) Lab. Invest. 16, 422—435
10 Low, M.G. and Finean, J.B. (1977) Biochem. J. 162, 235—240
11 McNamara, D.B., Sulakhe, P.V., Singh, J.N. and Dhalla, N.S. (1974) J. Biochem. 75, 795—803
12 Takeo, S., Duke, P., Taam, G.M.L., Singal, P.K. and Dhalla, N.S. (1979) Can. J. Physiol. Pharmacol. 57, 496—503
13 Avruch, J. and Wallach, D.F.H. (1971) Biochim. Biophys. Acta 233, 334—347
14 Lamers, J.M.J., Stinis, J.T., Kort, W.J. and Hulsmann, W.C. (1978) J. Mol. Cell. Cardiol. 10, 235—248
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
16 Bonting, S.L. and DePont, J.J.H.H.M. (1980) Biochem. Soc. Transact. 8, 40—42
17 Low, M.G. and Finean, J.B. (1978) Biochim. Biophys. Acta 508, 565—570