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SUBCELLULAR DISTRIBUTION OF PHOSPHOLIPID-SENSITIVE CALCIUM-DEPENDENT PROTEIN KINASE IN GUINEA PIG HEART, SPLEEN AND CEREBRAL CORTEX, AND INHIBITION OF THE ENZYME BY TRITON X-100.

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SUMMARY: The subcellular distribution of phospholipid-sensitive Ca^{2+}-dependent protein kinase in guinea pig heart was found to be: cytosol, 73%; microsome, 18%; plasma membrane, 9%; nuclei and mitochondria, < 0.1%. The enzyme in spleen and cerebral cortex was distributed nearly equally in the cytosolic and total (unfractionated) particulate fractions. The particulate enzyme in heart was released by EGTA (2.5 mM) alone but not by Triton X-100 (0.3%) alone, although a combination of the two was most effective. On the other hand, the particulate enzyme in spleen and cerebral cortex was released only by a combination of Triton X-100 and EGTA. Triton X-100 inhibited the enzyme, and this inhibition was reversed by phosphatidylserine (a phospholipid cofactor for the enzyme). The detergent, however, was without effect on cyclic AMP-dependent and cyclic GMP-dependent protein kinases.

Takai et al. (1) have reported in brain the presence of a Ca^{2+}-dependent protein kinase (protein kinase C) requiring phospholipid (such as phosphatidylserine), instead of calmodulin, as a cofactor. We have reported a ubiquitous occurrence of this phospholipid-sensitive Ca^{2+}-dependent protein kinase (PL-Ca-PK) in the animal kingdom (2), and the presence of its specific endogenous substrate proteins in cerebral cortex (3), heart (4,5), liver, pancreas, vas deferens and adrenal (6). In the present studies we report the subcellular distribution of PL-Ca-PK in guinea pig heart, spleen and cerebral cortex, and inhibition of the enzyme by Triton X-100.

EXPERIMENTAL PROCEDURES

Materials: Phosphatidylserine (bovine brain), cyclic nucleotides, lysine-rich histone (type III-S, corresponding to histone H1), mixed histone (type II), EGTA, and Triton X-100 were purchased from Sigma.

Methods: The procedures for the preparations of subcellular fractions (nuclei, mitochondria, plasma membrane, microsome, and cytosol) of guinea pig hearts are essentially the same as reported by others (7-11). These procedures yield the cyto-

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TABLE I

Distribution of PL-Ca-PK in cytosol and total particulate fraction of guinea pig heart, spleen and cerebral cortex, and effects of EGTA and/or Triton X-100 on release of the particulate enzyme.

Tissue	$CaCl_2$	Protein kinase activity (pmol P/min/g tissue)					
		Cytosol	Particulate[b]	Particulate treated with[a]			
				- EGTA - Triton	+ EGTA - Triton	- EGTA + Triton	+ EGTA + Triton
Heart	-	1,421	257	253	322	871	729
	+	2,746	290	232	685	895	1,443
Spleen	-	9,200	9,413	6,630	5,376	17,474	15,429
	+	14,508	9,644	7,006	8,216	18,328	27,020
Cerebral cortex	-	6,104	8,065	3,667	3,029	26,855	26,530
	+	22,975	7,821	5,553	7,270	28,843	48,136

The total particulate fraction (5.1-9.3 mg protein/ml) from tissues, obtained as described in Methods, was incubated in solution A, in the presence or absence of EGTA (2.5 mM) and/or Triton X-100 (0.3%), for 1 hr in ice. The protein released, recovered by centrifugation for 1 hr at 105,000 x g, was diluted 4-fold with solution A. Aliquots of cytosol (16-33 µg) and the untreated particulate (27-37 µg), both in solution A, and the solubilized particulate protein (2-16 µg) were assayed for the enzyme activity in 0.2 ml, in the presence of phosphatidylserine (5 µg), with or without $CaCl_2$ (0.1 µmol; 0.5 mM final concentration) as described in Methods. The final concentration, in the reaction mixture, of EGTA was 0.25 mM in all cases, and that of Triton X-100, if present, was 0.0075%. This concentration of Triton had no effect on the enzyme activity (Fig. 1). The values presented are averages of duplicate incubations, variations between assays being less than 5%. The data shown are typical of three experiments.

[a]Suspended in solution A and solubilized for 1 hr in ice under the indicated conditions, and the supernatant (105,000 x g, 1 hr) used as the enzyme source.

[b]Suspended in solution A and used directly as the enzyme source after incubation in ice for 1 hr.

solic fraction which may be too dilute for certain studies. In these cases, heart (also spleen or cerebral cortex) was directly homogenized in 3 volumes of solution A (0.25 M sucrose, 25 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$ and 50 mM mercaptoethanol) and the homogenate was centrifuged at 105,000 x g for 60 min. The resulting supernate (cytosol) and pellet (total particulate) were used as sources of PL-Ca-PK. Its activity was assayed as we reported recently (2-6,12). Briefly, the incubation system, in 0.2 ml, contained 5 µmol of Tris/HCl (pH 7.5), 2 µmol of $MgCl_2$, 0.05 µmol of EGTA, 40 µg of histone H1, 1 nmol of [γ-^{32}P]ATP (containing about 0.3-1.1 x 10^6 cpm), in the presence or absence of $CaCl_2$ (0.1 µmol) and phosphatidylserine (5 µg). The reaction was carried out at 30° for 5 min. PL-Ca-PK was purified from bovine heart extracts (over 15,000-fold and up to 95% homogeneous) through the step of phosphatidylserine-Affigel 102 affinity chromatography (12). Cyclic AMP-dependent protein kinase (A-PK) and cyclic GMP-dependent protein kinase (G-PK) were partially purified from bovine heart extracts and their activities were assayed as we reported previously (13). Protein was determined according to Lowry et al. (14) and [γ-^{32}P]ATP was prepared by the method of Post and Sen (15).

RESULTS AND DISCUSSION

Although the PL-Ca-PK activity in the cytosol of heart, spleen and cerebral cortex was readily detectable, the activity in the total particulate fraction of these

TABLE II
Subcellular distribution of PL-Ca-PK in guinea pig heart.

	Protein kinase activity (pmol P/min/g tissue)					
	Untreated[a]		Treated with EGTA and Triton			
			Supernate		Pellet	
	- Ca	+ Ca	- Ca	+ Ca	- Ca	+ Ca
Homogenate	4,557	4,722	4,312	5,814	2,739	2,753
Nuclei	371	378	45	47	148	148
Mitochondria	189	181	256	271	206	206
Microsome	179	178	299	621 (18)	140	160
Plasma membrane	221	177	285	452 (9)	237	233
Cytosol	2,432	3,726 (73)				

The subcellular fractions were diluted or suspended in appropriate volumes of solution A, treated with 2.5 mM EGTA and 0.3% Triton X-100 for 1 hr in ice, followed by centrifugation at 105,000 x g for 1 hr. The resulting pellets were taken up in the original volumes of the same solution. Aliquots (0.02 ml, containing 1-21 µg protein) were assayed for the enzyme activity in the presence of phosphatidylserine (5 µg/0.2 ml), with or without $CaCl_2$ (500 µM, in the presence of 250 µM EGTA) as indicated. The values shown in parentheses represent the percent distribution of the Ca^{2+}-dependent enzyme activity.

[a]Diluted or suspended in solution A.

tissues was not obvious unless it was treated with EGTA alone or with EGTA and Triton X-100 (Table 1). In heart, about one half of the particulate enzyme activity (stimulated by Ca^{2+} in the presence of phosphatidylserine) was released by 2.5 mM EGTA; 0.3% Triton X-100 was virtually ineffective. A maximal release of the enzyme, however, was achieved by a combination of EGTA and Triton X-100. The enzyme in the total particulate fraction of spleen and cerebral cortex, compared to that of heart, was only slightly released by EGTA alone. Although having little or no effect when used alone, Triton X-100 enhanced the ability of EGTA to release a large amount of the enzyme from the spleen and cerebral cortex particulate fraction. If one assumes a combination of EGTA and Triton X-100 could release all latent enzyme, the presence of the enzyme in heart seems to be largely localized in the cytosol, whereas that in spleen and cerebral cortex seems to be distributed nearly equally in both the cytosolic and particulate fractions (Table 1). A higher concentration (10 mM) of EGTA, used either singly or in combination with 0.3% Triton X-100, did not release additional enzyme activity (data not shown).

The enzyme in the total particulate fraction shown in Table 1 was found to be localized in microsomal and plasma membrane fractions (Table 2). Again, as in the

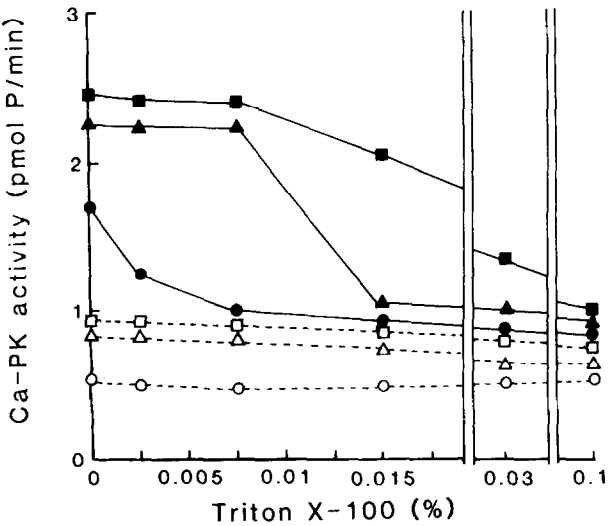

Fig. 1. Effect of phosphatidylserine on inhibition of PL-Ca-PK by Triton X-100. The enzyme, released from the guinea pig heart plasma membrane with 2.5 mM EGTA as in Table 2 (but without Triton X-100), was used. The enzyme (3 μg) was assayed (in 0.2 ml) in the presence (———) or absence(- - - -) of $CaCl_2$ (500 μM, in the presence of 250 μM EGTA), in the presence of 1 (○,●), 5 (△,▲) or 25 μg (□,■) of phosphatidylserine.

case of the total particulate fraction, the enzyme activity in these two subcellular organelles was not detected unless it was released by treatment with EGTA, or EGTA plus Triton X-100, but not Triton X-100 alone (Table 2; some data not shown). Takai et al. (1) have reported that PL-Ca-PK reversibly binds to membranes via Ca^{2+}, and this binding can be disrupted by EGTA. We reported that a similar situation also prevails for the interaction between the enzyme and phosphatidylserine-Affigel 102 (12). It is possible, therefore, that the enzyme released from the total particulate fraction or subcellular organelles by EGTA alone might be "extrinsic", whereas that released by a combination of EGTA and Triton X-100 might be "intrinsic", to the membranes.

During the course of investigation, we noted that PL-Ca-PK was inhibited by Triton X-100 used for solubilizing the particulate proteins. Further studies showed that the detergent inhibited the Ca^{2+}-stimulated activity of the enzyme from plasma membrane without affecting its basal activity, and furthermore, this inhibition was inversely related to the concentration of phospholipid cofactor present (Fig. 1). It should be mentioned here that in all assays in which Triton X-100 was present (Tables 1 and 2), its final concentrations in assay mixtures were below 0.0075%,

TABLE III

Comparative effects of Triton X-100 on various enzymes.

Triton X-100 (%)	Protein kinase activity (pmol P/min)					
	PL-Ca-PK		A-PK		G-PK	
	- Ca	+ Ca	- cAMP	+ cAMP	- cGMP	+ cGMP
0	2.9	17.5	2.4	19.5	1.8	8.1
0.1	2.8	3.1	2.4	17.2	1.8	8.0

Purified cardiac PL-Ca-PK (0.01 µg) was assayed in the presence of phosphatidylserine (5 µg/0.2 ml), with or without $CaCl_2$ (500 µM). Cardiac A-PK (10 µg) and G-PK (27 µg) were assayed with or without respective cyclic nucleotides (0.5 µM), as indicated.

which was not inhibitory to the enzyme normally assayed in the presence of 5 µg/0.2 ml of phosphatidylserine. Triton X-100 similarly inhibited the enzyme in the heart cytosol or purified from bovine heart, pig spleen and rat brain (data not shown). The detergent, however, did not inhibit A-PK or G-PK (Table 3).

Because a hydrophobic interaction between PL-Ca-PK and phosphatidylserine is presumably required for the Ca^{2+} activation of the enzyme, inhibition of the enzyme by detergents such as Triton X-100 was not totally unexpected. Nishizuka's and our groups have shown that certain lipophilic substances inhibit or, in some cases, stimulate the enzyme. Examples are phenothiazines (16-18), diolein (2,19), phospholipids (20), palmitoylcarnitine (4), adriamycin (5), W-7 (21), and melittin (22). The mechanism by which calmodulin activates many Ca^{2+}-dependent enzymes (e.g. certain phosphodiesterases and myosin light chain kinase) involves hydrophobic interactions between the cofactor (calmodulin) and the enzymes (23,24); phenothiazines (23) and W-7 (24,25) inhibit these enzymes and have been commonly referred to as calmodulin antagonists. It appears that the hydrophobic interaction is likely a common feature for the Ca^{2+}-dependent enzymes requiring either phospholipid or calmodulin as a cofactor, and that phenothiazines and W-7 are not selective inhibitors of calmodulin. This notion is further supported by our findings that palmitoylcarnitine (4), adriamycin (5), trifluoperazine (18) and melittin (22) all similarly inhibit Ca^{2+}-dependent phosphorylation, stimulated by phosphatidylserine or calmodulin, of an exogenous substrate (histone) or a variety of endogenous proteins. In addition,

Triton X-100 has been reported by others to inhibit calmodulin/Ca^{2+}-stimulated phosphodiesterase from heart (26), similar to its inhibition of PL-Ca-PK shown in the present studies.

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