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ACTIVATION OF THE CYCLIC NUCLEOTIDE PHOSPHODIESTERASE FROM RAT HEART CYTOSOL BY PHOSPHOLIPASE C

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SUMMARY : Phospholipase C (clostridium perfringens) significantly increased the cyclic nucleotide phosphodiesterase activity of a crude 105 000 g supernatant from rat heart. This activation only concerned the basal activity of cyclic GMP phosphodiesterase determined with 0.25 µM cyclic GMP as substrate, in the presence of EGTA, whereas stimulation was found to be independent of EGTA when phosphodiesterase activity was measured with 0.25 µM cyclic AMP. Similar qualitative results were found for the three cytosolic forms of phosphodiesterase separated from rat heart supernatant by isoelectric focusing. Supplementary experiments provided evidence that the activation of the cyclic AMP-specific phosphodiesterase was attributable to Phospholipase C activity and not to contaminating protease(s). In contrast, the stimulation of the cyclic GMP phosphodiesterase activity appeared to be largely dependent on the proteolytic activity of commercial Phospholipase C. Phosphatidic acid also significantly increased the cyclic AMP phosphodiesterase activity of the rat heart cytosol. These results suggest that the activation of cardiac cyclic AMP phosphodiesterase may be related to changes in phospholipid metabolism, notably the accumulation of phosphatidate, and relevant to physiological regulatory processes.

INTRODUCTION : It is now well documented that the calmodulin-dependent forms of cyclic 3'-5' nucleotide phosphodiesterase (EC 3.1.4.17) can be activated by proteolytic enzymes as well as by phospholipids and various fatty acids (1). Such a stimulation by phospholipids as lysolecithin or lysophosphatidylinositol has been frequently reported for crude enzyme preparations from rat brain (2) and for the activator-deficient phosphodiesterase purified from rat (3), bovine (4,5) and porcine (6) brain cytosolic fractions. Similarly, phosphatidylserine, used in well defined experimental conditions can activate the calmodulin-deficient phosphodiesterase from rat brain (7,8). In contrast, the cytosolic calcium -independent forms of phosphodiesterase from various organs (lung, liver) are not affected by phospholipids, while such a calcium-independent form, separated from bovine heart, is non-competitively inhibited by some phospholipids or by D-L Palmitoylcarnitine (9). More recently, the high affinity form of the membrane-bound cyclic AMP phosphodiesterase from rat brain (10) and from the microsomal fraction of rat adipocytes (11) was shown to be stimulated by lipidic extracts (10) or by phosphatidylserine (11). On the other hand, little is known about the

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effect of Phospholipase C on the activity of cyclic nucleotide phosphodiesterase. A two-fold increase in the membrane-bound phosphodiesterase activity under Phospholipase C treatment, was reported by Baba et al. (12) in the rat cerebral cortex. Pichard and Cheung (4) observed a stimulatory effect of Phospholipase C on the activator-deficient phosphodiesterase from bovine brain while Tai and Tai (5), Wolf and Brostrom (6), did not find any effect on similar preparations from bovine and porcine brain. These discrepant results and the lack of information concerning the effects of Phospholipase C on the other forms of cytosolic phosphodiesterase prompted us to investigate the influence of a Phospholipase C treatment on the cyclic nucleotide phosphodiesterase activity from rat heart cytosol. Since commercially available preparations of clostridium perfringens Phospholipase C are often contaminated by enzymatic proteins and especially by proteases (13), we have attempted to discriminate between the proteolytic and catalytic effects of Phospholipase C upon the phosphodiesterase activity. In addition, we have examined the effect of the products of Phospholipase C-catalyzed reaction on the cytosolic phosphodiesterase activity.

MATERIALS AND METHODS : Heart cytosolic fractions prepared as described in (14) were incubated in the presence of clostridium perfringens Phospholipase C (0.05 to 5 $U.mg^{-1}$ soluble proteins) in a reaction mixture containing 40 mM Tris-HCl buffer, pH 8.0, and 0.5 mM $CaCl_2$, at 30°C, for various lengthes of time. 1 mM EGTA in 40 mM Tris-HCl buffer, pH 8.0 was then added and phosphodiesterase activity was assayed immediatly after appropriate dilution of the reaction mixture. Alternatively, some experiments were performed without $CaCl_2$ and EGTA addition. Control preparations were treated identically with either boiled Phospholipase C or Tris-HCl buffer. In both cases, similar control values were obtained. In some experiments, the three main phosphodiesterase forms of cardiac cytosol were separated by preparative isoelectric focusing as in (14). Phospholipase C - pretreatment of the separated forms of phosphodiesterase was performed on 50-100 µl aliquots (1.2 µg proteins) with or without $CaCl_2$ and EGTA.

The protease activity of commercial Phospholipase C preparations was systematically determined, according to a general procedure described by New England Nuclear Co. (15), with [^{14}C]-labeled casein as substrate. Various dilutions of standard protease trypsin, or Phospholipase C, were incubated in 40 mM Tris HCl buffer pH 8.0 with substrate solution of casein (2 mg/ml) containing [methyl ^{14}C] methylated α-casein (2.5 nCi/assay), at 30°C, for 60 min, with or without protease inhibitors. The reaction was stopped by addition of 10 % TCA to precipitate unreacted substrate. After 20 min. at 4°C, the precipitate was removed by centrifugation (10.000 g for 10 min.). Aliquots of supernatant fractions were counted by a liquid scintillation technique. Blank values (without added protease) were usually less than 2 %. Linear relationships between the amount of added protease and the amount of non TCA-precipitable casein were found up to 30-40 % of the total casein . Phospholipase C was assayed by the spectrophotometric method of Kurioka and Matsuda (16) using p-nitrophenylphosphorylcholine as a chromogenic substrate in a 60 % glycerol medium, with or without 1-10 phenanthroline. Cyclic nucleotide phosphodiesterase activity was assayed as reported in (14,17) following a modified method based on the original procedure of Thompson and al. (18).

RESULTS AND DISCUSSION : The preincubation at 30°C of a crude 105 000 g supernatant fraction from rat heart with Phospholipase C (1 U/mg cytosolic proteins), induced a stimulation of phosphodiesterase activity (Fig. 1A-1B). When phospho-

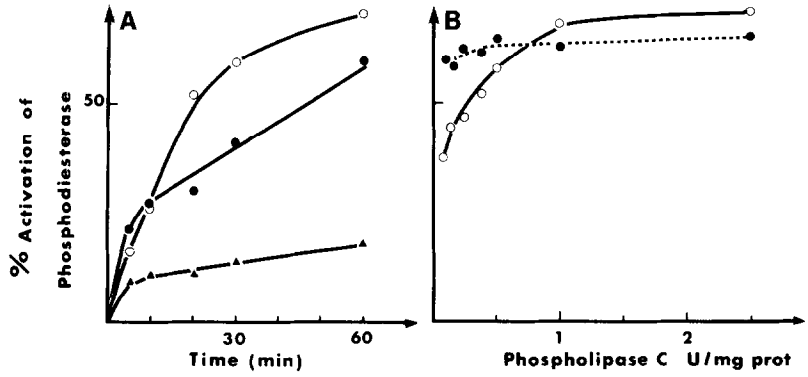

Fig. 1 : Effect of Phospholipase C pretreatment on the cyclic nucleotide phosphodiesterase activity from rat heart cytosol.
A : activation of cyclic nucleotide phosphodiesterase as a function of preincubation time (Phospholipase C: 1U/mg cytosolic proteins, temp.: 30°C). B : activation of cyclic nucleotide phosphodiesterase as a function of Phospholipase C concentration in the preincubation medium (preincubation time: 60 min, temp.: 30°C). Phosphodiesterase assays were performed in triplicate, in the presence of 1 mM EGTA, with : •—• 0.25 µM cyclic AMP, o—o 0.25 µM cyclic GMP, ▲—▲ 25 µM cyclic GMP ,as substrate.

diesterase activity was measured with 0.25 µM cyclic AMP as substrate, the stimulation (60-70 % after a 60 min. period,Fig. 1A), was observed either in the presence or in the absence of EGTA. With 0.25 µM cyclic GMP as substrate, this activation only was observable in the presence of EGTA. With 25 µM of either cyclic nucleotide as substrate the stimulation by Phospholipase C measured in the presence of EGTA did not exceed 10-15 % (Fig. 1A). The 105 000 g preparation, incubated at 30°C without Phospholipase C or with boiled Phospholipase, during the same time, did not show any significant variation of phosphodiesterease activity in each substrate condition. In addition, Phospholipase C preparation did not exhibit any phosphodiesterease activity. The relationship between the amount of Phospholipase C in the preincubation medium and phosphodiesterase activity was investigated over a 0.05 - 2.5 U per mg cytosolic proteins range. The stimulation of cyclic AMP phosphodiesterase activity did not appear to be dose-dependent, in this range of Phospholipase C concentrations, whereas the activation of cyclic GMP phosphodiesterase activity determined in the presence of EGTA was found to be dose-dependent (Fig. 1B). Higher concentrations of Phospholipase C usually gave lower activation of both cyclic AMP and cyclic GMP phosphodiesterase activities.

The rat heart cytosolic fraction possesses three distinct forms of cyclic nucleotide phosphodiesterase (14,19). As established in a previous work (14), assays performed with 0.25 µM cyclic AMP essentially reflect the activity of the cyclic AMP-specific form, assays performed with 0.25 µM cyclic GMP reflect the activity of both the calmodulin-dependent (pI 4.9) and the less substrate-specific form of pI 5.45, while assays performed with 25 µM of either substrate preferentially

traduce the activity of the pI 5.45 form. So, the activation of cytosolic cyclic nucleotide phosphodiesterase activity by Phospholipase C treatment reported in the present work seems to preferentially concern the cyclic AMP-specific form and the basal activity of the cyclic GMP-specific, calmodulin-dependent form of phosphodiesterase. The pI 5.45 form appears to be rather insensitive to Phospholipase C as indicatedby the lack of effect observed when phosphodiesterase was assayed with 25 µM cyclic nucleotide as substrate or with cyclic GMP (0.25 µM) in the absence of EGTA.

In order to confirm or infirm these results we further investigated the effect of Phospholipase C on the three forms of phosphodiesterase separated from rat heart cytosol by isoelectric focusing. The various cyclic-AMP-specific peaks focusing between pI 5.55 and 6 proved to be less sensitive (+ 15 %) to Phospholipase C pretreatment (30°C for 30 min.) than the cyclic AMP phosphodiesterase activity of the cytosol. The weakness of Phospholipase C effect on the separated cyclic AMP phosphodiesterase strongly suggests the occurrence of an additional metabolic step, necessary to phosphodiesterase activation, and requiring elements only present in crude preparations. The pI 5.45 form did not exhibit any sensitivity to Phospholipase C. The cyclic GMP-hydrolyzing peak of pI 4.9 showed a net increase in its basal phosphodiesterase activity (75 %), while no variation occurred in its fully calcium plus calmodulin-activated state, as observed with the unseparated supernatant. Thus, as shown in Fig. 2, Phospholipase C preatreat-

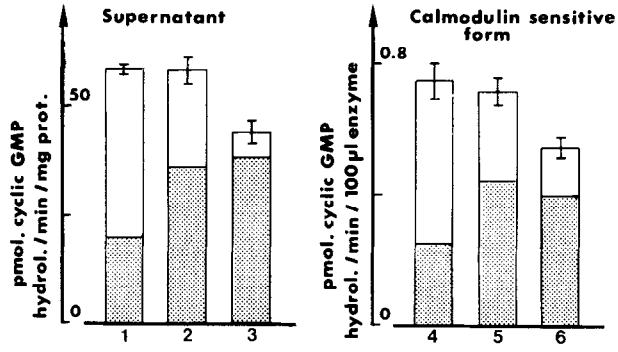

Fig. 2 : Comparative effects of phospholipase C and Trypsin on the cyclic GMP phosphodiesterase activity of a crude 105 000 g supernatant from rat heart and on the calmodulin-sensitive form of pI 4.9 separated by isoelectric focusing (assays with 0.25 µM cyclic GMP).
Aliquots of supernatant were preincubated with: (1) Tris-HCl buffer= control, (2) Phospholipase C: 5 U/mg cytosolic proteins, (3) Trypsin: 25 µg/mg cytosolic proteins. Aliquots of the calmodulin-sensitive form of phosphodiesterase (100 µl) were preincubated with: (4) Tris-HCl buffer = control, (5) Phospholipase C (0.32 U), (6) Trypsin (0.1 µg) Preincubations with Phospholipase C were performed for 30 min at 30°C (2,5). Preincubations with trypsin were performed for 5 min at 0°C and stopped by 0.1 mg Soybean trypsin inhibitor (3,6). Similar control values were obtained when preincubations with Tris-HCl buffer were performed for 30 min at 30°C or for 5 min at 0°C. Results are expressed as means of two separate experiments made in triplicate. The shaded part of diagrams indicates phosphodiesterase activity level measured in the presence of 1 mM EGTA

ment induced an apparent loss in EGTA sensitivity with the crude preparation as well as with the partly purified calcium-dependent form, in a manner reminiscent of the effect of trypsin already described for brain enzyme (20). In contrast with the proteolytic effect of trypsin, Phospholipase C did not affect the calcium plus calmodulin-level of cardiac phosphodiesterase.

Our use of commercial Phospholipase C preparations without further purification in the above experiments, raises question about the enzyme activity responsible for the phenomena described. To assess the possibility that a contaminating protease activity might have produced these results, several experimental approaches were taken. In a first approach, the proteolytic activity of Phospholipase C was determined, by a highly sensitive radioisotopic method, comparatively with that of a reference proteolytic enzyme : trypsin, and was found 5000 - 10 000 fold lower. Thus, 1 mg Phospholipase C was found to be equivalent to 0.16 $\pm$ 0.03 µg trypsin (n = 14). Several protease inhibitors were tested for their ability to overcome the proteolytic activity of both Phospholipase C and trypsin preparations. As shown in Table I, the mixture of protease inhibitors : PMSF 1 mM + TLCK 40 µM + Benzamidine 50 mM was able to entirely block the proteolytic activity of concentrated Phospholipase C solutions (6 mg i.e. 48 U/ml). It was checked that these inhibitors do not notably prevent the catalytic action of Phospholipase C (not shown). Bovine serum Albumin addition

Table I : Comparative effects of protease inhibitors on the proteolytic activity of Trypsin and Phospholipase C

Inhibitors	% residual proteolytic activity	
	Trypsin 1 µg/ml	Phospholipase C 6 mg/ml.
none	100	100
Benzamidine 5 mM	14	90
Benzamidine 20 mM	2	74
Benzamidine 50 mM	nd	49
PMSF 1 mM	87	60
Benzamidine 8 mM + PMSF 1 mM	nd	33
TLCK 4.5 µM	100	58
TLCK 9 µM	100	56
TLCK 30 µM	100	58
PMSF 1 mM + TLCK 30 µM	nd	27
Pepstatin 16.7 mg/ml	100	78
NaF 50 mM	nd	92
BSA 0.33 mg/ml	100	100
Benzamidine 20 mM + NaF 20 mM + PMSF 0.5 mM + TLCK 10 µM	0	32
Benzamidine 50 mM + PMSF 1 mM + TLCK 40 µM	0	1

The assays were performed in duplicate as described in Materials and Methods. Data are representative of two to five separate experiments.
nd : not determined; PMSF : phenyl methylsulfonyl fluoride; TLCK : Nα p-tosyl-L-lysin chloromethylketone; BSA : Bovine serum albumin.

Table II : Effect of protease inhibitors on the activation of rat heart cytosolic cyclic nucleotide phosphodiesterase by Phospholipase C

Pretreatment (30°C - 60 min)	Cyclic nucleotides hydrolyzed /min /mg proteins		
	cyclic AMP 0.25 µM	cyclic GMP 0.25 µM	
	- EGTA	- EGTA	+ EGTA (1mM)
control	63.5 ± 1.6	68.1 ± 1.4	26.6 ± 2.4
Phospholipase C 5 U/ mg prot.	91.0 ± 4	68.5 ± 2.1	39.9 ± 0.6
% activation	43.3	0	50
Benzamidine 50 mM + PMSF 1 mM + TLCK 40 µM	46.5 ± 1.5	58.1 ± 1	25.6 ± 3.3
Benzamidine 50 mM + PMSF 1 mM + TLCK 40 µM + Phospholipase C 5 U /mg prot.	69.0 ± 3	57.2 ± 1.7	25.7 ± 0.7
% activation	48.4	0	0

The mixture of protease inhibitors was added in the preincubation medium with or without Phospholipase C adjunction. Phosphodiesterase activity was then determined after appropriate dilutions. Results are expressed as the means of two separate experiments made in triplicate.

to the assay, although sometimes employed with this aim, did not prevent at all proteolysis (Table I). As indicated in Table II, the stimulation of cyclic AMP phosphodiesterase activity was not suppressed by the adjunction of proteolytic inhibitors. In contrast the activation of the basal level of cyclic GMP phosphodiesterase (in the presence of EGTA) was totally abolished. Thus, the activation of cyclic AMP phosphodiesterase, by Phospholipase C pretreatment is very likely to be due to the catalytic activity of Phospholipase C while its contaminating proteolytic activity appears more probably responsible for the increase in cyclic GMP phosphodiesterase activity (basal level). A final approach has involved the used of 1,10-phenanthroline to inhibit Phospholipase C activity through the chelation of the zinc ion, a cofactor essential for its enzymatic activity (16, 21). In preliminary experiments, we verified as described in Materials and Methods, that 2 mM 1,10-phenanthroline actually inhibit the catalytic activity of increasing amounts of Phospholipase C. A 90 % inhibition was observed in all the cases. When unseparated supernatant from rat heart was preincubated with various amounts of Phospholipase C in the presence of 1,10-phenanthroline, the activation of cyclic nucleotide phosphodiesterase activity was substantially overcome (Fig. 3). The decrease in the stimulation of phosphodiesterase activity proved evident when cyclic AMP 0.25 µM was used in the phosphodiesterase assay but was still observable with cyclic GMP 0.25 µM in the presence of EGTA. Although the stimulation of basal cyclic GMP phosphodiesterase appears essentially as the result of proteolysis (Table II), experiments performed in the presence of 1-10-phenan-

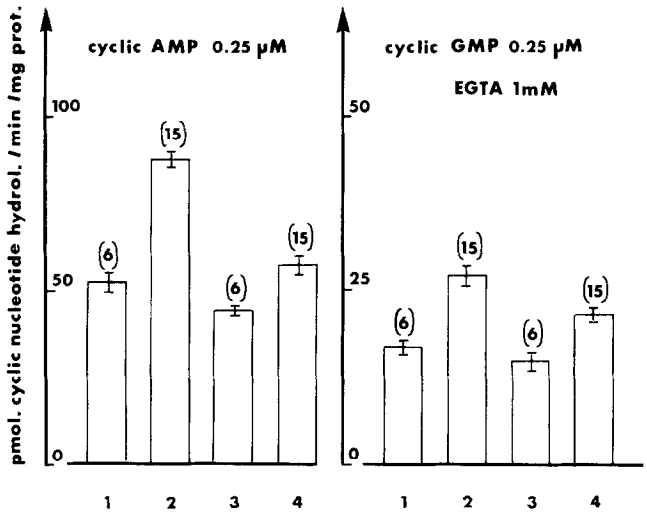

Fig. 3 : Effect of 1-10 phenanthroline on the activation of cardiac cyclic nucleotide phosphodiesterase by Phospholipase C.
Aliquots of supernatant were preincubated for 60 min at 30°C with: (1) Tris-HCl buffer = control, (2) Phospholipase C (1 U/mg cytosolic proteins), (3) 2 mM 1-10 phenanthroline, (4) 2 mM 1-10 phenanthroline + Phospholipase C (1 U/mg cytosolic proteins). Cyclic nucleotide phosphodiesterase activity was measured with 0.25 µM cyclic AMP or 0.25 µM cyclic GMP in the presence of 1 mM EGTA. Data between brackets indicate the number of experiments.

throline do not allow us to exclude a possible participation of the catalytic activity of Phospholipase C in enzyme activation.

In additional experiments we have examined the influence of products of Phospholipase C-catalyzed reaction on the phosphodiesterase activity of rat heart supernatant (Table III). Neither phosphorylcholine nor diacyl-glycerols exhibited any influence on cyclic AMP hydrolysis by the unseparated supernatant from rat heart. In contrast, phosphatidic acid induced a substantial activation of cyclic AMP phosphodiesterase activity, which became evident starting from 10 µM and reached 44 % at 500 µM. Cyclic GMP phosphodiesterase (assayed without EGTA) was not affected. Such influence of phosphatidic acid on the cyclic AMP-specific, calmodulin-independent form of the enzyme has never been reported before. Discrepant reports concerning the effect of phosphatidic acid on the calmodulin-sensitive forms of the enzyme have already appeared. Thus, Tai and Tai (5) did not find any effect of 100 µM phosphatidic acid on bovine brain phosphodiesterase activity while Nemecek and Honeyman (22) reported a 3-fold increase in the activity of a similar form purified from VA-13 fibroblasts. In our experiments, the calmodulin-sensitive cyclic GMP phosphodiesterase from rat heart supernatant assayed in the presence of EGTA proved to be only weakly sensitive to phosphatidic acid stimulation (Table III).

The specific stimulation of the cytosolic, cyclic AMP-specific, phosphodiesterase activity from rat heart by Phospholipase C pretreatment might

Table III : Effect of phospholipid metabolites and ATP on the phosphodiesterase activity of rat heart cytosol, in percent of control activity

Pretreatment or addition[a]	Substrate 0.25 µM		
	cyclic AMP	cyclic GMP	
	- EGTA	- EGTA	+ EGTA (1mM)[b]
control	100	100	43
Phospholipase C 5 U/mg prot.	143	106	61
ATP 1 mM	87 (100)[c]	87 (100)	37
Phospholipase C 5 U/mg prot. + ATP 1 mM	154 (177)	112 (129)	64
Diolein 50 µM	97	95	41
Dipalmitin 50 µM	106	94	42
Phosphatidic acid 10 µM	112	100	45
500 µM	144	65	54
Phosphorylcholine 500 µM	102	nd	nd

[a]ATP and Phospholipase C were added to the preincubation medium, in the same conditions as in Table II. Other reagents tested were directly added to the phosphodiesterase assay medium. [b]Activities in the presence of EGTA are expressed as percent of control value measured without EGTA. [c]Data under brackets are expressed as percent of plus ATP values. Phosphodiesterase activity was determined in triplicate.

involve the in situ formation of phosphatidic acid from diacylglycerol via endogenous diacylglycerolkinase as proposed by Honeyman et al. (23). Indeed, in additional experiments we observed a marked potentiation of Phospholipase C stimulation in the presence of 1 mM ATP (Table III). An alternative interpretation can be proposed. Phospholipase C might induce the formation of a metabolite which would activate a phosphorylating process via proteine kinase stimulation, with a resultant increase in phosphodiesterase activity. The existence of a phospholipid-sensitive calcium-dependent protein kinase activated by diacylglycerol in the rat heart cytosol (24) suggests the possibility that changes in diacylglycerol or phosphatidic acid are linked to various metabolic regulations and might participate to the control of phosphodiesterase activity. This proposal could explain the weakness of stimulation observed when Phospholipase C is directly added to the purified cyclic AMP-specific form of phosphodiesterase. Thus, the stimulation of the cyclic AMP-specific phosphodiesterase activity by Phospholipase C in the rat heart cytosol seems to occur through an indirect process which involves the participation of phosphatidic acid or possibly diacylglycerol, whereas the activation of calmodulin-sensitive cyclic GMP phosphodiesterase activity appears rather to depend on the contaminating proteolytic activity of commercial Phospholipase C. Although part of our proposed mechanism remains somewhat speculative it may serve as a useful working hypothesis for further experimental work.

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