Inactivation of Gastric and Pancreatic Lipases by Diethyl *p*-Nitrophenyl Phosphate

Hervé Moreau,[‡] André Moulin,[‡] Youssef Gargouri,[‡] Jean-Pierre Noël,[§] and Robert Verger*[,‡]

Centre de Biochimie et de Biologie Moléculaire, Centre National de la Recherche Scientifique (CNRS), 31 chemin Joseph Aiguier, 13402 Marseille, France, and Service des Molécules Marquées, Commissariat à l'Energie Atomique, Centre d'Etudes Nucléaires de Saclay, 91191 Gif sur Yvette Cedex, France

Received March 15, 1990; Revised Manuscript Received July 5, 1990

ABSTRACT: Reacting gastric and pancreatic lipases with mixed diethyl *p*-nitrophenyl phosphate/bile salt micelles resulted in a stoichiometric inactivation of these enzymes as tested on emulsified tributyroylglycerol and trioleoylglycerol as substrates. Diethyl *p*-nitrophenyl phosphate treated gastric lipases were also inactive on water-soluble *p*-nitrophenyl acetate, whereas the modified pancreatic lipase was still able to hydrolyze this water-soluble substrate. The binding of diethyl *p*-nitrophenyl phosphate modified pancreatic and gastric lipases to tributyroylglycerol/water interface was comparable to that of native lipases. The essential free sulfhydryl group of gastric lipases underwent no chemical changes due to the reaction with micellar diethyl *p*-nitrophenyl phosphate. All in all, these results indicate that, in both gastric and pancreatic lipases, the essential serine residue which was stoichiometrically labeled by this organophosphorus reagent is involved in catalysis and not in lipid binding.

Preduodenal lipolysis has been described in several mammals since the beginning of this century, but its physiological importance was underestimated for a long time. The authors of many studies undertaken from 1970 onward have shown an increasing interest however in the enzymes involved in preduodenal lipolysis (Hamosh, 1984; Gargouri et al., 1989). In vitro studies showed that HGL[1] prehydrolysis of intralipid emulsion facilitates its subsequent degradation by HPL (Gargouri et al., 1986). Four preduodenal lipases have been purified: rat lingual lipase (Field & Scow, 1983; Roberts et al., 1984), human gastric lipase (HGL) (Tirruppathi & Balasubramanian, 1982), bovine pharyngeal lipase (Bernbäck et al., 1985), and rabbit gastric lipase (RGL) (Moreau et al., 1988a)). Rat lingual lipase (Docherty et al., 1985) and HGL (Bodmer et al., 1987) were cloned and expressed in *Escherichia coli* or yeast. The amino acid sequences of these two lipases were deduced from the cDNA sequence, and a high degree of homology (up to 80%) was found to exist between these two enzymes; however, no sequence homology was detected with other mammalian or microbial lipases, except for a pentapeptide (Gly-X-Ser-X-Gly) which is conserved in all the known lipases (Komaromy & Schotz, 1987; Lowe et al., 1989; Shimida et al., 1989). The conserved serine within this pentapeptide corresponds to residue 152 in porcine pancreatic lipase (PPL) (De Caro et al., 1981) as well as in human pancreatic lipase (HPL) (Winkler et al., 1990) and was numbered 153 in the HGL and rat lingual lipase sequences.

In previous studies, it has been shown that organophosphorus compounds such as diethyl *p*-nitrophenyl phosphate (E600) are able to stoichiometrically inactivate PPL (Desnuelle et al., 1960; Maylié et al., 1972; Rouard et al., 1978). This chemical inactivation of pancreatic lipase occurred exclusively with mixed E600/bile salt micelles and in the presence of colipase (Rouard et al., 1978). Guidoni et al. (1981) have shown that Ser 152 in PPL can be specifically labeled with E600. Furthermore, it was reported by Chapus and Sémériva (1976) that E600-modified PPL (DP-lipase) had a decreased interfacial binding capacity onto siliconized glass beads but was still able to hydrolyze a water-soluble substrate such as *p*-nitrophenyl acetate. Without any restriction, these authors extrapolated their results obtained with siliconized glass beads to lipid/water interfaces and speculated that the essential serine 152 of PPL was involved in the lipid binding domain, and not in the catalytic site. From these initial speculations, it came to be generally assumed that the homologous pentapeptide Gly-X-Ser-X-Gly which is to be found in all the known lipase sequences might be part of the lipid binding domain of these lipases (Verger, 1984; Sikk et al., 1985; Desnuelle, 1986; Maraganore & Heinrikson, 1986; Komaromy & Schotz, 1987; Chapus et al., 1988; Lowe et al., 1989; Shimida et al., 1989). It did seem rather puzzling however that the only preserved stretch of sequence detected in all lipases was apparently the lipid binding domain and not the catalytic site. Finally, the first three-dimensional crystallographic structures of human pancreatic (Winkler et al., 1990) and *Mucor miehei* (Brady et al., 1990) lipases have recently been solved. From these two lipase structures, it is now clear that essential serine 152 is part of the classical Asp/His/Ser triad and probably constitutes the nucleophilic residue essential for catalysis. The spatial arrangement of the proposed catalytic triad in these lipases is very similar to the catalytic triad of classical serine esterases, such as trypsin. The widespread distribution of Gly-X-Ser-X-Gly pentapeptide in the primary structures of all the known lipases and the apparent contradiction between previous interpretations of the interfacial binding data and the three-dimensional structure of pancreatic lipase have brought the question of potential role of serine 152 very much to the forefront. We therefore reinvestigated the inactivation of PPL using radiolabeled E600 and extended this study to gastric

* Corresponding author.
‡ Centre National de la Recherche Scientifique.
§ Centre d'Etudes Nucléaires de Saclay.

[1] Abbreviations: PPL, porcine pancreatic lipase; HPL, human pancreatic lipase; HGL, human gastric lipase; RGL, rabbit gastric lipase; E600, diethyl *p*-nitrophenyl phosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); 4-PDS, 4,4′-dithiopyridine; DP-lipase, diethyl phospho lipase; TNB-lipase, thionitrobenzoyl lipase; TP-lipase, thiopyridyl lipase; SDS, sodium dodecyl sulfate; p-NPA, *p*-nitrophenyl acetate; NaTDC, sodium taurodeoxycholate; BSA, bovine serum albumin.

0006-2960/91/0430-1037$02.50/0

lipases such as HGL and RGL.

Materials and Methods

Products. ^{14}C-Uniformly radiolabeled E600 was prepared by the Commissariat à l'Energie Atomique (Saclay, France), with a specific radioactivity of 94.2 mCi/mmol.

HGL, RGL, and PPL containing saturating amounts of colipase were purified at our laboratory as described previously by Tirruppathi and Balasubramanian (1982), Moreau et al. (1988a), and Verger et al. (1969), respectively.

Enzymatic Assays. The lipolytic activity of these three lipases was measured titrimetrically at 37 °C with a pH stat (Radiometer TTT80), with tributyroylglycerol emulsion as substrate, under optimized conditions for each lipase: pH 6.0 in the case of gastric lipases (Moreau et al., 1988a) and pH 8.0 in the presence of colipase in the case of PPL (Rathelot et al., 1981). One unit corresponds to one micromole of fatty acid released per minute.

The esterase activity of the lipases was measured with *p*-nitrophenyl acetate (p-NPA) as substrate, according to a modified version of Erlanson's method (Erlanson, 1970). One milliliter of a p-NPA solution in methanol (10 mg/mL) was added to 50 mL of a 50 mM acetate buffer, pH 5.0 (solution A). The assays were performed either at pH 5.0 with gastric lipases (1 mL of solution A added to 2 mL of 0.5 M acetate buffer, pH 5.0, and 6 mM NaTDC) or at pH 7.4 with PPL (1 mL of solution A added to 2 mL of 0.5 M Tris buffer, pH 7.4, and 6 mM NaTDC). The enzymatic activity was estimated spectrophotometrically, using a molar absorption coefficient of 5000 at 348 nm or 16 000 at 400 nm, for assays at pH 5.0 and 7.4, respectively. One unit corresponds to one micromole of *p*-nitrophenol released per minute.

Inactivation of Lipases by Radiolabeled E600 (Rouard et al., 1978). The lipases (2 mg/mL final concentration, 40 μM) were dissolved in 50 mM acetate buffer, 50 mM NaCl, 25 mM $CaCl_2$, and 3 mM taurodeoxycholate, pH 6.0. Radiolabeled E600 at a final concentration of 42 mM (4 mCi/mL) was dissolved in acetonitrile. A 0.62-mL aliquot of a lipase solution (25 nmol) was incubated with 50 μL of radiolabeled E600 (2.1 μmol, 200 μCi) at 25 °C. The E600 to lipase molar ratio was 84.

The residual lipase activity was measured as a function of time by sampling from the incubation medium. After 1 h (RGL) or 5 h (HGL and PPL) of incubation time, gel filtration chromatography (FPLC Pharmacia, Superose 12 column) was performed to remove the excess radiolabeled E600. The protein concentration of the modified DP-lipases was determined spectrophotometrically at 280 nm, on the basis of their respective molar absorption coefficients ($E^{1\%}$ = 15.8 for gastric lipases, and $E^{1\%}$ = 13.5 for PPL). The ^{14}C radioactivity associated with the lipase was measured by using a β scintillation counter (Beckman, LS 3800). The stoichiometry of the radiolabeling by E600 was calculated by using a specific radioactivity of 94.2 Ci/mol.

Double Labeling of Gastric Lipases with Radiolabeled E600 and 4-PDS. HGL and RGL were first labeled by radiolabeled E600 as described above, and the excess free reagent was removed by gel filtration (FPLC Pharmacia, Superose 12 column, 20 mM Tris-HCl buffer, pH 8.0). Sulfhydryl group titration in gastric lipases was then performed spectrophotometrically (324 nm) using 4-PDS, as described previously (Gargouri et al., 1988; Moreau et al., 1988b). Excess of unreacted 4-PDS was removed by gel filtration (Pharmacia FPLC, Superose 12 column, 20 mM Tris-HCl buffer, pH 8.0), and the ^{14}C radioactivity associated with the lipase samples was measured. Finally β-mercaptoethanol (0.5 mM final concentration) was added in order to release the covalently bound thiopyridine residue from the gastric lipases. The thiopyridine released was recorded spectrophotometrically at 324 nm.

Table I: Inactivation and Radiolabeling of PPL, HGL, and RGL by ^{14}C-Radiolabeled E600[a]

lipase	incubation time (h)	radiolabeling stoichiometry (1)	inactivation level (2)	ratio (1)/(2)
PPL	5	0.84	0.75	1.15
		0.89	0.83	1.07
		0.76	0.75	1.01
HGL	5	0.71	0.85	0.83
	5	0.66	0.81	0.81
	1	0.34	0.46	0.74
RGL	1	0.76	0.86	0.88
		0.77	0.93	0.83
		0.79	1.00	0.79

[a] See Materials and Methods for the measurement of inactivation levels and radiolabeling stoichiometry. Ratio (1)/(2) expresses the labeling stoichiometry as a function of the inactivation level.

Interfacial Binding of Native and E600-Labeled Lipases. We adapted the original procedure developed by Borgström for describing the binding of PPL and colipase to tributyroylglycerol emulsion (Borgström, 1975). Native PPL, HGL, RGL, and their respective diethyl phospho derivatives (DP-lipases) were incubated under optimal assay conditions with a tributyroylglycerol emulsion (0.5 mL of tributyroylglycerol in a final volume of 15 mL). This incubation was performed at 37 °C for 1 min, under stirring. The reaction medium was then centrifuged at 10000*g* for 5 min, and the radioactivity or enzymatic activity associated with either the tributyroylglycerol phase (lower phase) or the aqueous phase (upper phase) was measured, which made it possible to determine the percentage of lipase bound to the tributyroylglycerol phase.

The binding of proteins, without any known triacylglycerol hydrolase activity (serum albumin, lyzozyme, pepsin, trypsin, and pancreatic phospholipase A2), was studied in control experiments. After incubating these proteins with a tributyroylglycerol emulsion as described above, the protein concentration was measured spectrophotometrically at 280 nm in the aqueous phase (supernatant) or estimated by SDS–polyacrylamide gel electrophoresis.

Results and Discussion

Inactivation of PPL, HGL, and RGL by Radiolabeled E600. Figure 1 gives the time course of inactivation of PPL, HGL and RGL incubated at pH 6.0 with radiolabeled E600/NaTDC mixed micelles at an E600 to lipase molar ratio of 84. PPL and HGL showed the same inactivation rates, reaching a plateau at a 20% remaining activity level after 5 h of incubation. This partial inactivation of PPL by E600 was previously reported by Rouard et al. (1978). By contrast, RGL inactivation proceeded at a faster rate, and the lipolytic activity was completely abolished after 60 min of incubation. From previous data on gastric lipase inactivation by sulfhydryl reagents, RGL can be said to have shown a faster reaction rate than HGL (Gargouri et al., 1988; Moreau et al., 1988b). This difference in reaction velocity might reflect a difference in active site accessibility.

After removing by gel filtration the excess micellar radiolabeled E600, the labeling stoichiometry of the inactivated lipases was determined and correlated with the percentage of lipase inactivation (Table I). One can note the existence of a direct correlation between the inactivation levels of the three digestive lipases tested and the stoichiometric labeling obtained

Table II: Double Labeling of Gastric Lipases by Radiolabeled E600 and 4-PDS[a]

	flowsheet of experiments			
gastric lipase	E600 labeling and gel filtration: labeling stoichiometry with E600	4-PDS labeling: sulfhydryl titration by 4-PDS	gel filtration: labeling stoichiometry with E600	β-mercaptoethanol addition: released 4-thiopyridine
HGL	0.31	1.08	0.35	0.97
RGL	0.70	0.90	0.76	0.92

[a] See Materials and Methods for experimental details. The values in this table are the average of data obtained in two different experiments.

Table III: Specific Activity of Native and E600-, DTNB-, or 4-PDS-Treated PPL, HGL, and RGL, Measured on p-NPA and Tributyroylglycerol as Substrates

lipase	specific activity on p-NPA	specific activity on tributyroylglycerol	specific activity ratio (tributyroylglycerol/ *p*-NPA)
PPL	3.5	8120	2320
HGL	1.2	1200	1000
TP-HGL	0	0	
TNB-HGL	0	0	
RGL	0.85	1215	1430
DP-RGL	0	0	
TP-RGL	0	0	
TNB-RGL	0	0	

[a] See Materials and Methods for the enzymatic assays. Specific activities are expressed as μmol/(min·mg of lipase).

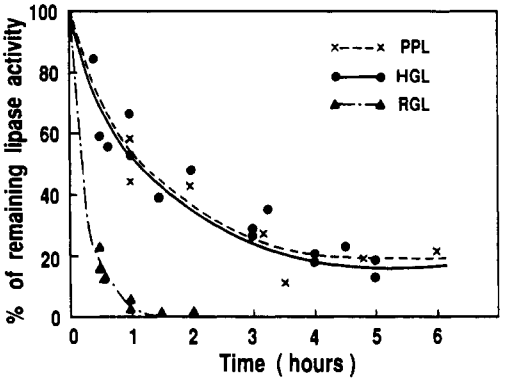

FIGURE 1: Time course of inactivation of PPL, HGL, and RGL during incubation with radiolabeled E600 (E600 to lipase molar ratio: 84). Lipases (25 nmol) were incubated with 2.1 μmol of E600 (200 μCi) at 25 °C in 50 mM acetate buffer, pH 6.0, 50 mM NaCl, 25 mM $CaCl_2$, and 3 mM NaTDC. Residual lipase activity was measured on tributyroylglycerol as substrate, as described under Materials and Methods.

with [^{14}C]E600. In the case of PPL, it has been clearly shown that the classical serine reagent (E600) inactivates the enzyme after reacting with the essential serine 152 (Guidoni et al., 1981). Using the same experimental conditions, we fully confirmed the inactivation and stoichiometric labeling of PPL and further assumed that serine 152 was the labeled residue. Furthermore, we showed for the first time that gastric lipases were also stoichiometrically inactivated by mixed E600/bile salt micelles under identical experimental conditions to those used with PPL.

Gastric lipases have been reported to be sulfhydryl enzymes, and chemical modification of the single cysteine residue induced complete lipase inactivation (Gargouri et al., 1988; Moreau et al., 1988b). In order to check whether the free sulfhydryl residue or a potential serine residue was reacting with E600, we performed double labeling experiments using successively [^{14}C]E600 and 4-PDS, a classical SH reagent (Table II). As detailed under Materials and Methods, gastric lipases were first stoichiometrically labeled by using [^{14}C]E600, and the excess reagent was removed by gel filtration. Using 4-PDS, one free sulfhydryl group was still titrable as in the native gastric lipases. The excess free 4-PDS was then removed by a second gel filtration, and we checked that 0.68 and 0.94 radiolabeled DP residues were still associated with HGL and RGL, respectively. Furthermore, the presence of a 4-thiopyridine residue was confirmed by checking whether it was released from the doubly labeled gastric lipases after β-mercaptoethanol treatment. From these data (Table II), one can deduce that E600 did not react with the free cysteine residue of gastric lipases and, furthermore, that the cysteine modification with a sulfhydryl reagent such as 4-PDS did not release the DP radical previously fixed by E600 treatment. It can thus be concluded that E600 and 4-PDS reacted independently from each other on different amino acid residues of the gastric lipases.

Table IV: Interfacial Binding to Tributyroylglycerol Emulsion of Native and Chemically Modified Digestive Lipases[a]

protein	protein detected in aqueous supernatant after SDS-PAGE (silver nitrate staining)	protein recovery in aqueous supernatant (%)			protein recovery in oil phase (%)	
		OD_{280nm}	enzymatic activity	radioactivity count (DP-lipases)	enzymatic activity[b]	radioactivity count (DP-lipase)
BSA	+	100				
lysozyme	+	89				
pepsin	+	98				
trypsin	+	99				
phospholipase A_2	−	nd	0		33	
native PPL	−	nd	1		45	
DP-PPL	−	nd		0		100
native HGL	−	nd	3		40	
DP-HGL	−	nd		4		100
native RGL	−	nd	0		35	
DP-RGL	−	nd		0		100

[a] See Materials and Methods for details of interfacial binding to tributyroylglycerol emulsion. Nonlipolytic proteins (BSA, lysozyme, pepsin, and trypsin) were used at final concentrations ranging from 100 to 800 nM. Native or E600-treated lipases and pancreatic phospholipase A_2 were used at final concentrations ranging from 0 to 150 nM. [b] The recovery of lipolytic activities found in the oil phase was partial, probably due to interfacial denaturation during the binding protocol.

Chemical modification of the single free sulfhydryl group of gastric lipases, with either 4-PDS or DTNB (Gargouri et al., 1988; Moreau et al., 1988b), induced a complete loss of activity, on both water-soluble p-NPA and emulsified tributyroylglycerol used as substrates (see Table III). Both catalytic activities were also abolished by treatment with a serine reagent such as E600, suggesting that p-NPA and tributyroylglycerol hydrolysis might take place at the same catalytic site of gastric lipases, in sharp contrast with PPL. Previous investigations (Bousset-Risso et al., 1985; De Caro et al., 1986) showed that, after a limited chymotrypsin treatment of PPL, two domains were cleaved with a concomitant loss of tributyroylglycerol activity. The isolated C-terminal domain of PPL (12 kDa) was still able to hydrolyze p-NPA and not tributyroylglycerol. The rate of hydrolysis of p-NPA was no longer affected by the presence of an interface. The recently published three-dimensional structure of HPL (Winkler et al., 1990) provides strong evidence that the essential Ser 152 is located in the larger N-terminal domain, at the C-terminal edge of a doubly wound, parallel β-sheet, and that it is part of an Asp-His-Ser triad. This putative hydrolytic site is covered by a surface loop and therefore rendered inaccessible to solvents. Thus interfacial activation, a characteristic property of lipolytic enzymes, is likely to involve a substantial conformational change during adsorption at the lipid/water interface. A second potential "catalytic site", located in the C-terminal domain and containing His and Asp residues, can be hypothesized to be responsible for the hydrolysis of p-NPA by pancreatic lipases. It should be stressed however that the specific activity of digestive lipases on p-NPA can be taken to be negligible since it amounts to less than one-thousandth of the catalytic activity measured on tributyroylglycerol as substrate (see Table III). The significance of p-NPA hydrolysis by lipases has been overestimated in previous investigations, and there is no justification for extrapolating triacylglycerol hydrolysis mechanisms from the p-NPA ones.

Interfacial Binding to Tributyrin Emulsion of Native and Chemically Modified Digestive Lipases (see Table IV). Several proteins (bearing lipolytic activity or otherwise) were incubated with a tributyroylglycerol emulsion, at concentrations ranging from 0 to 800 nM. After centrifugation of the emulsified system, it emerged that the nonlipolytic proteins were entirely located in the aqueous supernatants, whereas each protein bearing lipolytic activity was entirely located in the tributyroylglycerol phase. These data show that the experimental binding protocol in which a tributyroylglycerol emulsion is used and the oil and water phases are separated by centrifugation is an appropriate means of distinguishing between proteins able to specifically bind lipid/water interfaces such as lipolytic enzymes. With this experimental protocol, the binding of E600-modified lipases was found to be comparable to that of native lipases (see Table IV). Furthermore, when DP-PPL was used in the presence of a tributyroylglycerol emulsion, the interface-mediated labeling reaction with [^{3}H]sulfobenzoic cyclic anhydride was still possible (Moulin et al., 1989), which also indicates that DP-PPL was present at the lipid/water interface.

These data obtained with PPL are at odds with previous reports by Chapus and Sémériva (1976), based on data obtained with siliconized glass beads. Furthermore, these authors concluded that Ser 152 of PPL, labeled by E600 treatment, was involved in the interfacial binding site and not in catalysis as usually occurs with many other serine esterases. This misinterpretation was probably due to the implicit assumption that siliconized glass beads were a good substitute and a valid model for the "interfacial quality" of triglyceride/water interfaces. All in all, the present results suggest that, in both gastric and pancreatic lipases, an essential serine residue, which was stoichiometrically labeled with the organophosphorus reagent (E600), is involved in catalysis and not in lipid binding.

Acknowledgments

Thanks are due to the Commissariat à l'Energie Atomique (Professor P. Fromageot and Dr. A. Vanhove) for radiolabeling of E600 and to Dr. Jessica Blanc (Marseille) for correcting the manuscript.

References

Bernbäck, S., Hernell, O., & Blackberg, L. (1985) *Eur. J. Biochem. 148*, 233–238.

Bodmer, M. W., Angal, S., Yarranton, G. T., Harris, T. J. R., Lyons, A., King, D. J., Piéroni, G., Rivière, C., Verger, R., & Lowe, P. A. (1987) *Biochim. Biophys. Acta 909*, 237–244.

Borgström, B. (1975) *J. Lipid Res. 16*, 411–417.

Bousset-Risso, M., Bonicel J., & Rovery, M. (1985) *FEBS Lett. 182*, 323–326.

Brady, L., Brzozowski, A. M., Derewenda, Z. S., Dodson, E., Dodson, G., Tolley, S., & Turkenburg, J. (1990) *Nature 343*, 767–770.

Chapus, C., & Sémériva, M. (1976) *Biochemistry 15*, 4988–4991.

Chapus, C., Rovery, M., Sarda, L., & Verger, R. (1988) *Biochimie 70*, 1223–1234.

De Caro, J., Boudouard, M., Bonicel, J., Guidoni, A., Desnuelle, P., & Rovery, M. (1981) *Biochim. Biophys. Acta 671*, 129–138.

De Caro, J., Rouimi, P., & Rovery, M. (1986) *Eur. J. Biochem. 158*, 601–607.

Desnuelle, P., Sarda, L., & Ailhaud, G. (1960) *Biochim. Biophys. Acta 37*, 570–571.

Desnuelle, P. (1986) in *Molecular and Cellular Basis of Digestion* (Desnuelle, P., Sjöström, H., & Noren, O., Eds.) pp 275–296, Elsevier, Amsterdam.

Docherty, A. J. P., Bodmer, M. W., Angal, S., Verger, R., Rivière, C., Lowe, P. A., Lyons, A., Emtage, J. S., & Harris, T. J. R. (1985) *Nucleic Acids Res. 13*, 1891–1903.

Erlanson, C. (1970) *Scand. J. Gastroenterol. 5*, 333–336.

Field, R., & Scow, R. O. (1983) *J. Biol. Chem. 258*, 14563–14569.

Gargouri, Y., Moreau, H., Piéroni, G., & Verger, R. (1988) *J. Biol. Chem. 263*, 2159–2162.

Gargouri, Y., Moreau, H., & Verger, R. (1989) *Biochim. Biophys. Acta 1006*, 255–271.

Guidoni, A., Benkouka, F., De Caro, J., & Rovery, M. (1981) *Biochim. Biophys. Acta 660*, 148–150.

Hamosh, M. (1984) in *Lipases* (Borgström, B., & Brockman, H. L., Eds.) pp 49–81, Elsevier, Amsterdam.

Komaromy, M. C., & Schotz, M. C. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1526–1530.

Lowe, M. E., Rosemblum, J. L., & Strauss, A. W. (1989) *J. Biol. Chem. 264*, 20042–20048.

Maraganore, J. M., & Heinrikson, R. L. (1986) *Trends Biochem. Sci. 11*, 497–498.

Mayliè, M. F., Charles, M., & Desnuelle, P. (1972) *Biochim. Biophys. Acta 276*, 162–175.

Moreau, H., Gargouri, Y., Lecat, D., Junien, J. L., & Verger, R. (1988a) *Biochim. Biophys. Acta 960*, 286–293.

Moreau, H., Gargouri, Y., Piéroni, G., & Verger, R. (1988b) *FEBS Lett. 236*, 383–387.

Moulin, A., Fourneron, J. D., Piëroni, G., & Verger, R. (1989) *Biochemistry 28*, 6340–6346.
Rathelot, J., Julien, R., Bosc-Bierne, I., Gargouri, Y., Canioni, P., & Sarda, L. (1981) *Biochimie 63*, 227–234.
Roberts, I. M., Montgomery, R. K., & Carey, M. C. (1984) *Am. J. Physiol. 247*, G385–G393.
Rouard, M., Sari, H., Nurit, S., Entressangles, B., & Desnuelle, P. (1978) *Biochim. Biophys. Acta 530*, 227–235.
Shimida, Y., Sugihara, A., Tominaga, Y., & Tsunasawa, S. (1989) *J. Biochem. 106*, 383–388.
Sikk, P. F., Osa, A. V., & Aaviksaar, A. A. (1985) *FEBS Lett. 184*, 193–196.
Tiruppatthi, C., & Balasubramanian, K. A. (1982) *Biochim. Biophys. Acta 712*, 692–697.
Verger, R. (1984) in *Lipases* (Borgström, B., & Brockman, H. L., Eds.) pp 83–150, Elsevier, Amsterdam.
Verger, R., De Haas, G. H., Sarda, L., & Desnuelle, P. (1969) *Biochim. Biophys. Acta 188*, 272–282.
Winkler, F. K., D'Ary, A., & Hunziker, W. (1990) *Nature 343*, 771–774.

Mechanism of Protein Kinase C Activation by Phosphatidylinositol 4,5-Bisphosphate†

Myung-Ho Lee and Robert M. Bell*
Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710
Received July 16, 1990; Revised Manuscript Received October 19, 1990

ABSTRACT: The mechanism of protein kinase C (PKC) activation by phosphatidylinositol 4,5-bisphosphate (PIP_2), phosphatidylinositol 4-monophosphate (PIP), and phosphatidylinositol (PI) was investigated by using Triton X-100 mixed micellar methods. The activation of PKC by PIP_2, for which maximal activity was 60% of that elicited by *sn*-1,2-diacylglycerol (DAG), was similar to activation by DAG in several respects: (1) activation by PIP_2 and DAG required phosphatidylserine (PS) as a phospholipid cofactor, (2) PIP_2 and DAG reduced the concentration of Ca^{2+} and PS required for activation, (3) the concentration dependences of activation by PIP_2 and DAG depended on the concentration of PS, and (4) PIP_2 and DAG complemented one another to achieve maximal activation. On the other hand, PIP_2 activation of PKC differed from activation by DAG in several respects. With increasing concentrations of PIP_2, (1) the optimal concentration of PS required was constant at 12 mol %, (2) the maximal activity at 12 mol % PS increased, and (3) the cooperativity for PS decreased. PIP_2 did not inhibit [^{3}H]phorbol 12,13-dibutyrate (PDBu) binding of PKC at saturating levels of PS; however, at subsaturating levels of PS, PIP_2 enhanced [^{3}H]PDBu binding by acting as a phospholipid cofactor. PIP did not function as an activator but served as a phospholipid cofactor in the presence of PS. While PIP_2, PIP, and PI did not support DAG-dependent PKC activation as phospholipid cofactors, their presence reduced the amount of PS required for maximal activation to as low as 2 mol % from 8 mol %. These data establish that PIP_2, PIP, and PI can function to spare, in part, the PS phospholipid cofactor requirement of PKC, and they demonstrate that PIP_2 but not PIP and PI can function as a lipid activator of PKC by mechanisms distinct from those of DAG and phorbol esters.

Phosphatidylinositol 4,5-bisphosphate (PIP_2),[1] phosphatidylinositol 4-monophosphate (PIP), and phosphatidylinositol (PI) are components of the phosphatidylinositol cycle, which is stimulated by growth factors, neurotransmitters, and hormones. When cells are activated by these agents, PIP_2 is hydrolyzed by phospholipase C, generating two intracellular second messengers, inositol 1,4,5-trisphosphate (IP_3) and *sn*-1,2-diacylglycerol (DAG) (Nishizuka, 1984; Berridge, 1984). IP_3 stimulates the release of Ca^{2+} from intracellular storage sites, while DAG activates protein kinase C (PKC), a Ca^{2+}- and phospholipid-dependent protein kinase, by increasing its affinity for Ca^{2+} and its phospholipid cofactor, PS (Takai et al., 1979; Hannun et al., 1986). Tumor-promoting phorbol esters activate PKC by mechanisms similar to that of DAG (Castagna et al., 1982). DAG inhibits phorbol ester binding through a competitive manner (Sharkey et al., 1984), suggesting that their interaction sites are the same. Studies with DAG analogues (Ganong et al., 1986; Molleyres & Rando, 1988) demonstrated that certain structural features within DAG are required for PKC activation: (1) two fatty acyl chains, which allow interaction with the membrane surface, (2) the stereospecific *sn*-1 and *sn*-2 oxygen ester bonds, and (3) a free hydroxyl group in the *sn*-3 position. The phospholipid cofactor requirement of PKC was recently shown to be highly specific for structural features contained within phosphatidyl-L-serine (Lee & Bell, 1989).

O'Brian et al. (1987) showed that, in the presence of 1 mM Ca^{2+}, PI, PIP, or PIP_2 acted to some extent as a phospholipid cofactor for PKC. Chauhan and Brockerhoff (1988) reported that PIP_2, but not PIP, activated PKC directly in a Ca^{2+}- and

† This work was supported by National Institutes of Health Grant GM38737.
* To whom correspondence should be addressed.

[1] Abbreviations: PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-monophosphate; PI, phosphatidylinositol; IP_3, inositol 1,4,5-triphosphate; DAG, *sn*-1,2-diacylglycerol; PKC, protein kinase C; PS, phosphatidyl-L-serine; PA, phosphatidate; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PDBu, phorbol 12,13-dibutyrate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

0006-2960/91/0430-1041$02.50/0