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INTERACTION OF GLUTATHIONE-INSULIN TRANSHYDROGENASE (DISULFIDE INTERCHANGE ENZYME) WITH PHOSPHOLIPIDS *

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Summary

The effect of the addition of several phospholipids (lysophosphatidylcholine, α-lecithin, phosphatidylserine, phosphatidylethanolamine, lysophosphatidylethanolamine, sphingomyelin, and disphosphatidylglycerol and phosphatidic acid) and related compounds (glycerophosphocholine, α- and β-glycerophosphate, choline, serine, glycerol, dipalmitoylglycerol, and stearic acid) on the ability of purified (from beef pancreas) and microsomal (rat liver) glutathione-insulin transhydrogenase (Glutathione:protein-disulphide oxidoreductase, EC 1.8.4.2) to degrade insulin has been examined.

With purified enzyme, except for phosphatidic acid and phosphatidylserine, all other phospholipids tested caused a slight activation at low concentration with phosphatidylethanolamine causing the highest activation. Lysophosphatidylcholine and phosphatidic acid are the only agents which cause inhibition of activity. The reaction rate as a function of concentration of inhibitor is hyperbolic for phosphatidic acid ($[I]_{0.5}$ = 25 μM) and biphasic for lysophosphatidylcholine ($[I]_{0.5}$ = 270 μM). Kinetic studies show that the two phospholipids are noncompetitive versus both substrates (insulin and GSH). Further, the structures of the phospholipids are quite different from the substrates and products of the reaction catalyzed by the enzyme. These data, together with the data obtained with microsomes (see below), support the possibility that phospholipids, in particular lysolecithin and phosphatidic acid, might function by an interaction at an allosteric site or sites to bring about a conformational change in the enzyme.

With a microsomal fraction, four phospholipids (lysophosphatidylcholine, lysophosphatidylethanolamine, phosphatidylethanolamine, and phosphatidic acid) caused an increase in GSH-insulin transhydrogenase activity. At low concentration the addition of each of these phospholipids led to a 2.5-fold increase

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in GSH-insulin transhydrogenase activity. At higher concentration, lysophosphatidylcholine almost totally inhibited the microsomal GSH-insulin transhydrogenase activity, as it did with purified enzyme, while phosphatidic acid showed only a slight inhibition, in contrast to its effect on purified enzyme. With the microsomal fraction in which GSH-insulin transhydrogenase activity had been previously unmasked by Triton X-100 treatment, the addition of small amounts of lysophosphatidylcholine and phosphatidic acid produced, as expected, only slight increase in the transhydrogenase activity for both phospholipids; again, only lysophosphatidylcholine but not phosphatidic acid caused inhibition when higher levels were used. It is concluded that the four phospholipids and Triton X-100 increase the GSH-insulin transhydrogenase activity in the microsomes by unmasking the catalytic site without fully unmasking the allosteric site, the point of reaction with the phosphatidic acid.

Introduction

Glutathione-insulin transhydrogenase * (glutathione:protein-disulfide oxidoreductase, EC 1.8.4.2) catalyzes via sulfhydryldisulfide interchange [1,2] the inactivation of insulin by splitting the disulfide bonds of the hormone and forming A and B chains [3—9]. GSH-insulin transhydrogenase of beef pancreas is a glycoprotein and might be composed of three polypeptide chains or subunits [10]. The enzyme occurs ubiquitously [11]. Its action is the first and rate-controlling step in the sequence of insulin inactivation and degradation, both at physiologic and pharmacologic concentrations of insulin [12—15]. The enzyme catalyzes insulin degradation via a complex mechanism, either by a random mechanism or by an allosteric mechanism [16].

GSH-insulin transhydrogenase has been shown to have many similarities to another disulfide-interchange enzyme, "ribonuclease-reactivating enzyme" [17,18]. Few differences have been noted, although these differences might occur because of the organ and species differences from which various preparations were obtained. Thus, overall evidence strongly suggests that the two enzymes probably have identical catalytic activities; see ref. 10 for a review on this point.

The enzyme has been purified from a number of sources (see ref. 9). For the preparation of purified enzyme it has been necessary in each case to extract the enzyme after the treatment of a tissue with an organic solvent, acetone [19-20], or a detergent, deoxycholate [21], indicating an apparent association of lipid with the enzyme protein. In a more recent study with rat liver [22], the enzyme has been shown to be membrane-bound, probably as a phospholipoprotein complex. Most of it occurs in the microsomal fraction but small amounts are also present in the plasma membrane fraction [23]. About 80% of the microsomal enzyme is in a latent state, and is released by treatments such

* It has recently been suggested [34] that the enzyme or enzymes that catalyze the sulfhydryldisulfide interchange reaction should be considered as "thiol-transferase" rather than a "transhydrogenase"; the designation glutathione-insulin transhydrogenase has been retained for consistency with previous publications to avoid further nomenclature confusion.

as homogenization with Polytron homogenizer, sonication, freezing and thawing, alkaline pH, the nonionic detergent Triton X-100, phospholipase C, and phospholipase A [22]. The biological significance of the latency of GSH-insulin transhydrogenase activity is not clear. It is possible that the GSH-insulin transhydrogenase activity increases because of the removal (or dissociation) of phospholipids and other membrane factors that might be attached to (or interact with) the transhydrogenase protein in such a manner that they interfere with the action of the enzyme, or, because the products of phospholipase (i.e., phospholipids) activate the enzyme activity. It is also possible that the release mechanisms (i.e., phospholipase) themselves are affected by various physiological conditions and thus regulate the effective levels of GSH-insulin transhydrogenase.

In view of the above observations, the effect of the addition of several exogenous phospholipids on the ability of pure and of microsomal enzyme to degrade insulin was investigated. Results show that of the several phospholipids and other factors tested, lysolecithin and phosphatidic acid are the only agents which cause inhibition of GSH-insulin transhydrogenase activity, i.e., a reversal of the effect of phospholipases observed on microsomal GSH-insulin transhydrogenase. Kinetic data on purified GSH-insulin transhydrogenase and studies with the microsomal preparation suggest that the two phospholipids probably restrain GSH-insulin transhydrogenase activity by an interaction at an allosteric site or sites. A preliminary account of some of these observations has appeared [24].

Materials and Methods

The preparations of beef pancreatic GSH-insulin transhydrogenase, biologically active ^{125}I-labelled insulin (containing about 0.6 atom of iodine per insulin molecule) and its purification, and microsomes from rat liver [22] have been described in previous publications; for references see Chandler and Varandani [16]. The purified enzyme is a homogeneous material as judged by several criteria.

Highly purified phospholipids were purchased from Grand Island Biological Company; many were purchased also from Sigma Chemical Company, Nutritional Biochemicals Corp. and General Biochemical Company to provide an additional source for confirmation of the data. Phosphatidylserine and sphingomyelin were prepared from bovine brain; the rest of phospholipids were from egg. Organic solvents were first evaporated in a stream of nitrogen and phospholipids or other test agents were then suspended in 0.25 M sucrose 50 mM Tris buffer, pH 7.5, by sonication; the suspensions were sonicated at 0° C, three times, for 15 s each, with a Bronwill Biosonic II Sonic Oscillator using a needle tip, 5/32 inch diameter at 70% of the probe intensity. GSH-insulin transhydrogenase (1 μg) or rat liver microsomes (0.08 mg protein) in a volume of 0.2 ml of sucrose/Tris buffer were incubated for 30 min at room temperature with the indicated concentrations of phospholipids or other agents; these preincubation conditions were chosen arbitrarily. The ability of the purified enzyme or the microsomal fraction to degrade insulin was then determined in the presence of 1 mM GSH, as described previously [22,25]. Several preliminary experiments

were carried out; the assay procedure used was linear with respect to time and amount of enzyme employed (e.g., see ref. 26). A brief description of the assay is as follows: The buffer for all assay solutions was 0.1 M potassium phosphate/ 5 mM EDTA, pH 7.5. To each tube containing 0.2 ml of phospholipid-treated (or buffer-treated) enzyme solution 0.6 ml of buffer and 0.1 ml of GSH solution (1 μmol or as indicated) was added and the tubes were incubated for 5 min at 37° C. The reaction was started by the addition of 0.1 ml of insulin solution (final concentration 1 μM or as indicated) containing tracer amount of ^{125}I-labelled insulin and 3 mg of bovine serum albumin (fraction V), and was terminated after 5 min by the addition of 1 ml of 10% trichloroacetic acid. The precipitates were collected by centrifugation and washed once as previously described. All enzyme activities reported have been corrected for non-enzymatic insulin degradation, which was determined by running matched control tubes with enzyme protein omitted.

Kinetic analysis was carried out using a nonlinear least-squares method as described in detail previously [16]; the actual fits were made to $v/[S]$, rather than v, versus substrate in order to satisfy the requirement of equal variance in the experimental velocities [27]. When the double reciprocal plots ($1/v$ vs. $1/[S]$) of the data were linear, fits were made to equation 1:

$$v = V[S]/(K + [S]) \quad (1)$$

and to equation 2 when they were parabolas:

$$v = V/(1 + b_1/[S] + b_2/[S]^2) \quad (2)$$

Results

Studies with purified beef pancreatic glutathione-insulin transhydrogenase

The variation in the activity of purified enzyme caused by phospholipids is shown in Fig. 1. Except for phosphatidic acid and phosphatidylserine, all other phospholipids tested caused a slight activation at low concentration; phosphatidyl ethanolamine caused the highest activation (about 40%), while the activation with others was between 10 and 20%. At higher concentrations, only lysophosphatidylcholine and phosphatidic acid caused marked inhibition. Control experiments in which lysophosphatidylcholine and phosphatidic acid were added to the reaction mixture just prior to termination of the reaction with trichloroacetic acid showed no change in the enzymic rates of insulin degradation from those without the phospholipids. Therefore, the specificity of inhibition and the results of control experiments indicate that addition of lysophosphatidylcholine and phosphatidic acid do not interfere with the assay procedure employed. The dose-response curve with lysophosphatidylcholine is biphasic, whereas that with phosphatidic acid is hyperbolic. There is no activation phase with phosphatidic acid when several lower concentrations than those shown in Fig. 1 were tested (lowest concentration tested, 14.5 nM). The concentration causing 50% inhibition for lysophosphatidylcholine is 270 μM, while that of phosphatidic acid is 25 μM indicating that the phosphatidic acid is about 10 times more potent than lysophosphatidylcholine. The physiological concentration of lysophosphatidylcholine and of phosphatidic acid in rat liver can be calculated from the data reported by Ray et al. [28] to be about 2—3 mM for

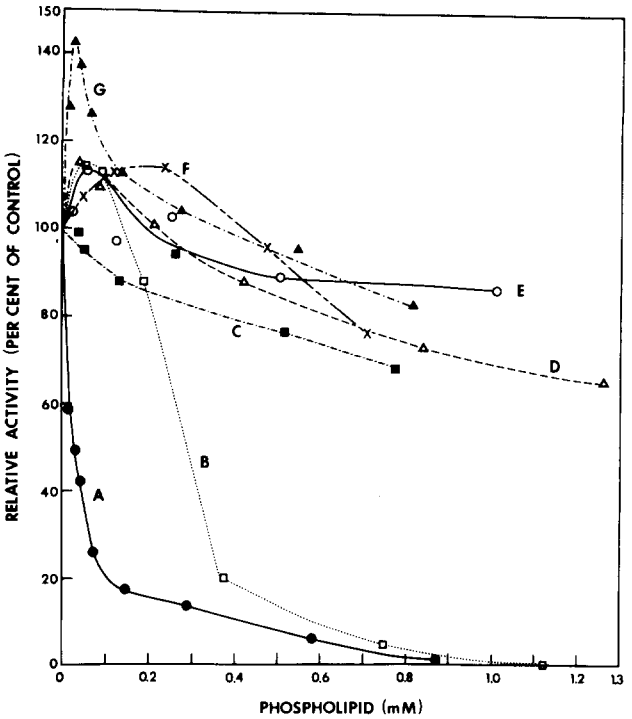

Fig. 1. Effect of variation of phospholipid concentration on catalysis of insulin degradation by purified glutathione insulin transhydrogenase. The assay system contained 80 mM potassium phosphate pH 7.5, 4 mM EDTA, 0.3% bovine serum albumin, 1 mM GSH, 1 μM insulin, a tracer amount of ^{125}I-labelled insulin, and 1 μg (5 enzyme units) of purified glutathione insulin transhydrogenase preincubated with the concentration of phospholipid as indicated, in a total volume of 1.0 ml. See text for other details. In the figure, all velocities shown are net enzymic values and are expressed relative to a control tube which was run under identical conditions with phospholipid omitted. Each point is the average of a minimum of two determinations. A, phosphatidic acid (691); B, lysophosphatidylcholine (536); C, phosphatidylserine (777); D, lysophosphatidylethanolamine (476); E, α-phosphatidylcholine (793); F, sphingomyelin (850); G, phosphatidylethanolamine (733). Values in parentheses are the molecular weight of each lipid assumed for calculation.

each, assuming: a uniform distribution within the cell; 71% water content and a lipid content of 15.2% of the dry weight [29]. Thus, the $I_{0.5}$ values for both lysophosphatidylcholine and phosphatidic acid are well below their physiological concentrations.

The effect of phospholipids on GSH-insulin transhydrogenase purified from rat liver was also determined using three phospholipids (lysophosphatidylcholine, lysophosphatidylethanolamine and phosphatidylethanolamine). The results obtained were similar to those obtained with the beef pancreatic enzyme.

Studies were undertaken to determine the chemical structures(s) of lysophosphatidylcholine and phosphatidic acid essential to their effect. Glycerophosphocholine (highest concentration tested, 1.2 mM), diphosphatidyl glycerol (1.2 mM), α- and β-glycerophosphate (1.2 mM each), choline chloride (34.2 mM), serine (45.6 mM) and glycerol (52.2 mM) were found to be ineffective; dipalmitoylglycerol (1 mM) and stearic acid (2.1 mM) (although poorly

suspended), or stearic acid complexed to bovine serum albumin [30], were also ineffective.

Further studies were performed to determine the type of inhibition by phosphatidic acid and lysophosphatidylcholine at inhibiting concentrations. Previous studies have shown [16] that Lineweaver-Burk plots of initial rate of insulin degradation as a function of insulin concentration are linear, whereas those as a function of GSH concentration are parabolic.

When either phosphatidic acid or lysophosphatidylcholine was studied as an inhibitor of the reaction at varying concentrations of insulin and in the presence of a fixed concentration of GSH, linear reciprocal plots ($1/v$ vs.$1/$ [insulin]) of the data were observed in the absence or presence of phosphatidic acid (Fig. 2) or lysolecithin (Fig. 3). The obvious effects on the ordinate inter-

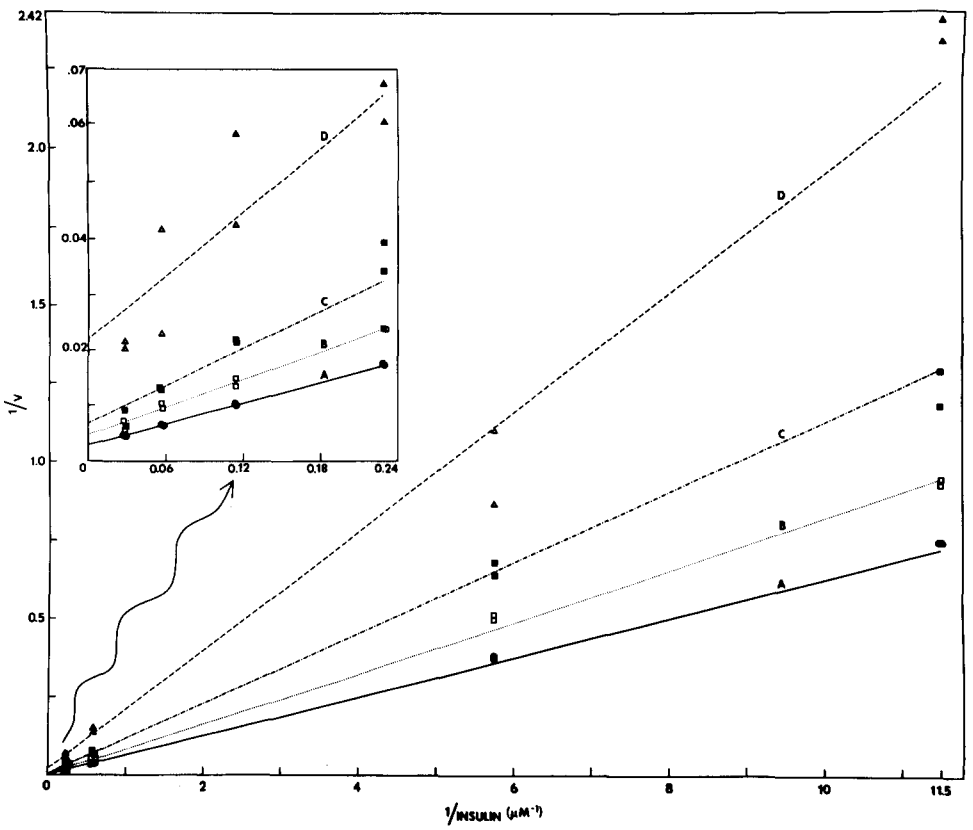

Fig. 2. Phosphatidic acid inhibition of insulin degradation by purified glutathione insulin transhydrogenase measured as a function of the concentration of insulin. Reciprocal plots of the initial velocity (pmol of insulin degraded per min per μg of glutathione insulin transhydrogenase). The points are experimental; the lines were derived from fits of the data at each level of phosphatidic acid to the Michaelis-Menten equation (eqn. 1). Concentrations of phosphatidic acid were: curve A, none; curve B, 14.5 μM; curve C, 29 μM; curve D, 58 μM. Eight levels of insulin concentration varying from 0.017 to 34.8 μM were used; however, in the figure the lowest level of insulin (0.017 μM) is not shown because of problems of scale. GSH was present at 1 mM; other experimental details were as given in Fig. 1. The apparent V values (pmol/min/μg) are 34 ± 2, 21 ± 2, 15 ± 2, and 5 ± 0.8; and the apparent K_m (insulin) values (μM) are 21.3 ± 1.5, 17.2 ± 1.6, 16.5 ± 2.9, and 8.7 ± 1.7 for curves A, B, C, and D, respectively.

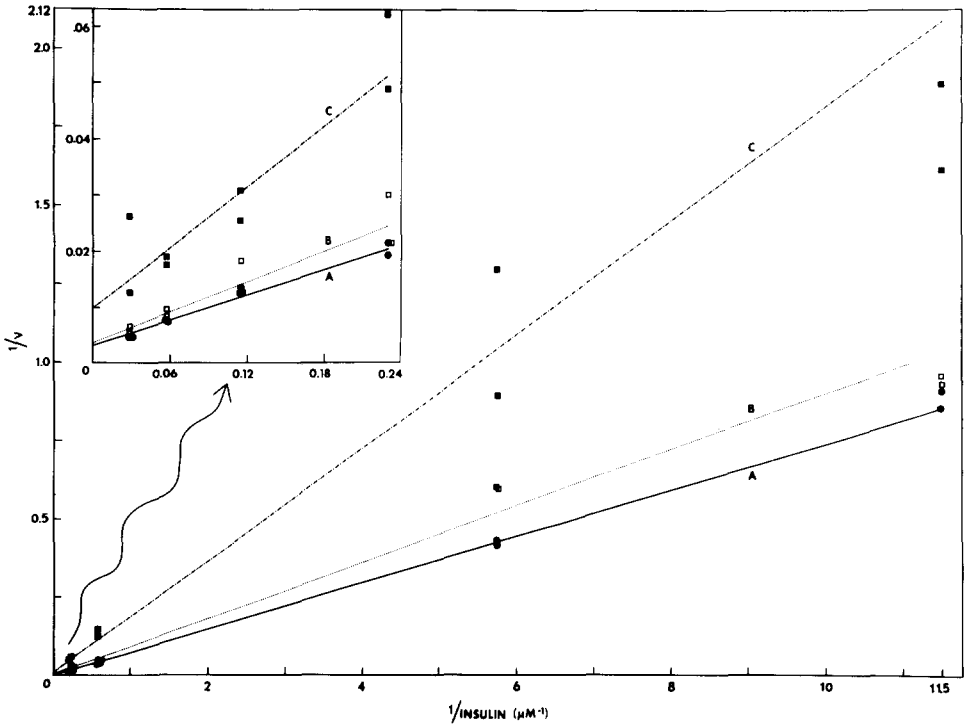

Fig. 3. Lysophosphatidylcholine inhibition of insulin degradation by purified glutathione insulin transhydrogenase measured as a function of the concentration of insulin. Lysophosphatidylcholine concentrations were as follows: curve A, none; curve B, 224 μM; curve C, 299 μM; other experimental details are the same as described in Fig. 2. The apparent V values (pmol/min/μg) are 31 ± 3, 28 ± 6, and 10 ± 2.6; and the apparent K_m (insulin) values (μM) are 23.4 ± 2.3, 25.6 ± 6.1, and 18.6 ± 5.4 for curves A, B, and C, respectively.

cepts ($1/V$) and the intersecting nature of these plots demonstrate that the inhibition by both phospholipids is noncompetitive against insulin.

When the inhibition data of the two phospholipids were examined with GSH as a variable substrate at a fixed concentration of insulin, the patterns shown in Figs. 4 and 5 were observed. The plots at different levels of inhibitors approach the ordinate at different $1/V$ values, indicating that the inhibition is not competitive against GSH.

Studies with the rat liver microsomal fraction

It has been previously demonstrated [22] on the basis of the results obtained after treating the liver microsomal fraction with several physical and enzymatic methods that GSH-insulin transhydrogenase occurs bound to membrane protein. Consistent with this conclusion, successive extraction of the microsomal fraction with 0.14 M NaCl, 1.0 M NaCl, and 0.1 M $NaHCO_3/Na_2CO_3$ in accordance with the procedure of Weihing et al., [31] also showed that more than 90% of the GSH-insulin transhydrogenase activity remains associated with the membrane protein even though more than 75% of the RNA was removed.

The change in GSH-insulin transhydrogenase activity in the microsomal preparation after the addition of several different phospholipids is shown in Fig. 6.

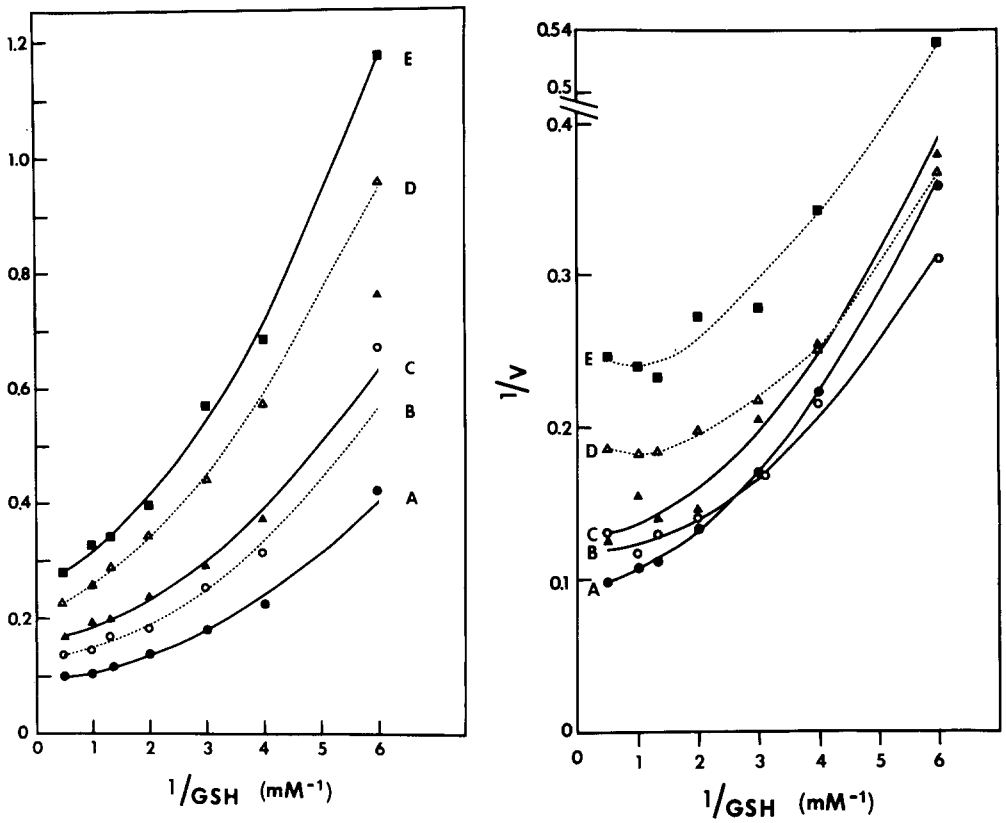

Fig. 4. Phosphatidic acid inhibition of insulin degradation by purified glutathione insulin transhydrogenase measured as a function of the concentration of GSH. Reciprocal plots of the initial velocity (pmol of insulin degraded per min per μg of glutathione insulin transhydrogenase). The points are experimental and for brevity only the average points of a minimum of two determinations are shown; the lines are derived from fits of the data at each level of phosphatidic acid to the equation for a parabola (eqn. 2). Concentrations of phosphatidic acid were: curve A, none; curve B, 11.6 μM; curve C, 23.2 μM; curve D, 57.9 μM; curve E, 80.9 μM. Insulin was present at 0.435 μM; other experimental details were as given in Fig. 1. The apparent V_I values (pmol/min/μg) are 10.7 ± 0.5, 7.6 ± 0.5, 6.2 ± 0.4, 4.9 ± 0.2, and 3.9 ± 0.2 for curves A, B, C, D, and E, respectively.

Fig. 5. Lysolecithin inhibition of insulin degradation by purified glutathione insulin transhydrogenase measured as a function of the concentration of GSH. Lysophosphatidylcholine concentrations were as follows: curve A, none; curve B, 175 μM; curve C, 200 μM; curve D, 225 μM; curve E, 250 μM; other experimental details are the same as described in Fig. 4. Solid lines A, B, and C have been drawn from fits to the equation for a parabola (eqn. 2). Dotted lines D and E are fitted by eye. The apparent V_I values (pmol/min/μg) are 10.6 ± 0.3, 8.4 ± 0.3, 7.7 ± 0.3, approx. 6.2 and approx. 4.8 for curves A, B, C, D, and E, respectively.

α-phosphatidylcholine, phosphatidylserine, and sphingomyelin had no effect. Lysophosphatidylethanolamine, phosphatidylethanolamine, lysophosphatidylcholine, and phosphatidic acid increased the transhydrogenase activity about 2.5-fold. At higher concentrations, as with the purified enzyme, only lysophosphatidylcholine caused inhibition ($I_{0.5}$ = 0.41 mM), bringing about almost total inactivation. However, phosphatidic acid, which inhibited the purified enzyme with potency far greater than lysophosphatidylcholine, caused only slight in-

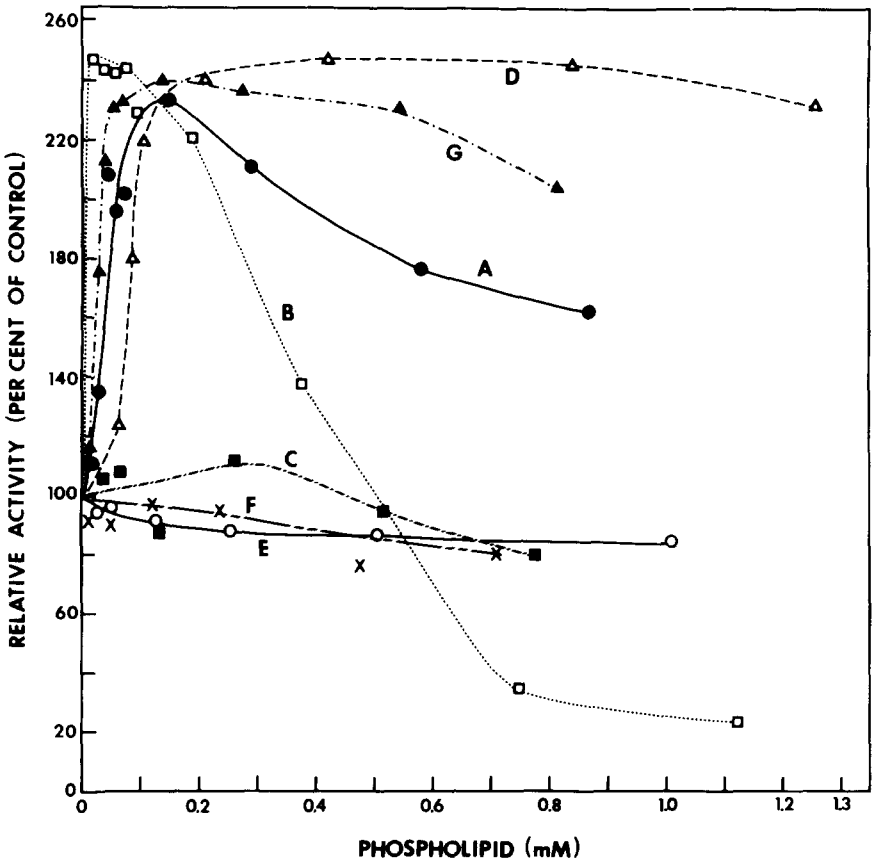

Fig. 6. Effect of variation of phospholipid concentration on catalysis on insulin degradation by rat liver microsomes. The assay system contained 80 mM potassium phosphate pH 7.5, 4 mM EDTA, 0.3% bovine serum albumin, 1 mM GSH, 1 μM insulin, a tracer amount of ^{125}I-insulin, and 0.08 mg (9.2 enzyme units) of microsomal protein preincubated with the concentration of phospholipid as indicated, in a total volume of 1.0 ml. See text for other details. In the figure, all velocities shown are net enzymic values and are expressed relative to a control tube which was run under identical conditions with phospholipid omitted. Each point is the average of a minimum of two determinations. Symbols are explained in Fig. 1.

hibition of the microsomal fraction. Apparently the four effective phospholipids unmask the catalytic site fully, but only partially unmask the allosteric site at which the phosphatidic acid appears to interact (see Discussion).

The increase in the GSH-insulin transhydrogenase activity in microsomes caused by the phospholipids is probably analogous to that caused by the detergent, Triton X-100, since the addition of lysophosphatidylcholine and phosphatidic acid to the microsomal fraction in which the transhydrogenase activity had been previously unmasked by Triton X-100 treatment (activity increased 1.7-fold) showed that while the increase in the transhydrogenase activity by both phospholipids (Fig. 7) was, as expected, much smaller than the increase obtained with non Triton-treated microsomes (Fig. 6), again only lysophosphatidylcholine but not phosphatidic acid could cause inhibition (Fig. 7). Thus, treatment with Triton, like treatment with phospholipids, apparently results in the unmasking of the catalytic site, leaving the allosteric site masked. Since

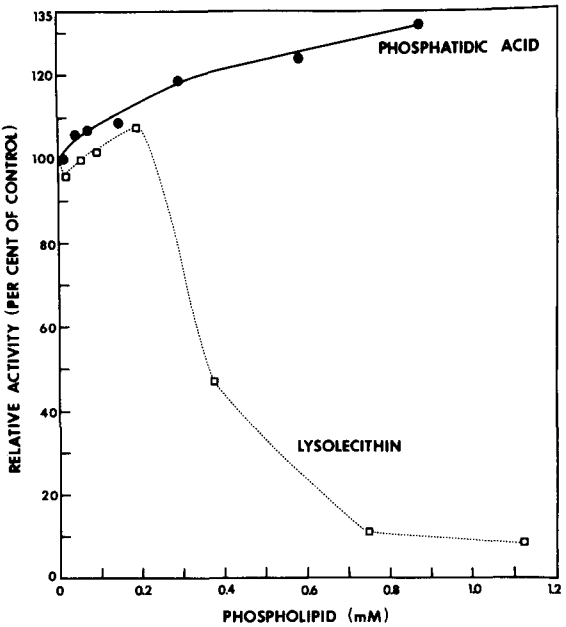

Fig. 7. Effect of lysophosphatidylcholine and phosphatidic acid on catalysis of insulin degradation by rat liver microsomes which had been previously treated with Triton X-100. First, the microsomal protein was incubated with 0.2% Triton X-100 for 30 min at room temperature. The Triton-treated microsomal protein was then incubated with the indicated concentrations of phospholipid and the assay for the glutathione insulin transhydrogenase activity (final concentration, 0.05 mg of protein/ml; 0.002% of Triton X-100) was carried out. Other experimental details are the same as described in Fig. 6. In the figure, all velocities shown are net enzymatic values and are expressed relative to a control tube (i.e. using Triton-treated microsomes) which was run under identical conditions with phospholipid omitted. Triton X-100 treatment increased the glutathione insulin transhydrogenase activity in the microsomal fraction 1.7-fold. Each point is the average of two determinations.

the purified enzyme is readily inhibited by phosphatidic acid (Figs. 1, 2 and 4), in fact with a potency far greater than lysophosphatidylcholine, these data indicate that while the catalytic site can easily be unmasked by some phospholipids and Triton (although there appears to be a requirement for some special environment since only a few phospholipids could increase the activity), the unmasking of the allosteric site requires more drastic conditions such as those employed during the purification of GSH-insulin transhydrogenase.

Discussion

From the preceding data, it appears that the simplest phospholipid responsible for eliciting inhibition of GSH-insulin transhydrogenase activity is probably phosphatidic acid. Apparently the presence of the fatty acyl moieties is essential for this effect since glycerophosphates, glycerophosphocholine, and disphosphatidylglycerol were ineffective. The potency of phosphatidic acid and the mode of its action is modified by the type of base with which it is coupled. The combinations of different fatty acyl and different base moieties on phosphatidic acid would yield different net charges or configurations, thus

imparting the selectivity and variable potencies to various phospholipids which can cause activation (e.g., phosphatidylethanolamine), or inhibition (lysophosphatidylcholine) of the enzymic activity, or which have no effect on the enzymic activity.

While the effects of phospholipids on the enzymic activity are clearly demonstrated, the nature of underlying events is not apparent. Since phosphatidic acid and lysophosphatidylcholine are quite dissimilar in structure from the substrates (insulin and GSH) and products of reaction catalyzed by GSH-insulin transhydrogenase, these data can best be interpreted by postulating that the two phospholipids interact with the enzyme at an allosteric site or sites (i.e. a site other than the catalytic site) thus exerting their effect by modifying the conformation of the enzyme [32]; the phospholipid interaction might be via binding and/or via alteration of environment. Consistent with this inference are the findings of noncompetitive inhibition by the two phospholipids against both substrates (insulin and GSH) indicating that the two lipids interact at site or sites other than the activity site. Although the gross mechanism with the two lipids is the same (i.e., noncompetitive), the difference in the pattern of the dose-response curves (Fig. 1) between lysophosphatidylcholine (biphasic) and phosphatidic acid (hyperbolic) suggests that the exact mechanism by which the two phospholipids function may not be identical; this illustrates the complex nature of the system. Finally, the differential effects of lysophosphatidylcholine and phosphatidic acid on the activity of microsomal enzyme (Fig. 6 and 7) also support the suggestion of two distinct allosteric sites on GSH-insulin transhydrogenase apparently separated by compartmentation. In view of the complexity of the system, these conclusions should be confirmed by additional, more direct evidence, such as by measurement of conformational changes and/or binding of labelled phospholipids.

The lipid structure of the membranes is an important determinant for the correct functioning of several membrane-bound enzymes (for a review, see ref. 33). Coleman [33] lists more than 20 such mammalian enzymes and enzyme systems, and the list is continually growing. Phospholipids have been found both to support and to restrain the maximal activities of enzymes. However, almost nothing is understood or known, at the molecular level, about how phospholipids support or restrain enzyme activities, whether they act by different mechanisms in different situations, or what direct effects phospholipids have on the enzyme conformation (or organization of the system). The specificity of inhibition and the small amounts required in comparison with their physiological concentrations suggests that constraint on GSH-insulin transhydrogenase by lysophosphatidylcholine and phosphatidic acid could serve as an important physiological control mechanism.

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