Cysteine-less glycosylphosphatidylinositol-specific phospholipase C is inhibited competitively by a thiol reagent: evidence for glyco-mimicry by \(p\)-chloromercuriphenylsulphonate

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Glycosylphosphatidylinositol (GPI)-specific phospholipases are highly valuable for studying the structure and function of GPIs. GPI-specific phospholipase C (GPI-PLC) from Trypanosoma brucei and phosphatidylinositol-specific phospholipase C (PI-PLC) from Bacillus cereus are the most widely studied of this class of phospholipases C. Inhibition of protein activity by thiol reagents is indicative of the participation of cysteine residues in biochemical events. The thiol reagent \(p\)-chloromercuriphenylsulphonate (\(p\)-CMPS) inhibits \(T.\) brucei GPI-PLC, which has eight cysteine residues. Surprisingly, we found that the activity of \(B.\) cereus PI-PLC is also blocked by \(p\)-CMPS, although the protein does not contain cysteine residues. Inhibition of \(B.\) cereus PI-PLC was reversed when \(p\)-CMPS was size-separated from a preformed \(p\)-CMPS \(\cdot\) PI-PLC complex. In contrast, no activity was recovered when \(T.\) brucei GPI-PLC was subjected to a similar protocol. Equimolar \(\beta\)-mercaptoethanol (\(\beta\)-ME) reversed the inhibition of PI-PLC activity in a \(p\)-CMPS \(\cdot\) PI-PLC complex. For \(T.\) brucei GPI-PLC, however, ultrafiltration of the \(p\)-CMPS \(\cdot\) PI-PLC complex and addition of a large excess of \(\beta\)-ME was necessary for partial recovery of enzyme activity. Thus \(T.\) brucei GPI-PLC is susceptible to inactivation by covalent modification with \(p\)-CMPS, whereas PI-PLC is not. Kinetic analysis indicated that \(p\)-CMPS is a competitive inhibitor of PI-PLC when a GPI was a substrate. Curiously, with phosphatidylinositol as substrate, inhibition was no longer competitive. These data suggest that \(p\)-CMPS is a glyco-mimetic that occupies the glycan binding site of PI-PLC, from where, depending on the substrate, it inhibits catalysis allosterically or competitively.

Key words: covalent modification, enzyme inhibition, GPI.

INTRODUCTION

Glycosylphosphatidylinositol (GPI)-specific phospholipases have intriguing effects on cell physiology [1–4]. Phosphatidylinositol-specific phospholipase C (PI-PLC) from Bacillus spp. and GPI-specific phospholipase C (GPI-PLC) from Trypanosoma brucei are widely used for studying the biochemistry and cell biology of GPIs both in vitro and in vivo [5–8]. Consequently, understanding the biochemical and physiological roles of these enzymes is important. PI-PLC from \(B.\) cereus lacks cysteine residues [9], whereas GPI-PLC from \(T.\) brucei has eight cysteines [10, 11].

Thiol reagents are routinely used for determining whether (or not) cysteine residues are important for protein activity. For polypeptides that contain Cys residues, it is generally thought that inactivation of their function arises from covalent modification of the polypeptide. While these concepts may be true in many instances, other possibilities may be worthy of consideration.

First, inactivation of a protein by a thiol reagent may not necessarily mean that a Cys residue is essential for function. A case in point is GPI-PLC from \(T.\) brucei; the enzyme is inactivated by \(p\)-chloromercuriphenylsulphonate (\(p\)-CMPS) [12, 13], yet there is no evidence that a Cys is critical for catalysis [14].

Recently, Cys-80 was identified as the putative target of \(p\)-CMPS [15]. Coupled with the fact that Gln-81 mutants of GPI-PLC were inactive, two factors were thought to account for the inactivation of GPI-PLC by \(p\)-CMPS: (1) the proximity of Cys-80 to the putative active site, and (2) the conversion of Cys-80 into a bulky cysteinylmercuriphenylsulphonate adduct that could sterically hinder the active site [15].

Secondly, thiol reagents could inhibit the function of proteins that do not contain cysteine residues. An example of such a protein is PI-PLC from \(B.\) cereus spp. [18]. A logical interpretation of such data would be that the thiol reagent reacted with a non-cysteine side chain in the polypeptide.

We found that \(B.\) cereus PI-PLC is inhibited by \(p\)-CMPS, as reported previously [18]. To explore how a thiol reagent inhibited a Cys-less protein, numerous hypotheses were examined. Several lines of evidence lead to a conclusion that \(p\)-CMPS is a glyco-mimetic that competitively inhibits PI-PLC by binding to the glycan recognition site on the enzyme. In related studies, we established that \(p\)-CMPS inactivates \(T.\) brucei GPI-PLC by covalent modification.

MATERIALS AND METHODS

Reagents

\(p\)-CMPS, CuSO\(_4\), HgCl\(_2\), and \(\beta\)-mercaptoethanol (\(\beta\)-ME) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). BSA was from New England BioLabs (Beverly, MA, U.S.A.).

Abbreviations used: DMG, dimyristoylglycerol; GPI, glycosylphosphatidylinositol; GPI-PLC, glycosylphosphatidylinositol-specific phospholipase C; \(\beta\)-ME, \(\beta\)-mercaptoethanol; mfVSG, membrane-form variant surface glycoprotein; \(p\)-CMPS, \(p\)-chloromercuriphenylsulphonate; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C.

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GPI-PLC assays

Recombinant T. brucei GPI-PLC from was purified from Escherichia coli [19]. B. cereus PI-PLC (600 units/mg) was a gift from Dr Mary Roberts (Boston University, Boston, MA, U.S.A.). Membrane-form variant surface glycoprotein (mVSG) labelled with [3H]myristate in its GPI (i.e. [3H]mfVSG) devoid of SDS was isolated from T. brucei [9,19,20] and initially solubilized in 0.1% Nonidet P-40. It was then diluted 40-fold in either a GPI-PLC assay buffer (50 mM Tris/HCl, pH 8.0, 5 mM EDTA, 1.0% Nonidet P-40) or PI-PLC buffer (25 mM Hepes/KOH, pH 7.5, 0.1% sodium deoxycholate) before use. Enzyme assays were performed in 30 μl (GPI-PLC) or 40 μl (PI-PLC) with 2 μg of [3H]mfVSG from 10 μl of appropriate assay buffer. Reactions were incubated at 37 °C for 30 min unless otherwise stated.

Preincubation of pCMPS with PI-PLC or GPI-PLC

B. cereus PI-PLC (0.85 ng in 10 μl of PI-PLC buffer) or T. brucei GPI-PLC (1 ng in 10 μl of GPI-PLC buffer) was incubated with (2 mM or 5 mM respectively) or without pCMPS in 20 μl at 37 °C for 10 min. [pCMPS stock (100 mM) was prepared in 100 mM NaOH.]

Removal of unreacted pCMPS

To prepare Centricon-10 ultrafiltration units for use, sites for non-specific protein adsorption were blocked with 1 ml of BSA (50 μg/ml in water). After incubation of the phospholipase with pCMPS (in 20 μl), the mixture was diluted with 980 μl of the appropriate buffer. The diluted mixture was centrifuged (5000 g, 4 °C, 2.5 h) through a Centricon-10 membrane. Approx. 50 μl of ‘retentate’ was obtained for each enzyme, 5 μl of which was used per assay.

Modification of [3H]mfVSG with pCMPS

[3H]mfVSG (20 μg) was incubated with (or without) pCMPS (2.5 mM final) in 20 μl of PI-PLC buffer at 37 °C for 10 min, after which 980 μl of chilled GPI-PLC buffer was added. [3H]mfVSG was recovered in the retentate after ultrafiltration through a Centricon-10 membrane (5000 g, 4 °C, 2.5 h). A sample of 2 μg of pCMPS-treated [3H]mfVSG was used in specified GPI-cleavage assays.

Inhibition of PI-PLC and GPI-PLC

B. cereus PI-PLC (0.3 ng) was added to pCMPS (0.0625–0.625 mM) in PI-PLC buffer and kept at 37 °C for 10 min. [3H]mfVSG was introduced, and the incubation was continued at 37 °C for 30 min. Released [3H]dimyristoylglycerol ([3H]DMG) was quantified by liquid scintillation counting. For inhibition of GPI-PLC by SDS, the final volume of detergent (diluted from a 10% stock solution in assay buffer; see above) was between 0.005% and 0.03%.

[3H]PI substrate

To prepare [3H]PI substrate (10 mM final concentration; 3000 d.p.m./μl), 54 μl of [3H]PI (55 500 d.p.m./μl) was added to 8.57 mg (9.99 μmol) of soybean PI in 100 μl of chloroform [9]. The mixture was dried under nitrogen gas and the PI was resuspended in 1 ml of PI-PLC buffer. The suspension was sonicated for 5 min in a Vibra Cell sonicator (Sonics and Materials, Pittsburgh, PA, U.S.A.) with amplitude at 20, and the pulser off. The substrate was stored at −20 °C and thawed just before use.

[3H]PI digestion by PI-PLC and its inhibition by pCMPS, CuSO4, and HgCl2

B. cereus PI-PLC (0.053 ng in 10 μl of PI-PLC buffer; 0.15 nM final concentration) was incubated with pCMPS (0.5–20 μl of 10 mM stock, in PI-PLC buffer) at 37 °C for 10 min [9]. (The
amount of PI-PLC used was determined empirically to be within the linear region of the enzyme assay.) Then 10 μl of [3H]PI (10000 d.p.m.) was added and the mixture (adjusted to a final volume of 40 μl with PI-PLC buffer) was incubated at 37 °C for 30 min. The reaction was terminated with 250 μl of chloroform/methanol/HCl (66:33:1, by vol.)[9,21]. Following centrifugation for 2 min (14000 g, 25 °C), 75 μl of the upper phase containing myo-[3H]inositol phosphate was recovered for scintillation counting. For inhibition by heavy metals, PI-PLC phase containing the experiments with PI-PLC buffer, and reactants were processed as described for centrifugation for 2 min (14000 g, 25 °C). A 400 μl aliquot of the upper phase was recovered for scintillation counting.

Results

pCMPS inhibits a cysteine-less PI-PLC from B. cereus

PI-PLC from B. cereus does not contain cysteine residues [18], yet the enzyme’s digestion of GPI was inhibited by the thiol reagent pCMPS (Figure 1A). The IC50 for the inhibition of PI-PLC by pCMPS was 0.3 mM, and 90% inhibition (IC90) was obtained with 1 mM pCMPS.

Since [3H]mVSG contains cysteine residues within the protein portion [24], inhibition of PI-PLC activity could arise from

Reversal of PI-PLC inhibition by EDTA and β-ME

EDTA (5–50 μM) or β-ME (10–100 μM) was added before incubation of PI-PLC with either pCMPS or HgCl2. Following addition of [3H]mVSG, the mixture was incubated at 37 °C for 30 min. The reaction (40 μl final volume) was terminated by addition of 500 μl of water-saturated n-butanol, and centrifuged for 2 min (14000 g, 25 °C). A 400 μl aliquot of the upper phase was recovered for scintillation counting.

Interfacial kinetics

The scooting model of kinetic analysis (reviewed in [22]) was used because the substrates, [3H]mVSG and [3H]PI, are cleaved at the interface between the aqueous solution and detergent micelles. The effective inhibitor concentration at the micelle interface was expressed as a ratio of the mole fraction of inhibitor (X) to the mole fraction of the remainder of the components of the reaction mixture (1−X), including substrate, inhibitor and detergent. Plots of V/i against X/i (where V is the reaction rate in the absence of inhibitor and V0 is the reaction rate in the presence of inhibitor) were obtained. Competitive inhibitors are identified by two features: (1) data on the graph can be fitted by a linear equation [with a coefficient of determination (r2) of > 0.90]; and (2) when X0 = 0, V0/V0 is close to 1 [22,23]. If either condition is not met, the points are fitted with an exponential curve function of Cricket Graph III version 1.5.3 (Computer Associates International, Inc., Islandia, NY, U.S.A.), and the inhibition is characterized as not competitive.
reaction of pCMPS with the substrate instead of with PI-PLC. This possibility was checked with two approaches. In the first protocol, pCMPS-treated [3H]mfVSG was used as substrate for the enzyme. pCMPS-treated [3H]mfVSG was as good a substrate for PI-PLC as unmodified [3H]mfVSG (results not shown). This observation suggested that PI-PLC, not [3H]mfVSG, was the target of pCMPS. In a second strategy, [3H]PI, which does not contain cysteine residues, was used as substrate. PI-PLC digestion of [3H]PI was inhibited by pCMPS (Figure 1B). The IC_{50} of pCMPS was 0.3 mM (Figure 1B), similar to the value obtained when the GPI-anchored [3H]mfVSG was substrate (Figure 1A). The simplest interpretation of these data is that pCMPS interacts directly with PI-PLC during inhibition of enzyme activity.

Inhibition of a Cys-less PI-PLC by a thiol reagent was intriguing. Therefore we investigated possible mechanisms by which pCMPS might act on the phospholipase C. We tested a hypothesis that inhibition of PI-PLC resulted from non-covalent interactions between the enzyme and pCMPS. For this, the protein was pre-exposed to the inhibitor, and residual pCMPS (0.415 kDa) was removed by ultrafiltration before assay of the ‘modified’ enzyme. Therefore we investigated possible mechanisms by which pCMPS might act on the phospholipase C. We tested a hypothesis that inhibition of PI-PLC resulted from non-covalent interactions between the enzyme and pCMPS. For this, the protein was pre-exposed to the inhibitor, and residual pCMPS (0.415 kDa) was removed by ultrafiltration before assay of the ‘modified’ enzyme. In principle, enzyme activity would be inhibited if an adduct had formed between PI-PLC and pCMPS, whereas little loss of activity would be observed if no covalent adduct was created during the pre-incubation. Ultrafiltration of the PI-PLC: pCMPS complex resulted in loss of inhibition: PI-PLC activity was stimulated 10-fold (compare activity before and after filtration of pCMPS-treated PI-PLC; Figure 2). These data indicate that pCMPS inhibits PI-PLC without covalently modifying the enzyme.

**GPI-PLC from T. brucei: removal of unreacted pCMPS fails to reverse inhibition**

GPI-PLC from T. brucei contains eight cysteine residues [10,11], one of which appears to be at the enzyme active site [15]. The enzyme activity was inhibited completely by 5 mM pCMPS (Figure 1A). The IC_{50} and IC_{90} for pCMPS were 0.3 mM and 2 mM respectively.

We assessed whether inhibition of GPI-PLC was correlated with covalent modification of the enzyme by pCMPS. When GPI-PLC was pretreated with pCMPS, which was subsequently ‘removed’ by ultrafiltration, no re-activation of enzyme activity occurred (Figure 3A). Hence pCMPS is likely to have modified GPI-PLC covalently. Nevertheless, it was important to test whether modification of the substrate ([3H]mfVSG) contributed significantly to loss of the GPI cleavage activity of GPI-PLC. For this, two substrates, pCMPS-treated [3H]mfVSG and untreated [3H]mfVSG, were used separately. pCMPS treatment slightly affected the suitability of [3H]mfVSG as a substrate, causing 33% loss of enzyme activity (compare ‘untreated’ lanes in Figures 3A and 3B). This degree of inhibition is expected if the filtered [3H]mfVSG contained 13 μM untreated pCMPS (Figure 1A; estimated from an initial 5 mM pCMPS, dilution before the filtration, and the final volume in the retentate [detailed in the Materials and methods section]). We surmise that GPI-PLC, and not mfVSG, is the major target of pCMPS.

**B. cereus PI-PLC: equimolar β-ME reverses inhibition by pCMPS**

Further evidence for the non-covalent nature of the interaction between B. cereus PI-PLC and pCMPS was acquired by examining the effect of β-ME on enzyme inhibition (β-ME can react with pCMPS). PI-PLC was treated in two temporal stages, first with pCMPS (2 mM final) and then with β-ME (2 mM). Substrate was added in the third stage, and GPI digestion was determined.

Although introduced after exposure of the enzyme to pCMPS, β-ME reversed the inhibition of PI-PLC by the thiol reagent (Figure 4A). [By itself β-ME does not activate PI-PLC (Figure 4A).] To test whether β-ME could protect PI-PLC from inhibition by pCMPS, PI-PLC was preincubated with β-ME before the addition of PI-PLC and substrate digestion. Equimolar β-ME (i.e. with respect to pCMPS) counteracted pCMPS completely (Figure 4B), but less than stoichiometric amounts failed to protect PI-PLC. This observation suggests that a complex formed between β-ME and pCMPS cannot inhibit PI-PLC (Figure 4B).

**T. brucei GPI-PLC: excess β-ME is required to protect the enzyme from pCMPS**

The possibility that β-ME might reverse the inactivation of T. brucei GPI-PLC by pCMPS was investigated. GPI-PLC was treated with 5 mM pCMPS prior to the addition of various
Figure 5 Effect of β-ME on pCMPS-mediated inhibition of T. brucei GPI-PLC

(A) Equimolar β-ME fails to reverse inhibition of T. brucei GPI-PLC by pCMPS. GPI-PLC (2.5 nM) was incubated with 5 mM pCMPS before addition of different concentrations of β-ME. [3H]mfVSG was added, and the reaction was incubated at 37 °C for 10 min. Released [3H]DMG was quantified.

(B) Equimolar β-ME partially protects GPI-PLC from inhibition by pCMPS. pCMPS (5 mM) was first incubated with different concentrations of β-ME. GPI-PLC (2.5 nM) was then added, followed by [3H]mfVSG, whose digestion was carried out at 37 °C for 10 min. [3H]DMG was quantified after extraction into n-butanol. β-ME acts on pCMPS-modified GPI-PLC to re-activate enzyme activity. T. brucei GPI-PLC (25 nM) was preincubated with or without 5 mM pCMPS at 37 °C for 10 min. The mixture was filtered through Centricon-10 (stage 1). GPI-PLC (25 nM) from the Centricon-10 retentate was exposed to various concentrations of β-ME at 37 °C for 10 min (stage 2). [3H]mfVSG was added, and the reaction was incubated at 37 °C for 10 min for GPI cleavage. Released [3H]DMG was quantified.

Amounts of β-ME ([β-ME] alone stimulated GPI-PLC 2–3-fold (Figure 5A).] An equimolar amount (i.e. with respect to pCMPS) of β-ME failed to restore GPI-PLC activity (Figure 5A). A 10-fold excess of β-ME led to recovery of 50% of the maximal GPI-PLC activity (i.e. with β-ME). When pCMPS was preincubated with β-ME before addition to GPI-PLC, equimolar β-ME could not protect GPI-PLC from inhibition by pCMPS (Figure 5B): only 13% of the maximal activity was recovered. A 2-fold excess, at least, of β-ME was needed in preincubations with pCMPS to fully protect GPI-PLC (Figure 5B).

Figure 6 Nature of the pCMPS-mediated inhibition of B. cereus PI-PLC

(A, B) [3H]mfVSG as substrate. (A) Inhibition by pCMPS of [3H]mfVSG digestion. Various concentrations of pCMPS were incubated with B. cereus PI-PLC at 37 °C for 10 min. Following addition of [3H]mfVSG, the mixture was incubated at 37 °C for 10 min. Released [3H]DMG was determined (see Figure 1A). The ratio of PI-PLC reaction rate in the absence of inhibitor (V_o) to that in the presence of inhibitor (V_i) is plotted against the ratio of the mole fraction of inhibitor (X_i) to the mole fraction of the remainder of the components of the reaction mixture (1 – X) (see the Materials and methods section for details). (B) Inhibition of GPI-PLC by SDS. SDS was added to the reaction mixture during [3H]mfVSG digestion (see the Materials and methods section), and the reaction analysed as described for (A). (C) [3H]PI as substrate. PI-PLC assays were performed and quantified as described in Figure 1A. V_o/V_i again plotted against X_i/(1 – X_i).
We tested a hypothesis that the \( \beta \)-ME-mediated reversal of \( p \)CMPS-dependent inhibition was the result of reaction of the reducing agent with \( p \)CMPS-modified GPI-PLC. To this end, GPI-PLC was first treated with \( p \)CMPS (5 mM) and the complex was filtered through a Centricon-10 membrane. GPI-PLC in the retentate was treated with various amounts of \( \beta \)-ME (Figure 5C). \( \beta \)-ME at 1 mM restored 50% of GPI-PLC activity, and 5 mM was sufficient for 100% activity. In the presence of 50 mM \( \beta \)-ME, GPI-PLC activity was stimulated slightly (i.e. activity was above the basal level observed in the absence of \( p \)CMPS) (Figure 5C).

When GPI-PLC was treated first with \( \beta \)-ME and the filtered enzyme studied, 1 mM \( p \)CMPS inhibited the enzyme by 80% (results not shown). Thus \( \beta \)-ME does not permanently alter the biochemical properties of recombinant GPI-PLC; protection conferred by \( \beta \)-ME against \( p \)CMPS requires the continued presence of the reducing agent.

Together, these data indicate that \( p \)CMPS, in contrast with its mode of action on \( B. \) cereus PI-PLC, modifies \( T. \) brucei GPI-PLC covalently. Consistent with this conclusion, a large excess of reducing agent is needed to restore partial enzyme activity to a \( p \)CMPS \( \cdot \) GPI-PLC adduct.

\( p \)CMPS is a competitive inhibitor of \( B. \) cereus PI-PLC

The absence of covalent modification during the inhibition of PI-PLC by \( p \)CMPS made it possible to determine the nature of the inhibition by the thiol reagent. Interfacial kinetic analysis (reviewed in [22]) was chosen for this aspect of the work, since PI-PLC interacts with its substrates at the boundary between detergent micelles and the aqueous medium. The effective inhibitor concentration at the micellar interface was expressed as the ratio of the mole fraction of inhibitor (\( X_i \)) to the mole fraction of the remainder of the components of the reaction mixture (\( 1 - X_i \)). Plots of \( V_i / V \) against \( X_i / (1 - X_i) \) (where \( V_i \) is the reaction rate in the absence of inhibitor and \( V \) is the reaction rate in the presence of inhibitor) [25] are presented (Figure 6).

Competitive inhibitors are identified by two features in the plots: (i) a successful fit of the data points to a linear equation (with \( r^2 > 0.90 \)); and (ii) when \( X_i / (1 - X_i) = 0 \), the value of \( V_i / V \) approximates 1 (see the Materials and methods section). This approach cannot distinguish between non-competitive and uncompetitive inhibition.

\( p \)CMPS proved to be a competitive inhibitor of the PI-PLC-catalysed digestion of \( [\text{H}] \text{mfVSG} \) (Figure 6A). In control experiments, inhibition of \( T. \) brucei GPI-PLC by SDS did not occur competitively; a linear curve fit to the data failed to satisfy the required conditions (see above). An exponential curve fit for the inhibition by SDS is presented (Figure 6B). Surprisingly, when \( [\text{H}] \text{PI} \) was used as substrate, the linear equation obtained for the data was \( y = 6.105x - 1.831 \), and \( r^2 = 0.886 \) (i.e. less than 0.9) (graph not shown). When \( X_i / (1 - X_i) = 0 \), \( V_i / V = -1.831 \) (this value deviates significantly from the expected value of 1.0). A much better fit of the data was obtained with the exponential equation \( y = 0.893 \times 10^{(-0.358x)} \) \( r^2 = 0.994 \) (Figure 6C). From the curve, \( V_i / V = 0.893 \) (i.e. close to 1) when \( X_i / (1 - X_i) = 0 \). We conclude that when \( [\text{H}] \text{PI} \) is the substrate, \( p \)CMPS is not a competitive inhibitor of PI-PLC.

**EDTA distinguishes between \( p \)CMPS- and \( \text{Hg}^{2+} \)-mediated inhibition of \( B. \) cereus PI-PLC**

Mercury is a component of \( p \)CMPS. Consequently we considered the possibility that inhibition of PI-PLC by the thiol reagent was due to \( \text{Hg}^{2+} \) contamination of commercial \( p \)CMPS (the manufacturer’s product analysis sheet did not report the presence of \( \text{Hg}^{2+} \)). We presumed that between 0.1% and 1% of \( \text{Hg} \) in \( p \)CMPS was \( \text{Hg}^{2+} \) ion. Since 2 mM \( p \)CMPS inhibited \( B. \) cereus PI-PLC completely (Figures 1A and 1B), we tested whether 2 mM \( \text{Hg} \) \( \equiv\text{Hg}^{2+} \) (i.e. 0.1% of the \( p \)CMPS concentration), presented as the chloride salt, would inhibit PI-PLC. (Micromolar chloride ions, as the sodium salt, do not inhibit PI-PLC activity.)

\( \text{HgCl}_2 \) was a potent inhibitor of PI-PLC; 0.6 mM inhibited PI-PLC by 50% (Figure 7A), as reported previously [18]. In control experiments, \( \text{CuSO}_4 \) inhibited PI-PLC feebly (Figure 7B). Although 5 mM \( \text{CuSO}_4 \) decreased PI-PLC activity by 50%, the susceptibility of the enzyme to \( \text{Cu}^{2+} \) tapered off; significant ac-
tivity remained in 30 μM CuSO$_4$ (results not shown). Thus Cu$^{2+}$ is not as good an inhibitor of PI-PLC as Hg$^{2+}$.

EDTA binds to metal ions. We therefore tested whether it could counteract the effects of Hg$^{2+}$ on PI-PLC. When PI-PLC was preincubated with 10 μM Hg$^{2+}$, equimolar EDTA blocked the inhibition of PI-PLC (Figure 7C). This result is consistent with successful chelation of Hg$^{2+}$ by EDTA.

The effect of EDTA on the pCMPS-mediated inhibition of PI-PLC was different from that reported for Hg$^{2+}$-mediated inhibition (Figure 7C). Quantities of EDTA (5 μM and above) similar to those that rescued PI-PLC from Hg$^{2+}$-mediated inhibition failed to shield PI-PLC from pCMPS (Figure 7C). If the effect of pCMPS on PI-PLC was due to contamination by 0.1 %, Hg$^{2+}$, 5 μM EDTA would reverse the inhibition. Yet 50 μM EDTA could not prevent the pCMPS-mediated inhibition of PI-PLC. These results indicate that the pathway of Hg$^{2+}$-mediated inhibition of PI-PLC is distinct from the mechanism employed by pCMPS. More importantly, the data indicate that inhibition of PI-PLC by pCMPS is not the result of Hg$^{2+}$ contamination of the thiol reagent.

**DISCUSSION**

GPI-specific phospholipases are used routinely for the demonstration of GPI anchoring of proteins and polysaccharides to biological membranes [26], and as membrane-impermeable ‘probes’ of GPI synthesis in vivo and in vitro [7,27–29]. The enzymes have been used to construct transgenic GPI-deficient cells to explore the biological roles of GPIs [5,6,30]. These diverse uses of this unique class of phospholipases call for clear understanding of the conditions that affect their activity in whole cells and in vitro.

The participation of cysteine residues in biochemical and biological events is frequently revealed initially by susceptibility to thiol reagents (e.g. pCMPS or N-ethylmaleimide). Some recent cases involving membrane-active proteins highlight interesting developments in the field. Calcium-insensitive cytosolic phospholipase A$_2$, is inhibited by N-ethylmaleimide, yet mutagenesis studies indicate that none of the nine cysteine residues in the protein are critical for activity [31]. GPI-PLC from *T. brucei* is inhibited by thiol reagents [12,32,33], but no single cysteine is indispensable for enzyme activity [12,33,34]. Similarly, Cys residues in Na$^+$/dicarboxylate co-transporter-1 are not needed for function, although activity of the protein is blocked by p-chloromercuribenzenesulphonate [35]. At the other end of the spectrum, a Cys-less PI-PLC can be inhibited by a thiol reagent (Figure 1) [18].

In an attempt to gain understanding of how pCMPS affects *B. cereus* PI-PLC, our first objective was to determine whether the inhibition required covalent modification of the enzyme. In experiments where a preformed PI-PLC · pCMPS complex was resolved by ultracentrifugation into enzyme (in the retentate) and free pCMPS (in the filtrate), most of the enzyme activity was recovered (Figures 2 and 4). This finding suggested that inhibition of PI-PLC by pCMPS did not result from covalent modification. In a control experiment with *T. brucei* GPI-PLC, activity in a pCMPS-treated enzyme could not be recovered in the retentate by following the protocols described for PI-PLC (Figure 3).

Further evidence for the absence of covalent modification during inhibition of *B. cereus* PI-PLC activity by pCMPS was obtained from staged incubations of the enzyme with either pCMPS or the reducing agent β-ME. After pretreatment of PI-PLC with pCMPS, the amount of β-ME needed to ‘reverse’ the inhibition was equimolar to the concentration of pCMPS (Figure 4). One would not expect such low (relative) stoichiometry for the reducing agent if the enzyme had been modified covalently (see data on *T. brucei* GPI-PLC below). These data support the earlier conclusion (from the ultracentrifugation experiments) that pCMPS does not modify *B. cereus* PI-PLC covalently.

With regard to the use of PI-PLC for analysis of GPIs, our observations suggest the following. First, it may be beneficial to avoid pCMPS in the preparation of substrates from cell lysates. Secondly, inclusion of EDTA (50 μM) in PI-PLC buffers will be beneficial; EDTA is likely to keep the enzyme active in cases where small amounts of metal ions may interfere with its action on GPIs. Thirdly, if the use of thiol reagents is unavoidable in substrate preparation, investigators are encouraged to include β-ME in the reaction buffer to reverse inhibition by the thiol reagents.

Unlike *B. cereus* PI-PLC, *T. brucei* GPI-PLC displayed properties of a protein that was covalently modified by pCMPS. Following exposure to pCMPS, a 5–10-fold excess of β-ME was needed to recover significant enzyme activity (Figure 5A). A preformed pCMPS · GPI-PLC complex had no activity even after ultrafiltration to remove pCMPS (Figure 5C). Recovery of activity in the filtered enzyme (2.5 mM in the reaction) required a 2.5 × 10$^{-3}$-fold excess (1 mM) of β-ME (Figure 5C). Even then, 50 mM β-ME failed to restore full activity to GPI-PLC (results not shown).

The nature of the inhibition of *B. cereus* PI-PLC by pCMPS (Figure 6) is captivating. pCMPS has affinity for the GPI binding site of PI-PLC, since it is a competitive inhibitor of mfVSG digestion (Figure 6A). Curiously, with PI as a substrate the type of inhibition changes; it is no longer competitive (Figure 6B). The major difference between the two substrates (i.e. PI and GPI) is the presence of the ethanolamine (EtN)-phosphoglycan (EtN-P-Man$_2$-GlcN) group [27] on the GPI of mfVSG. So how does one reconcile the different modes of pCMPS-mediated inhibition?

PI-PLC binds to both the inositol and the glycan moieties of the substrate [36,37], and a GPI is a better substrate for PI-PLC than PI [38–40]. Scission of the phosphodiester bond at the inositol 1-position is influenced by glycan recognition. Surprisingly, data from crystal structures indicate that the myo-inositol headgroup of PI is not recognized within the same pocket where the glycan is bound to PI-PLC. myo-Inositol makes several contacts within a relatively deep groove in PI-PLC [37], but the glycan recognition site extends on shallow grooves along the surface of the protein [reviewed in [41]]. From these considerations, an inhibitor that occupies the myo-inositol site need not interfere with another molecule binding to the glycan binding site. Our data suggest that pCMPS binds the glycan recognition site on PI-PLC. Thus, when a GPI is the substrate, the mode of inhibition is competitive, because of the glycan on that substrate. However, PI is recognized solely at the myo-inositol site, meaning that PI does not compete with pCMPS, which, acting as a glycomimetic compound, is at the glycan recognition site of PI-PLC. Thus, when PI is the substrate, pCMPS occupancy of the glycan binding site lowers rates of catalysis allosterically.

Our data on *B. cereus* PI-PLC and similar results [17] call for caution in the interpretation of observations from inhibition of protein function by thiol reagents. Where possible, supporting evidence for covalent modification (e.g. resuscitation with a reducing agent after removal of the inhibitor) can strengthen conclusions about how the thiol reagents act on the polypeptides. The experimental strategies employed in the present study point to some rapid biochemical approaches for distinguishing between covalent and non-covalent inhibition of protein function by thiol reagents. Eventually, these biochemical studies can be
complemented with mutagenesis of the cysteine residues when genes for the proteins in question become available.

This study was supported by NIH grant AI33383 to K.M.-W.

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Received 4 March 2002/10 May 2002; accepted 15 May 2002
Published as BJ Immediate Publication 15 May 2002, DOI 10.1042/BJ20020367

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