A Receptor and G-protein-regulated Polyphosphoinositide-specific Phospholipase C from Turkey Erythrocytes

II. PZy-PURINERGIC RECEPTOR AND G-PROTEIN-MEDIATED REGULATION OF THE PURIFIED ENZYME RECONSTITUTED WITH TURKEY ERYTHROCYTE GHOSTS*

(Received for publication, December 27, 1989)

Andrew J. Morris, Gary L. Waldo, C. Peter Downes‡, and T. Kendall Harden§

From the Department of Pharmacology, the University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599 and the Department of Biochemistry, Medical Sciences Institute, University of Dundee, Dundee DD1 4HN, Scotland

The preceding paper describes purification and properties of a 150-kDa polyphosphoinositide-specific phospholipase C from a cytosolic fraction of turkey erythrocytes (Morris, A. J., Waldo, G. L., Downes, C. P., and Harden, T. K. (1990) J. Biol. Chem. 265, 13501-13507). Turkey erythrocytes express a PZy-purinergic receptor that employs an unidentified G-protein to activate phospholipase C (Boyer, J. L., Downes, C. P., and Harden, T. K. (1989) J. Biol. Chem. 264, 884-890; Cooper, C. L., Morris, A. J., and Harden, T. K. (1989) J. Biol. Chem. 264, 6202-6206). This paper describes receptor and G-protein regulation of the purified turkey erythrocyte phospholipase C after reconstitution of the enzyme using [3H]inositol pre-labeled turkey erythrocyte ghosts as acceptor membranes. These membranes contain polyphosphoinositides labeled to high specific radioactivity and display reduced responsiveness of their endogenous phospholipase C to PZy-purinergic receptor agonists and guanine nucleotides. Reconstitution of purified enzyme had no effect on basal inositol phosphate production, but markedly increased PZy-purinergic receptor agonist and guanine nucleotide-dependent accumulation of inositol phosphates. Reconstitution of 5 ng of purified phospholipase C with 10 μg of acceptor membrane protein produced half-maximal effects, and maximal activity was observed with reconstitution of 100 ng of purified enzyme. Agonist and guanine nucleotide-regulated phospholipase C activity measured using a reconstitution assay co-purified with phospholipase C activity detected using exogenously provided phosphatidylinositol 4,5-bisphosphate during purification of the 150-kDa protein. Only the maximal rate of inositol phosphate formation attained upon activation was increased in the presence of the purified phospholipase C. K0.5 values for adenosine 5'-O-(2-thiodiphosphate), guanosine 5'-3' O-(thio)triphosphate, and AlF4- activation of the purified enzyme were the same as for the endogenous phospholipase C activity of the acceptor membranes.

Inositol lipid-specific phospholipase C (PLC) plays a central role in the signal transduction mechanism employed by a large group of cell surface receptors. There have been recent parallel advances in understanding of both the mechanisms receptors utilize to regulate PLC activity and of the enzymology of the PLC family (reviewed in Refs. 1 and 2). However, none of the identified PLC isoenzymes has been directly implicated in receptor-mediated inositol lipid hydrolysis.

Three distinct lines of experimental evidence support a role for a G-protein in receptor-mediated regulation of PLC. Thus, agonist binding to PLC-coupled receptors is modulated by guanine nucleotides (3, 4), and pertussis toxin, a bacterial toxin known to inhibit function of a number of G-proteins, has been shown, in certain cases, to inhibit receptor-mediated activation of PLC (5, 6 but see Refs. 7 and 8). The most direct evidence for the involvement of a G-protein in receptor regulation of PLC comes from studies using cell-free preparations where receptor-mediated activation of PLC has been found to be absolutely dependent on the presence of guanine nucleotides (see Ref. 1 for review).

Exogenously provided phospholipid substrates, often presented as components of mixed phospholipid and detergent micelles, have been used to follow PLC activity during purification (2, 12). Several laboratories have reported receptor-mediated, guanine nucleotide-dependent activation of PLC when phosphoinositide substrates were presented in this form to membrane preparations from several cell types (9-11). However, these observations have not been extended to systems containing purified proteins. Conversely, the most detailed examinations of receptor-mediated regulation of PLC have used cell-free preparations in which the sources of substrates were endogenously labeled inositol lipids (see Ref. 1 for review). While clearly of greater physiological relevance than that of assays requiring exogenously provided substrates, this approach has several drawbacks. The most prominent of these relates to the inherent inability to vary substrate concentration and substrate-specific radioactivity without coincidently producing a parallel change in the amounts of the proteins responsible for receptor-stimulated inositol lipid hydrolysis.

* This work was supported by United States Public Health Service Grants GM 29563 and GM 38215 and by the Patrick Mitchell Research Fellowship from the American Heart Association North Carolina Affiliate (to A. J. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence and reprint requests should be addressed.
G-protein-regulated Phospholipase C from Turkey Erythrocytes

The preceding paper describes the purification and properties of a PLC from turkey erythrocytes (12). In this report, we describe the regulation of the purified PLC when reconstituted with [H]inositol-labeled turkey erythrocyte ghosts. The results demonstrate that this isoenzyme of PLC can function as a catalytic component of the machinery used by cell surface receptors and their associated G-proteins to generate inositol lipid-derived second messengers.

EXPERIMENTAL PROCEDURES

Purification of PLC—PLC was purified from turkey erythrocytes exactly as described previously (12). The mass of the purified PLC was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and staining with Coomassie Brilliant Blue.

[3H]Inositol Labeling of Turkey Erythrocytes—Turkey erythrocytes were labeled with [3H]inositol using the method described (13) except that chicken serum was omitted from the incubations. One ml of washed, packed cells was incubated with 2 mCi of [3H]inositol for 24 h. Ghosts were prepared from the labeled cells by hypotonic lysis and repeated centrifugation as described below (14).

PLC Assay—PLC activity against 3H-labeled inositol lipids of turkey erythrocyte ghosts was determined as described (13). Briefly, the ghosts were prepared by hypotonic lysis of 1 ml of turkey erythrocytes in 40 ml of buffer containing 5 mM KH2PO4, 5 mM MgCl2, 1 mM DTTA, followed by three washes of treated centrifugation and resuspension in this buffer. The ghosts were finally resuspended by homogenization in 40 ml of 10 mM HEPES, pH 7.4, sedimented by centrifugation, and resuspended in 1 ml of 10 mM HEPES, pH 7.4. The assays were started by the addition of prewarmed [3H]inositol-labeled turkey erythrocyte ghosts from polyethylene tubes to borosilicate glass tubes containing warmed assay buffer plus drugs. In some cases, the ghosts were held on ice, and purified turkey erythrocyte PLC was combined with them before dilution with 10 mM HEPES, pH 7.4, warming, and addition to the assay medium. The assays were performed at 30 °C, and the incubation time was 5 min unless otherwise stated. Incubations were terminated with ice cold 7% perchloric acid and neutralized (20). [3H]Inositol phosphates were quantitated by liquid scintillation spectrometry after anion exchange chromatography on Bio-Rad AG1-X8 (200-400 mesh) as described (13). PLC activity against exogenously provided PtdIns(4,5)P2 was determined as described (12).

Protein Assay—Protein was determined by the method of Bradford (21) using bovine serum albumin as standard. Particulate material was warmed in 0.1 M NaOH and neutralized before assay.

RESULTS

Reconstitution of Purified Turkey Erythrocyte PLC with [3H]inositol-labeled Turkey Erythrocyte Ghosts—Ghosts prepared from turkey erythrocytes labeled to high specific radioactivity with [3H]inositol (average specific radioactivity of PtdIns(4,5)P2 = 0.5 Ci/mmol) were used as acceptor membranes for the reconstitution of turkey erythrocyte PLC purified as described in the accompanying manuscript (12). PLC activity was typically determined in incubations containing 10 μg of ghost protein. This amount of protein is 10 to 20 times less than that used in our previous studies of the receptor and G-protein-regulated PLC of turkey erythrocyte ghosts (13, 14, 22, 23). In contrast to these reports, under the conditions used for the experiment presented in Table I, there was little measurable stimulation of PLC activity by 10 μM GTPγS alone, and 10 μM GTPγS in combination with a maximally effective concentration of the P2Y-purinergic receptor agonist ADP/3S elicited a comparatively modest activation of PLC. In the experiment illustrated in Table I, addition of 10 ng of purified turkey erythrocyte PLC to the ghosts had no effect on the formation of inositol phosphates determined under basal conditions or in the presence of ADP/3S alone. We have occasionally noted small increases in basal PLC activity upon reconstitution of the purified PLC (see Fig. 8). However, in the presence of the purified 150-kDa turkey erythrocyte PLC, inositol phosphate formation in response to GTPγS was markedly enhanced and was dramatically amplified in the combined presence of ADP/3S and GTPγS. Purified turkey erythrocyte PLC that had been inactivated by boiling prior to reconstitution had no effect on either GTPγS or GTPγS plus ADP/3S-promoted inositol phospholipid hydrolysis when combined with the ghosts.

Dependence of PLC Reconstitution on Acceptor Membrane Concentration—[3H]Inositol-labeled turkey erythrocyte ghosts were prepared, and their protein content was determined. The amount of ghost protein in a 200-μl final assay volume was varied from 1 to 100 μg. [3H]Inositol phosphate release from the ghosts was determined under basal conditions (B—B) or during stimulation with 10 μM ADP/3S and 10 μM GTPγS (O—O). In some cases (A—A), purified turkey erythrocyte PLC (10 ng/assay) was added to the ghosts. The data presented are means of triplicate determinations expressed as a percentage of the total amount of [3H]inositol-labeled lipid originally present in each incubation. A zero time value which ranged from 0.05 to 0.2% of the total [3H]inositol-labeled lipid present in each incubation has been subtracted from the data shown.

![FIG. 1. Dependence of PLC reconstitution on acceptor membrane concentration. [3H]Inositol-labeled turkey erythrocyte ghosts were prepared, and their protein content was determined. The amount of ghost protein in a 200 μl final assay volume was varied from 1 to 100 μg. [3H]Inositol phosphate release from the ghosts was determined under basal conditions (B—B) or during stimulation with 10 μM ADP/3S and 10 μM GTPγS (O—O). In some cases (A—A), purified turkey erythrocyte PLC (10 ng/assay) was added to the ghosts. The data presented are means of triplicate determinations expressed as a percentage of the total amount of [3H]inositol-labeled lipid originally present in each incubation. A zero time value which ranged from 0.05 to 0.2% of the total [3H]inositol-labeled lipid present in each incubation has been subtracted from the data shown.](image-url)
ulated release of [3H]inositol phosphates from the ghosts expressed as a percentage of total [3H]inositol labeled lipids decreased with increasing dilution of the ghost membranes. Agonist and guanine nucleotide-stimulated PLC activity was reduced markedly with less than 10 μg of protein in the assays and was maximal with 100 μg of ghost protein. Reconstitution of 10 ng of purified PLC resulted in a substantial increase in agonist and guanine nucleotide-stimulated inositol lipid hydrolysis under conditions where no activation of endogenous PLC was observed (1–10 μg of membrane protein) and resulted in augmentation of the [3H]inositol phosphate formation observed with amounts of acceptor protein in the range of 20–50 μg. Activity of the reconstituted PLC was only expressed in the presence of ADPβS and GTPγS. That is, basal activity was identical in both the presence and absence of the purified PLC (Fig. 1).

**Effect of Purified PLC on the Concentration Dependence for Agonist and Guanine Nucleotide-stimulated Inositol Lipid Hydrolysis**

Effect of Purified PLC on the Concentration Dependence for Agonist and Guanine Nucleotide-stimulated Inositol Lipid Hydrolysis was investigated by the reconstitution of increasing amounts of enzyme (0.01–200 ng) with 10 μg of [3H]inositol-labeled turkey erythrocyte ghosts. GTP was used instead of GTPγS in these experiments since, in the presence of this nucleotide, receptor activation of PLC occurs more rapidly than with GTPγS, yet the steady state rate of PLC activity attained is lower with GTP than that attained with GTPγS (13). This strategy was used to minimize substrate limitation of PLC activity. ADPβS in the presence of GTP produced a negligible accumulation of inositol phosphates in the absence of added enzyme (Fig. 2). An increase in ADPβS plus GTP-stimulated inositol lipid hydrolysis was apparent with reconstitution of as little as 1 ng of the purified PLC. Agonist and guanine nucleotide-stimulated inositol phosphate formation increased with increasing amounts of the purified PLC. This effect was half-maximal with 5 ng of the purified PLC and maximal with 100–200 ng. In the absence of agonist plus GTP, the purified PLC did not significantly increase inositol phosphate formation at any of the concentrations tested (data not shown).

**Activators of G-protein-regulated PLC**—Concentration-effect curves for the activation of PLC in turkey erythrocyte ghosts were determined for ADPβS in the presence of 1 mM GTP (Fig. 3), for GTPγS (Fig. 4), and for NaF (assayed in the presence of 25 mM AlCl3, data not shown). An amount of acceptor protein was chosen that gave a measurable endogenous PLC activity (50 μg, see Fig. 1), and this was compared with the activity expressed in the presence of the purified PLC (50 ng/assay). The magnitude of inositol phospholipid hydrolysis was increased in the presence of the purified PLC (Figs. 3 and 4), but the Kₐ values for each of the activators was the same irrespective of whether they were determined in the presence or the absence of the purified PLC.

**Kinetics of Activation of the Reconstituted Purified PLC**—Boyer et al. (13) demonstrated that P₂Y-purinergic receptor agonists increased the rate of activation of PLC by GTPγS in a concentration-dependent and saturable manner. Experi-
ments were performed to compare the kinetics of activation of the endogenous PLC activity of turkey erythrocyte ghosts with that of the purified PLC when reconstituted with these ghosts. [3H]Inositol-labeled turkey erythrocyte ghosts (20 μg of protein per assay) were incubated with and without 100 μM ADPβS and 10 μM GTPγS in the presence and absence of the purified PLC (90 ng/assay). The time course of activation of PLC was determined. The rate of activation of PLC was the same in each case. Only the maximal rate attained was increased in the presence of the purified PLC (Fig. 5 and inset). A series of similar experiments demonstrated that the first order rate constant (kmax) for the activation of PLC was independent of the amount of PLC added to the ghosts (kmax = 0.86 ± 0.06 min⁻¹, mean ± S.E. of five replicate determinations using increasing amounts of the purified PLC in the range of 5–100 ng). GDPβS blocks guanine nucleotide-dependent activation of PLC in turkey erythrocyte ghosts (13). Although we did not attempt a precise quantitative analysis of this phenomenon, we also found that the rate of inactivation of PLC observed on addition of competing concentrations of GDPβS following preactivation with agonist plus GTP or GTPγS was similar for endogenous PLC and the purified 150-kDa PLC reconstituted into acceptor membranes. Thus, return of PLC to basal levels of activity was rapid with agonist plus GTP, but much slower following preactivation with agonist plus GTPγS (not shown).

Co-purification of the Receptor and G-protein-regulated PLC with PLC Activity against Exogenously Provided Substrates—We used the reconstitution assay described above to follow PLC activity during purification from turkey erythrocyte cytosol (12). Although PLC activity against exogenously provided substrates was readily detectable in both unfractionated turkey erythrocyte cytosol and in the resuspended fraction obtained following precipitation with 226 g/liter (NH₄)₂SO₄, we could not demonstrate a restoration of receptor and G-protein-regulated PLC activity when this material or fractions containing PLC activity following chromatography on Q-Sepharose or hydroxyapatite were combined with [3H]inositol-labeled turkey erythrocyte ghosts. By comparison with reconstitution experiments using the purified PLC, the large dilution and high salt content of the PLC preparation at these early steps makes it impossible to introduce sufficient enzyme into the reconstitution assay to produce a readily detectable receptor-stimulated accumulation of inositol phosphates. By the third and subsequent chromatographic steps (heparin-Sepharose, Sephacryl S-300, and Mono Q), reconstitution of agonist-stimulated inositol phosphate accumulation could be detected. Receptor-regulated PLC activity and PLC activity directed against exogenously provided substrates were coincident during elution in each of these column steps (not shown for the heparin-Sepharose and Sephacryl S-300 steps). The chromatographic behavior of receptor-stimulated PLC activity and PLC activity detected using exogenously provided substrate are illustrated in Fig. 6 for the final purification step of anion exchange chromatography on Mono Q.

Ca²⁺ Dependence of Reconstituted PLC Activity—In the absence of hormonal activators, PLC activity of turkey erythrocyte ghosts is markedly insensitive to activation by Ca²⁺ in the range 10⁻⁸ to 10⁻³ M, and GTPγS-activated PLC in this membrane preparation is potentiated by Ca²⁺ in the range 0.02–1 μM, although the Ca²⁺ dependence of P₂γ-purinergic receptor-promoted activation of PLC has not been investigated (22). Fig. 7 illustrates the dependence of polyphosphoinositide hydrolysis on Ca²⁺ determined under basal conditions or during activation with 10 μM GTPγS alone or in combination with 10 μM ADPβS using 40 μg of turkey erythrocyte ghosts per assay (left panel) or the same amount of ghosts reconstituted with approximately 50 ng of purified PLC (right panel). In the native ghosts, basal PLC activity was unaltered by increasing the calcium concentration from 20 nM to 1 mM, although in the presence of the purified PLC a small Ca²⁺-dependent increase in basal PLC activity was observed (see above). In both cases, GTPγS and GTPγS- plus ADPβS-stimulated polyphosphoinositide hydrolysis was markedly dependent on Ca²⁺, with Ca²⁺ having both stimulatory and

FIG. 6. Co-purification of receptor and G-protein-regulated PLC with PLC activity against exogenously provided substrates. PLC was purified from turkey erythrocyte cytosol as described previously (13). The pooled fractions containing PLC activity obtained after the second step of gel filtration chromatography on Sephacryl S-300 were applied to a Pharmacia Mono Q HR 10/10 anion exchange fast protein liquid chromatography column, and the column eluted with a gradient of NaCl as described (12). Fractions (0.3 ml) of the eluant were collected and assayed for PLC activity using exogenously provided PtdIns(4,5)P₂ as described. The fractions also were assayed for their capacity to reconstitute P₂γ-purinergic receptor-stimulated PLC activity when combined with [3H]inositol-labeled turkey erythrocyte ghosts using 2 μl of each fraction per assay combined with approximately 20 μg of ghost protein and conditions which were otherwise identical to those previously described (Table 1). The values presented for the exogenous substrate assay are means of duplicate determinations while the reconstitution assays were performed in triplicate. Endogenous agonist plus guanine nucleotide-stimulated PLC activity of the acceptor ghosts has been subtracted from the data shown.
inhibitory effects on enzyme activity. The concentration dependence for the stimulatory effect of Ca\(^{2+}\) was similar for both activating conditions, with half-maximal activation occurring at around 50 mM Ca\(^{2+}\) and maximal activity attained between 0.5 and 2.0 mM. Higher concentrations exerted an inhibitory effect on PLC activity. This inhibitory effect was half-maximal with approximately 100 mM Ca\(^{2+}\) and again, both GTPyS and GTP\(\gamma\)S-plus ADP\(\beta\)S-stimulated activity showed a similar Ca\(^{2+}\) concentration dependence. These results indicate that the Ca\(^{2+}\) dependence of the endogenous PLC activity of turkey erythrocyte ghosts and of the purified PLC reconstituted with turkey erythrocyte ghosts are very similar. In the turkey erythrocyte system, hormonal activation of PLC seems to occur without a change in the Ca\(^{2+}\) dependence of the enzyme which is in contrast to the reported effects of agonists and guanine nucleotides on PLC activity observed in other cell types (1, 11). In addition, we note that dependence of the purified PLC on Ca\(^{2+}\) for activity when assayed with exogenously provided polyphosphoinositide substrates (12) parallels the stimulatory requirement for Ca\(^{2+}\) shown by the reconstituted PLC acting on membrane phospholipid substrates.

Substrate Selectivity of the Reconstituted PLC—The selectivity of the reconstituted PLC for the three inositol lipids was assessed by anion exchange high performance liquid chromatography analysis of the water-soluble inositol phosphates formed in response to maximally effective concentrations of ADP\(\beta\)S and GTP\(\gamma\)S. Use of an amount of acceptor ghosts which themselves expressed PLC activity (20 \(\mu\)g, see Fig. 2) permitted a direct comparison of the reconstituted PLC with its endogenous counterpart. Consistent with our previous observations of the substrate selectivity of the endogenous PLC activity of turkey erythrocyte ghosts (22) and with the high specificity of the purified turkey erythrocyte PLC for the polyphosphoinositides when assayed with exogenously provided substrates (12), the only inositol phosphates formed were Ins\(\{1,4\}\)P\(_2\) and Ins\(\{1,4,5\}\)P\(_3\) (data not shown). The Ins\(\{1,4\}\)P\(_2\) may be derived by either direct PLC-catalyzed hydrolysis of PtdIns4P or by the subsequent dephosphorylation of PLC-generated Ins\(\{1,4,5\}\)P\(_3\) catalyzed by Ins\(\{1,4,5\}\)P\(_3\)-5-phosphatase also present in the ghosts (24).

**DISCUSSION**

PLC-catalyzed inositol lipid hydrolysis is an important mechanism of signal transduction employed by many cell surface receptors. A number of isoenzymes of PLC have been identified and purified from several different sources, yet their roles in the intact cell remain undefined. A conundrum which has contributed to this confusion concerns the subcellular distribution of PLC. When assayed with exogenously provided substrates, PLC activity has been found to be predominantly cytosolic in many different tissues (see Ref. 2 for review). The identified isoenzymes of PLC, including the turkey erythrocyte-derived enzyme, are stable soluble proteins that can be extracted from tissues and purified without exposure to detergents. Although membrane-associated PLC activity has been observed in many tissue preparations, some degree of extraction often could be effected by washing the membranes with salt (for example, Ref. 25). The most extensive study of this type (26) demonstrated by structural and immunological criteria that a PLC isoenzyme associated with bovine brain membranes (PLC-\(\beta\) and a truncated form of this protein) was identical with a previously purified soluble protein (see also Refs. 27 and 28). Consistent with these observations, the known primary sequences of three PLC isoenzymes do not reveal any regions of sufficient hydrophobicity to suggest that they may function in membrane insertion or anchoring of the proteins (28-31).

These observations are surprising because the phospholipid substrates of PLC and the cell surface receptors that control PLC activity are clearly integral components of the plasma membrane. The observation of guanine nucleotide-dependent decreases in agonist affinity of PLC-coupled receptors in washed membrane preparations (1, 3, 4) and the reported effects of G-protein \(\beta\)\(\gamma\) subunits on receptor regulation of PLC (23, 32) support a role for a heterotrimeric, plasma membrane-localized G-protein in the activation of PLC. Thus, either receptor-regulated PLC is stably associated with the plasma membrane yet has evaded purification, or a soluble form of PLC interacts with the plasma membrane and is indeed regulated by G-protein-linked cell surface receptors.

The results presented in this manuscript provide evidence in support of the latter proposal. That is, an isoenzyme of PLC has been purified from the cytosolic fraction of turkey erythrocytes and, when reconstituted with turkey erythrocyte ghosts, its catalytic activity against endogenously labeled polyphosphoinositides is regulated by G-protein-linked \(P_{\gamma}\)\(\gamma\)-purinergic receptors. The idea that a cytosolic PLC might be regulated by receptors is not a new one. Irvine et al. (33, 34) originally suggested that a stimulus-provoked association of a soluble PLC with the plasma membrane was a possible means by which cell surface receptors controlled inositol lipid hydrolysis. More recently, several groups have reported that guanine nucleotides activate crude preparations of soluble PLC from platelets, bovine brain, and calf thymocytes (35-37). In the studies using platelets and thymocytes, evidence has been presented for the physical association of soluble PLC with low molecular weight GTP-binding proteins (37, 38). The role of cell surface receptors in the regulation of these enzymes is not yet clear. Furthermore, studies using purified isoenzymes of PLC suggest that nucleotides may have direct effects on PLC activity expressed against exogenously provided substrates (39). Finally, one report suggests a role for soluble PLC in receptor-stimulated formation of inositol phosphates from plasma membrane-localized inositol lipids. Baldassare and Fisher (35) observed an amplified thrombin-stimulated guanine nucleotide-dependent inositol lipid hydrolysis when platelet cytosol was combined with \([^{3}H]\)inositol prelabeled platelet plasma membranes. Further work with more defined preparations of platelet PLC has not been reported. By contrast, we have been unable to demonstrate
guanine nucleotide regulation of PLC activity in turkey erythrocyte ghosts, and, as discussed above, our evidence suggests a plasma membrane localization of the P2Y-purinergic receptor-linked G-protein in this tissue (23, 40).

Our findings raise several important questions about the factors that determine the subcellular distribution of PLC in turkey erythrocytes. The data obtained imply that the interaction of PLC with the plasma membrane is a dynamic process. Central to our ability to reconstitute the purified turkey erythrocyte PLC with turkey erythrocyte ghosts is the observation that the endogenous PLC activity of the ghosts does not decrease proportionately when the ghosts are diluted into a fixed assay volume. These results imply that under the conditions used for preparation of the ghosts the putative components of the inositol lipid-dependent signalling system (that is, the PLC, its substrates and the receptor and associated G-protein that regulate it) do not behave as physically associated entities. The restoration of agonist and guanine nucleotide-regulated inositol lipid hydrolysis by the addition of the purified turkey erythrocyte PLC suggests that the lesion responsible for the observed reduction in agonist-stimulated inositol lipid hydrolysis involves a loss of PLC from the ghost membranes. However, we do not have any unambiguous experimental evidence to support this idea. Unfortunately at present we can only quantitate receptor-regulated PLC by measuring its catalytic activity expressed against endogenously labeled substrates, and this constraint makes it impossible to assess the partitioning of PLC between membranes and the soluble phase during receptor activation or during the preparation of acceptor ghosts for reconstitution experiments.

The catalytic and regulatory properties of the purified PLC when reconstituted with turkey erythrocyte ghosts and activated by the G-protein-linked P2Y-purinergic receptor are identical with those of the endogenous PLC activity of turkey erythrocyte ghosts (13, 22). This raises the possibility that the ghost-associated PLC and the purified cytosolic PLC are the same protein. Pertinent to this question we note that [3H]inositol-labeled turkey erythrocyte plasma membranes prepared using the nitrogen cavitation method employed to generate the cytosolic fraction from which the PLC was purified do not show hormonally regulated PLC activity and contain less than 1% of cellular PLC activity determined using exogenously provided substrates (12). Reconstitution of the purified 150-kDa turkey erythrocyte PLC with these membranes restores G-protein-regulated polyphosphoinositide hydrolysis, although the relative efficiencies of G-protein activators are somewhat different from those observed with the ghost reconstitution system. Finally, the precise nature of PLC membrane interaction remains obscure. In vitro experiments using phospholipid vesicles and purified PLC isoenzymes support a simple model in which PLC association with membranes is a substrate-directed process (41, 42). However, it is equally plausible that plasma membrane association of PLC is mediated through an as yet undefined but specific interaction with a protein or non-substrate lipid constituent of the plasma membrane. The preparation of specific antisera against the turkey erythrocyte PLC should provide an activity-independent means of detecting the enzyme and ultimately provide answers to some of the above questions.

Many questions remain. Can other isoenzymes of PLC also be regulated by the P2Y-purinergic receptor in the turkey erythrocyte reconstitution system? Will similar strategies prove suitable for examining receptor regulation of PLC in other cell types? Although supported by several lines of evidence, our supposition that P2Y-purinergic receptor regulation of PLC in turkey erythrocyte occurs by mechanisms analogous to other better understood G-protein-linked second messenger generating systems is still marred by our inability to identify the relevant G-protein. We hope that our identification of a G-protein-linked, receptor-regulated PLC will allow us to design a functional assay to facilitate the purification of the putative P2Y-purinergic receptor-linked G-protein from turkey erythrocytes. Finally, the ability to simply achieve functional reconstitution of an effector protein with the regulatory components of a G-protein-controlled system should eventually lead us to a better understanding of G-protein-effector coupling.

Acknowledgments—We would like to thank Drs. Jose Boyer and Phil Hughes for much valuable advice and assistance. We are indebted to Ron Freen and Carolina Turkeys Inc., Mount Olive, NC, for providing turkey blood.
G-protein-regulated Phospholipase C from Turkey Erythrocytes