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Purification of an AlF_4^- and G-protein $\beta\gamma$-Subunit-regulated Phospholipase C-activating Protein*

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A 150-kDa phospholipase C has previously been purified from turkey erythrocytes and has been shown by reconstitution with turkey erythrocyte membranes to be a receptor- and G-protein-regulated enzyme (Morris, A. J., Waldo, G. L., Downes, C. P., and Harden, T. K. (1990) *J. Biol. Chem.* 265, 13501–13507; Morris, A. J., Waldo, G. L., Downes, C. P., and Harden, T. K. (1990) *J. Biol. Chem.* 265, 13508–13514). Combination of this 150-kDa protein with phosphoinositide substrate-containing phospholipid vesicles prepared with a cholate extract from purified turkey erythrocyte plasma membranes resulted in conferrence of AlF_4^- sensitivity to the purified phospholipase C. Guanosine 5′-3-*O*-(thio)triphosphate also activated the reconstituted phospholipase C in a manner that was inhibited by guanosine 5′-2-*O*-(thio)-diphosphate. The magnitude of the AlF_4^- stimulation was increased with increasing amounts of plasma membrane extract, and was also dependent on the concentration of purified phospholipase C. Using reconstitution of AlF_4^- sensitivity as an assay, the putative G-protein conferring regulation to the 150-kDa phospholipase C was purified to near homogeneity by sequential chromatography over Q-Sepharose, Sephacryl S-300, octyl-Sepharose, hydroxylapatite, and Mono-Q. Reconstituting activity co-purified with an approximately 43-kDa protein identified by silver staining; lesser amounts of a 35-kDa protein was present in the final purified fractions, as was a minor 40-kDa protein. The 43-kDa protein strongly reacted with antiserum against a 12-amino acid sequence found at the carboxyl terminus of G_q and G_{11}, the 35-kDa protein strongly reacted with G-protein β-subunit antiserum, and the 40-kDa protein reacted with antiserum that recognizes G_{i3}. Immunoprecipitation of the 43-kDa protein resulted in loss of phospholipase C-stimulating activity of the purified fraction. The idea that this is a phospholipase C-regulating G-protein is further supported by the observation that co-reconstitution of G-protein $\beta\gamma$-subunit with the purified phospholipase C-activating fraction resulted in a $\beta\gamma$-subunit-dependent inhibition of AlF_4^--stimulated phospholipase C activity in the reconstituted preparation.

Cell surface receptors for a broad range of stimuli regulate a diverse group of effector proteins, *e.g.* adenylyl cyclase, ion channels, cyclic GMP phosphodiesterase, through activation of intermediary G-proteins (1, 2).[1] The inositol lipid signalling cascade represents one of the major second messenger pathways (3), but identification of its molecular components has proven difficult. Litosch *et al.* (4) and Cockcroft and Gomperts (5) first reported data that directly implicated a G-protein in receptor regulation of inositol lipid-specific phospholipase C. Subsequent work has pointed out kinetic similarities between the guanine nucleotide-dependent regulation of phospholipase C and that of other effector proteins (6, 7), and evidence that the phospholipase C-regulating G-protein is a heterotrimeric protein analogous to known members of the G-protein family has been presented (8, 9). Encouraging progress has been made recently in its identification.

Taylor and co-workers (10) have purified a rat liver protein that increases activity of a partially purified phospholipase C preparation from rat liver plasma membranes that were preactivated with GTPγS prior to solubilization. This protein does not react with antisera selective for known G-protein α-subunits, but antiserum against a concensus sequence found in most G-proteins recognizes this 42,000-dalton protein. Sternweis and co-workers (11) have developed an affinity matrix consisting of G-protein βγ-subunits linked to a solid support. Passage of Lubrol extracts of brain plasma membranes over this affinity matrix and subsequent elution with the G-protein activator, AlF_4^- (20 μM $AlCl_3$, 10 mM NaF), resulted in purification of a protein, G_q, with internal amino acid sequence identity (12) to sequence predicted by a G-protein cDNA cloned from a mouse brain library (13). This protein, when combined with a partially purified phospholipase C from bovine brain, resulted in stimulation of polyphosphoinositide hydrolysis in an AlF_4^--dependent fashion (14).

P_{2Y}-purinergic receptor- and G-protein-regulated inositol lipid hydrolysis has been studied in detail in turkey erythrocyte membranes (9, 15–18). A 150-kDa phospholipase C recently has been purified from turkey erythrocytes (19), and when reconstituted with turkey erythrocyte ghosts or plasma membranes devoid of phospholipase C activity, the purified 150-kDa protein acquires receptor- and G-protein-regulated enzyme activity that is indistinguishable from that of native

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[1] The abbreviations used are: G-protein, guanine nucleotide-binding regulatory protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns-4P, phosphatidylinositol 4-phosphate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; fplc, fast protein liquid chromatography; BSA, bovine serum albumin; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; GDPβS, guanosine 5′-(2-*O*-(thio)diphosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

membranes (20). This protein has been differentiated from previously purified/cloned mammalian phospholipases C by immunoreactivity and by partial internal amino acid sequence.[2] We now report that this purified G-protein-regulated phospholipase C can be used in an assay to identify and purify a 43,000-dalton protein in Na cholate extracts from turkey erythrocyte plasma membranes that when reconstituted with substrate-containing phospholipid vesicles confers AlF_4^-- and G-protein $\beta\gamma$-subunit sensitivity to the reconstituted purified phospholipase C.

EXPERIMENTAL PROCEDURES AND RESULTS[3]

Development of a reliable assay for screening putative phospholipase C-regulatory G-proteins has proven difficult. Efforts to selectively inactivate the endogenous turkey erythrocyte phospholipase C-regulatory G-protein by bacterial toxins or through chemical modifications have failed, thus making difficult the use of ghosts or membranes containing endogenous phospholipase C and PtdIns(4,5)P_2 substrate as an acceptor system in a G-protein reconstitution assay. Furthermore, there are no available cell lines deficient in the phospholipase C regulatory G-protein as has been the case for the G_s-deficient cyc$^-$ S-49 mutant cell line. The recent purification (19) of a 150-kDa G-protein-regulated phospholipase C from turkey erythrocytes (see Fig. 1*A* for a Ag-stained SDS-polyacrylamide gel of the purified phospholipase C) allowed development of an artificial phospholipid substrate preparation into which detergent extracts containing putative phospholipase C-regulatory G-protein could be introduced prior to reconstitution with the purified 150-kDa phospholipase C. Na cholate has been used to efficiently extract G-proteins from plasma membranes of a variety of tissues, but, as previously shown in cell-free assays (19), relatively low concentrations of Na cholate (0.2%, w/v) stimulate the catalytic activity of turkey erythrocyte phospholipase C. Thus, a procedure was adapted (see "Experimental Procedures") in which dispersed phospholipids were combined with Na cholate extract of turkey erythrocyte plasma membranes followed by gel filtration on Sephadex G-50 to remove Na cholate from the extract/phospholipid mixture and to form unilamellar phospholipid vesicles containing [^{3}H]PtdIns(4,5)P_2. These phospholipid vesicles containing detergent extract were combined with purified 150-kDa phospholipase C and incubated in the presence of GTPγS or AlF_4^- (Fig. 1*B*). GTPγS consistently produced a small stimulation of polyphosphoinositide hydrolysis which was blocked by GDPβS (Fig. 1*B*); addition of AlF_4^- markedly stimulated [^{3}H]PtdIns(4,5)P_2 hydrolysis. The stimulatory effect of AlF_4^- on [^{3}H]PtdIns(4,5)P_2 hydrolysis required reconstitution of phospholipid vesicles with both Na cholate extract and purified phospholipase C. There was no effect of AlF_4^- on polyphosphoinositide hydrolysis when either phospholipase C or Na cholate extract were omitted from the assay (data not shown). Heat inactivation of the Na cholate extract from plasma membranes prior to its combination with phospholipids and the purified phospholipase C abolished the effects of AlF_4^-, as did heat inactivation of the purified phospholipase C prior to reconstitution with substrate-containing vesicles (data not shown, but see Fig. 5). Finally, dephosphorylation of polyphosphoinositide substrates is minimal under

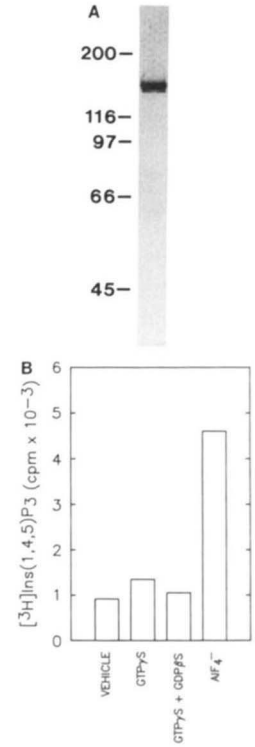

FIG. 1. **Reconstitution of purified turkey erythrocyte phospholipase C with Na cholate extract-containing phospholipid vesicles.** *A*, a silver stained SDS-polyacrylamide gel of 400 ng of purified turkey erythrocyte PLC. *B*, purified turkey erythrocyte plasma membranes were extracted with Na cholate as described under "Experimental Procedures." AMF was omitted from the extraction buffer. The extract was combined with dispersed phospholipids containing [^{3}H]PtdIns(4,5)P_2. Phospholipid vesicles were formed by desalting the dispersed phospholipids/Na cholate extract on Sephadex G-50 (see "Experimental Procedures"). The Na cholate extract-containing phospholipid vesicles were reconstituted with approximately 100 ng of purified phospholipase C and incubated with 10 mM Hepes (vehicle), 50 μM GTPγS, 50 μM GTPγS, and 300 μM GDPβS or 10 mM NaF, 20 μM $AlCl_3$ (AlF_4^-). The results are representative of those obtained in seven separate experiments.

the conditions of these reconstitution assays, and therefore, the effects of AlF_4^-, a potential phosphatase inhibitor, cannot be explained by preservation of an otherwise labile phospholipase C substrate (data not shown).

A partially purified fraction of reconstituting activity was prepared by passage of the Na cholate extract over a Q-Sepharose column (see "Experimental Procedures") prior to the reconstitution steps described above. As shown in Fig. 2, the capacity of the eluate to confer AlF_4^- sensitivity to phospholipase C was restricted to a narrow range of fractions eluting as a sharp peak of activity. Fractions of reconstituting activity were pooled and used to examine further the properties of the phospholipase C-regulating activity.

The effects of AlF_4^- on phospholipase C were dependent on the concentration of AlF_4^- (Fig. 3) and, in agreement with our previous observations of the substrate specificity of the 150-kDa phospholipase C (19), occurred irrespective of whether [^{3}H]PtdIns(4,5)P_2 or [^{3}H]PtdIns-4P was utilized as substrate (data not shown). The effect of AlF_4^- on inositol lipid hydrolysis was dependent on the amount of purified 150-kDa phospholipase C reconstituted with the vesicles made with the Q-Sepharose pool (Fig. 4). In the absence of AlF_4^- essentially no inositol lipid hydrolysis was observed even with the reconstitution of 300 ng of the purified 150-kDa phospholipase C per assay (Fig. 4). When a constant amount of purified phospho-

[2] Waldo, G. L., Morris, A. J., Klapper, D. G., and Harden, T. K. (1991) *Mol. Pharmacol.*, in press.

[3] Portions of this paper (including "Experimental Procedures" and Figs. 2 and 7–10) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

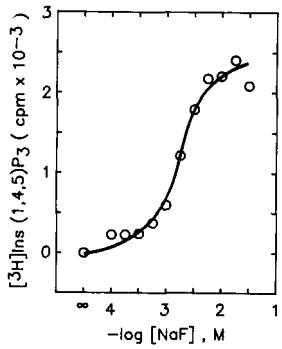

FIG. 3. **NaF dependence of phospholipase C-regulating protein reconstituted with purified phospholipase C.** Turkey erythrocyte plasma membranes (25 mg) were extracted with 1.2% Na cholate in the absence of AMF and the phospholipase C-regulating protein was partially purified by chromatography on a 1-ml column of Q-Sepharose as described under "Experimental Procedures." AMF was omitted from all chromatography buffers. Column fractions containing the peak activity of the phospholipase C-regulating protein were pooled (4 ml) and reconstituted (10 μl/assay) with purified phospholipase C (100 ng/assay) in phospholipid vesicles as described under "Experimental Procedures." Phospholipase C activity was measured in the presence of 20 μM $AlCl_3$ and the indicated concentrations of NaF.

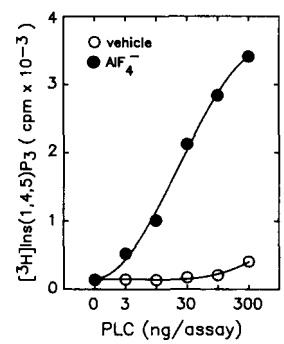

FIG. 4. **Concentration dependence of phospholipase C (*PLC*) for AlF_4^--stimulated polyphosphoinositide hydrolysis.** A pool of phospholipase C-regulating protein was prepared from the eluate of a Q-Sepharose column as described in the legend to Fig. 3. The indicated amount of purified phospholipase C was reconstituted in phospholipid vesicles with 10 μl of the phospholipase C regulating protein per assay. Phospholipase C activity was determined in the presence of AlF_4^- (●) or 10 mM Hepes (○).

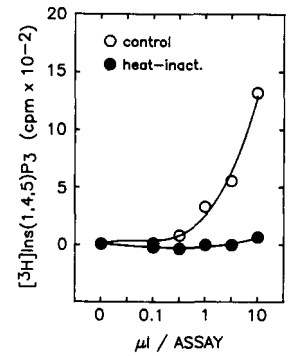

FIG. 5. **Concentration dependence of partially purified Na cholate extract for AlF_4^--stimulated polyphosphoinositide hydrolysis.** A pool of phospholipase C-regulating protein was prepared from the eluate of a Q-Sepharose column as described in the legend to Fig. 3. The indicated volumes of the pool (~0.25 mg of total protein/ml of pool) were reconstituted with purified phospholipase C (100 ng/assay) in phospholipid vesicles as described under "Experimental Procedures." Phospholipase C activity was determined in the presence of AlF_4^- for control (○) or for vesicles reconstituted with partially purified extract that was heat-inactivated prior to reconstitution (●). Phospholipase C activity observed in the absence of AlF_4^- has been subtracted.

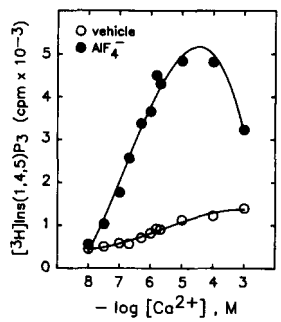

FIG. 6. **Ca^{2+} dependence of polyphosphoinositide hydrolysis in phospholipid vesicles prepared with partially purified Na cholate extract.** A pool of phospholipase C-regulating protein was prepared from the eluate of a Q-Sepharose column as described in the legend to Fig. 3. The phospholipase C-regulating protein (10 μl/assay) was reconstituted with purified phospholipase C (100 ng/assay) in phospholipid vesicles as described under "Experimental Procedures." Phospholipase C activity was determined in the presence of AlF_4^- (●) or 10 mM Hepes (○) at the indicated free Ca^{2+} concentrations.

lipase C was reconstituted with vesicles prepared with increasing amounts of the Q-Sepharose pool, a protein-dependent increase in AlF_4^--stimulated polyphosphoinositide hydrolysis was observed (Fig. 5). Reconstitution of 2.5 μg of the Q-Sepharose pool under the conditions described under "Experimental Procedures" resulted in an AlF_4^--stimulated rate of hydrolysis of 0.33 μmol/min/mg of 150-kDa phospholipase C. Under identical conditions in the absence of AlF_4^-, the rate of polyphosphoinositide hydrolysis was 0.01 μmol/min/mg of phospholipase C. Heat inactivation of the Q-Sepharose pool prior to formation of vesicles abolished the stimulation of the purified phospholipase C (Fig. 5).

Ca^{2+} previously has been shown to increase the activity of phospholipase C enzymes (including the turkey erythrocyte enzyme) against exogenous inositol lipid substrate, although the reasons for this enhanced activity are not understood (26). As illustrated in Fig. 6, a Ca^{2+}-dependent increase in phosphoinositide hydrolysis was observed when vesicles prepared with Q-Sepharose pool were incubated with purified 150-kDa phospholipase C. Incubation of these vesicles with AlF_4^- had little effect on the concentration dependence of Ca^{2+} for promoting inositol lipid hydrolysis, but markedly increased the maximal activity observed. These results are remarkably similar to those previously observed with native turkey erythrocyte membranes (16), or with purified turkey erythrocyte membranes in which purified 150-kDa phospholipase C has been reconstituted (20).

The results presented above suggest that a protein exists in turkey erythrocyte plasma membranes that confers sensitivity of phospholipase C to activators of G-proteins. Experiments were initiated to purify this protein. Purified turkey erythrocyte plasma membranes were prepared and a Na cholate extract generated as described under "Experimental Procedures." It was found in preliminary experiments that there was a rapid time-dependent loss of capacity of the Na cholate extract to confer AlF_4^- sensitivity to purified 150-kDa phospholipase C, and that maintenance of the extract in AMF (see "Experimental Procedures") greatly reduced this loss of activity. Thus, the extraction step and all subsequent purification steps were carried out in buffer containing AMF. The Na cholate extract from plasma membranes was passed sequentially over Q-Sepharose, Sephacryl S-300, octyl-Sepharose,

hydroxylapatite (where the elution buffer contained CHAPS rather than Na cholate), and fast protein liquid chromatography Mono-Q as described under "Experimental Procedures." Substrate-containing phospholipid vesicles were reconstituted with column fractions and purified 150-kDa phospholipase C as described above for Figs. 1–6 and is presented in detail under "Experimental Procedures." As with the Q-Sepharose column step (Fig. 2), the capacity to confer AlF_4^- sensitivity to the phospholipase C was restricted to a sharp peak of activity eluting from the Sephacryl S-300 (Fig. 7) column, and this activity did not coelute with the majority of the protein. Reconstituting activity eluted as a much broader band of activity from the octyl-Sepharose column (Fig. 8), and elution of activity from the hydroxylapatite column (Fig. 9) occurred as a sharp peak that also coincided with elution of a sharp peak of protein. Elution of reconstituting activity from the Mono-Q column (Fig. 10) also occurred as a sharp peak.

A concentration of protein-dependent increase in AlF_4^--stimulated polyphosphoinositide hydrolysis was observed when phospholipid vesicles prepared with increasing amounts of purified protein from the Mono-Q column were reconstituted with a fixed amount of purified phospholipase C (Fig. 11). As little as 0.5 ng of purified protein resulted in marked AlF_4^- stimulation of purified phospholipase C (70 ng under the conditions for the experiment depicted in Fig. 11). This corresponds to a "concentration" of approximately 1×10^{-10} M of a putative molecular mass 50,000 phospholipase C-activating G-protein and 2×10^{-9} M for the 150-kDa purified phospholipase C. Reconstitution of 3.3 ng of purified protein per assay resulted in a AlF_4^--stimulated rate of hydrolysis of 0.36 μmol/min/mg of purified phospholipase C at subsaturating concentrations of polyphosphoinositide substrate. In the absence of AlF_4^- the rate of polyphosphoinositide hydrolysis was 0.02 μmol/min/mg of purified phospholipase C. Until more is known concerning the percent of reconstituted G-protein available for activation, the extent to which the phospholipase C interacts with vesicle-associated G-protein, and the kinetic particulars of these interactions, it is not possible to make confident conclusions concerning the activities observed under these conditions at subsaturating substrate concentration.

Mono-Q column fractions expressing the reconstituting activity depicted in Fig. 10 were subjected to SDS-polyacrylamide gel electrophoresis; proteins were stained with silver (Fig. 12). The major protein coeluting with reconstituting activity was a 43-kDa protein. A 35-kDa protein was also observed, and a less prominent band of approximately 40-kDa was present.

The identity of proteins in the Mono-Q column eluates conferring AlF_4^- sensitivity to the purified phospholipase C was investigated with G-protein- and sequence-selective antisera. Antisera against the amino acid sequence ILQLNLKEYNLV, which represents the 12 amino acids at the carboxyl terminus of the recently identified G-protein α-subunits, G_q and G_{11} (13, 22), strongly reacted with the prominent approximately 43-kDa protein that coeluted with the peak of reconstituting activity from the hydroxylapatite (data not shown) and Mono-Q columns (Fig. 13). Antibody WO82, which was generated against an internal sequence in G_q (12), also recognized the 43-kDa protein but with less sensitivity. Antibody EC, which has been reported to show selectivity for α_{i3} (23), recognized the minor protein of approximately 40-kDa observed in some of the fractions conferring AlF_4^- sensitivity, but did not react with the 35-kDa protein and weakly reacted with the major 43-kDa protein (Fig. 13). No protein was identified by the antisera AS which has been reported to recognize G_t, $G_{i\alpha1}$, and $G_{i\alpha2}$ (Fig. 13). The 35-kDa protein is apparently G-protein β-subunit based on its strong immunoreactivity with antisera against this subunit (Fig. 13).

Based on its coelution with reconstituting activity and the fact that it is the most prominent Ag-stained protein observed, it seems likely that the 43-kDa protein is the species that confers AlF_4^- sensitivity to the purified phospholipase C. Furthermore, this is the approximate size of the protein isolated from liver membranes that activates phospholipase C (10) and of G_q, the protein shown by Smrcka *et al.* (14) to stimulate partially purified phospholipase C from bovine brain. The strong immunoreactivity of the 43-kDa protein with antisera to a carboxyl terminus amino acid sequence found in G_q and predicted from two cDNAs that encode G-protein α-subunits very similar in structure to G_q supports the idea that this is a

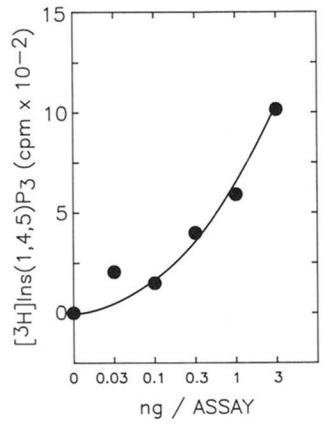

FIG. 11. **Concentration dependence of purified phospholipase C-regulating protein for AlF_4^--stimulated phosphoinositide hydrolysis.** The indicated amounts of purified phospholipase C-regulating protein from the Mono-Q column were reconstituted with purified phospholipase C (70 ng/assay) in phospholipid vesicles as described under "Experimental Procedures:" Phospholipase C activity was determined in the presence of AlF_4^-. Phospholipase C activity observed in the absence of AlF_4^- has been subtracted.

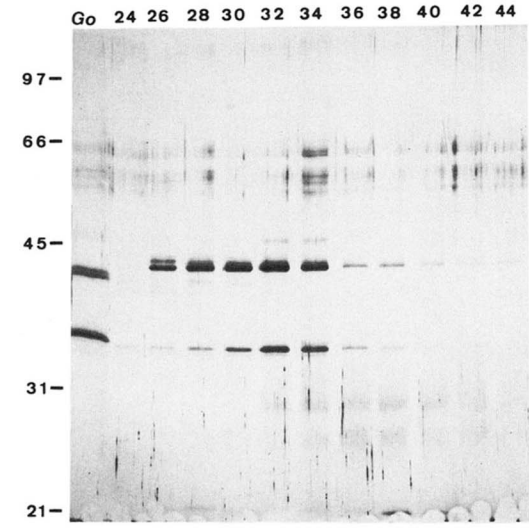

FIG. 12. **SDS-polyacrylamide gel electrophoresis of the phospholipase C regulating protein fractions eluting from the Mono-Q column.** Fractions from the Mono-Q column were analyzed by SDS-PAGE as described under "Experimental Procedures." Molecular weight standards are indicated on the left and column fraction numbers are listed across the top. For reference, bovine brain G_o α- and β-subunits were electrophoresed in the left most lane. The gel was stained with silver.

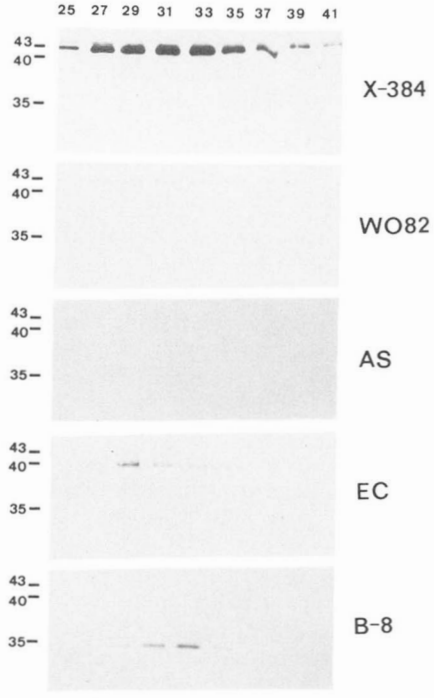

FIG. 13. **Immunoblot analysis of the phospholipase C-regulating protein fractions eluting from the Mono-Q column.** Fractions from the Mono-Q column were subjected to SDS-PAGE and transferred to nitrocellulose for Western blot analysis as described under "Experimental Procedures." The 30–45-kDa region for each immunoblot is shown. The specificities of the G-protein antisera are as follows: X384 was raised against a carboxyl terminus sequence in α_q and α_{11}; WO82 was raised against an internal amino acid sequence in α_q; AS was raised against α_t but also recognizes α_{i1} and α_{i2}; EC was raised against a carboxyl terminus sequence in α_{i3}; β-8 recognizes the G-protein β-subunit. The antisera used for immunodetection are indicated on the right. Molecular weight markers are indicated on the left and fraction numbers are listed across the top.

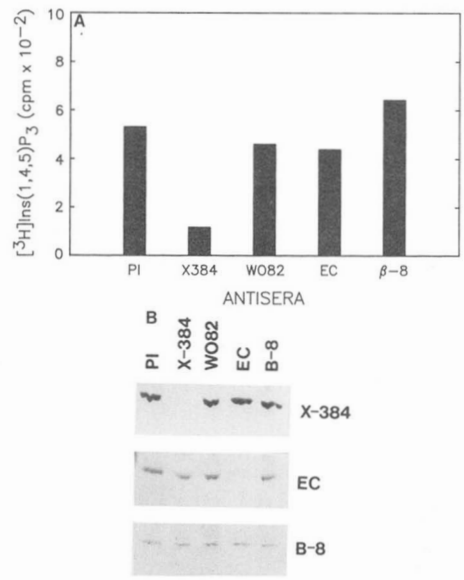

FIG. 14. **Immunoprecipitation of the purified phospholipase C-regulating protein with G-protein antisera.** Purified phospholipase C-regulating protein (200 ng) from the Mono-Q column was immunoprecipitated with the indicated G-protein antisera as described under "Experimental Procedures." The immunoprecipitated protein was removed by centrifugation and the capacity of the supernatant (3.3 ng/assay of phospholipase C-regulating protein in the preimmune sample) to confer AlF_4^- sensitivity to purified phospholipase C (22 ng/assay) was examined in a reconstitution assay (*panel A*). Phospholipase C activity was determined in the presence of AlF_4^-. The G-protein content of the supernatant was examined by immunoblot analysis (*panel B*). The antisera used for immunoprecipitation are listed across the top. The antisera used for immunodetection are indicated on the right.

G-protein α-subunit. A number of experiments addressed this possibility. First, experiments were performed to assess the capacity of G-protein selective antisera to remove reconstituting activity by immunoprecipitation. The purified fraction was treated with preimmune sera or with antisera X384, WO82, EC ($G_{i\alpha3}$), or β-8 (β-subunit) antisera, and antigen-antibody complexes immunoprecipitated with protein A-Sepharose as described under "Experimental Procedures." Immunoprecipitation with immune X384 antisera, but not preimmune sera, removed the 43-kDa protein from the supernatant and reduced reconstituting activity by 70–80% (Fig. 14). In contrast, although antibody to $G_{i\alpha3}$ immunoprecipitated the 40-kDa protein, it had no effect on the capacity of the purified fraction to confer AlF_4^- sensitivity, indicating that this protein is not involved in regulation of the purified turkey erythrocyte phospholipase C. Furthermore, reconstitution of up to 500 ng of purified bovine brain G_o/G_i α-subunit under the conditions used here did not result in AlF_4^- promoted activation of phospholipase C (data not shown). WO82 and β-8 antisera did not immunoprecipitate their respective antigens and had no effect on reconstitution of the purified fraction (Fig. 14).

We previously have shown that reconstitution of G-protein $\beta\gamma$-subunits with turkey erythrocyte membranes has marked effects on phospholipase C activity; suggesting the involvement of a phospholipase C-regulating heterotrimeric G-protein (9). Although based on Ag staining there is clearly less β-subunit than the 43-kDa protein in activating fractions, the above data suggest some co-purification of β-subunit with reconstituting fractions. This could be fortuitous or could represent purification of a phospholipase C-regulating G-protein as an oligomer in spite of its maintenance under supposed activating conditions, *i.e.* 50 μM $AlCl_3$, 10 mM $MgCl_2$, and 10 mM NaF. Thus, the effects of purified $\beta\gamma$-subunits on the responses illustrated above were examined. Reconstitution of $\beta\gamma$-subunit with the purified phospholipase C-activating fraction resulted in a marked decrease in the stimulatory effects of AlF_4^- on the purified phospholipase C (Fig. 15). This effect was dependent on the concentration of $\beta\gamma$-subunit. It occurred with as little as 7 ng of $\beta\gamma$-subunit and a 60–70% inhibition was observed under conditions in which 175 ng of $\beta\gamma$-subunit were reconstituted with 6.6 ng of phospholipase C-activating protein. Inhibitory effects were observed irrespective of whether the $\beta\gamma$-subunit was combined with reconstituting activity fractions prior to (Fig. 15) or after (data not shown) formation of phospholipid vesicles. Purified $\beta\gamma$-subunit had no effect on purified phospholipase C reconstituted with phospholipid vesicles prepared in the absence of the purified phospholipase C-activating fraction (data not shown). Likewise, heat inactivation of $\beta\gamma$-subunit prior to reconstitution with the purified fraction that confers AlF_4^- sensitivity resulted in failure to modify AlF_4^- promoted activation of the purified phospholipase C (Fig. 15).

DISCUSSION

Knowledge of the regulatory properties of the receptor- and guanine nucleotide-activated phospholipase C of turkey erythrocyte membranes allowed us to purify a 150-kDa phospholipase C (19), and show by reconstitution assay that the purified protein would fully reconstitute properties of the signalling response observed in native membranes to membranes that

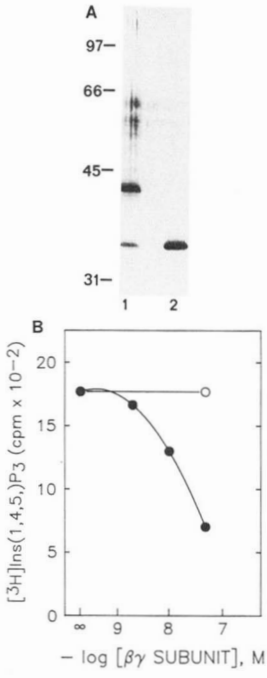

FIG. 15. **Effect of purified G-protein $\beta\gamma$-subunit on reconstituted phospholipase C-regulating activity.** *A*, a silver-stained SDS-polyacrylamide gel of the purified phospholipase C-regulating protein (*lane 1*) and purified turkey erythrocyte G-protein $\beta\gamma$ subunit (*lane 2*). *B*, the indicated amounts of purified turkey erythrocyte $\beta\gamma$-subunit (50 nM = 175 ng of $\beta\gamma$/assay) were combined with purified phospholipase C-regulating protein (6.6 ng/assay) from the Mono-Q column and reconstituted with purified phospholipase C (70 ng/assay) into phospholipid vesicles as described under "Experimental Procedures." Phospholipase C activity was determined in the presence of AlF_4^- for native $\beta\gamma$ subunit (●) or $\beta\gamma$ subunit heat-inactivated prior to reconstitution (○). Phospholipase C activity in the absence of AlF_4^- has been subtracted.

were devoid of such activities (20). Thus, we have purified the receptor and G-protein-regulated phospholipase C of turkey erythrocytes, and this has provided a necessary reagent for the purification of an activating G-protein based solely on its capacity to activate the effector phospholipase. The data presented here suggest that this goal has been accomplished.

By combining purified phospholipase C with phospholipid vesicles that contain polyphosphoinositide substrate and proteins from a Na cholate extract of plasma membranes devoid of phospholipase C activity, we have been able to measure the capacity of components in the extract to confer regulatory activities on the purified phospholipase C that are representative of those observed in native membranes. The extent to which the phospholipase C-activating protein is incorporated, embedded, or otherwise associated with the bilayer of these vesicles is currently unknown. Simple combination of the purified phospholipase C with substrate-containing vesicles prepared with Na cholate extract from plasma membranes did not result in an increase in polyphosphoinositide hydrolysis by the purified enzyme. However, addition of AlF_4^- to this reconstituted preparation markedly increased phospholipase C activity with properties analogous to those observed with activation in native membranes. The fact that GTPγS also produced a stimulation supports the idea that responsiveness was conferred to the phospholipase C by a G-protein in the Na cholate extract, but we have been troubled by the relatively minor response to guanine nucleotide that has been observed. The small guanine nucleotide effect could indicate that stimulation by AlF_4^- is not through a G-protein, but we doubt this is the case. Kinetic analyses of the activation process in crude membranes suggest that the involved G-protein does not readily turn over bound GDP (17). Preliminary studies with vesicles of phospholipid composition different from that utilized in the studies described here and with longer times of activation, suggest that conditions can be identified, whereby guanine nucleotides activate the G-protein and its associated phospholipase C to an extent similar to that observed with AlF_4^-.[4]

Assuming that the response of phospholipase C to AlF_4^- conferred by the Na cholate extract was provided by the phospholipase C-regulating G-protein, we used the reconstitution assay to purify this protein. The chromatographic steps used were adapted from those originally applied to purify the adenylyl cyclase-regulating G-proteins (28–31) and G_o (32, 33). The reconstituted response to AlF_4^- was used to identify the putative phospholipase C-regulating G-protein rather than [^{35}S]GTPγS binding or immunoblots with G-protein-selective or G-protein-common antibodies, and the goal here was to purify the phospholipase C-related G-protein to homogeneity rather than to provide any comparison of the elution properties of this protein to other GTP-binding proteins. We do know, however, from [^{35}S]GTPγS binding studies that the bulk of GTPγS binding activity does not co-purify with reconstituting activity over most of the column steps.[4] Our assumption from activity measurements is that the phospholipase C-regulating G-protein will not readily bind [^{35}S]GTPγS, at least under the conditions routinely used to measure conferrence of regulated activity to purified phospholipase C. This idea is supported by the observation that the final purified fraction binds less than 0.1 mol of [^{35}S]GTPγS/mol of 50,000 M_r protein.[4] Although we do not think this to be the case, inactivation of the G-protein during purification could also explain these results, as could a gross overestimation of the level of purity of the final preparation.

The most highly purified fraction of reconstituting activity consisted of an approximately 43-kDa protein and less than stoichiometric amounts of a 35-kDa protein that, based on immunoreactivity, is a G-protein β-subunit. Protein also ran with the dye front of SDS-PAGE gels suggesting that a G-protein γ-subunit is present, although appropriate SDS-PAGE conditions or G-protein γ-subunit antisera have not been applied to prove that a γ-subunit has co-purified with the 43- and 35-kDa proteins. The 43-kDa protein is present in excess of the 35-kDa protein, and the extent to which reconstituting activity purifies in the form of a G-protein heterotrimer is not known. All of the purification steps were in the presence of AMF (50 μM $AlCl_3$, 10 mM $MgCl_2$, 10 mM NaF), which theoretically should maintain the G-protein in an activated state. Whether this would involve dissociation of a species that exists as a heterotrimer in its native state is not known and is not really addressed by our studies. The α- and β-subunits of G_s co-purified over similar column steps and conditions similar to those used here (28), including the presence of AlF_4^-; furthermore, the phospholipase C-activating protein purified from GTPγS-preactivated rat liver membranes also co-purified with $\beta\gamma$-subunit (10).

Selective immunoprecipitation of the species recognized by antisera (EC) against a carboxyl terminus sequence in $G_{i\alpha 3}$ failed to alter reconstituting activity of the purified fraction leading to the conclusion that phospholipase C-regulating activity does not reside in this protein, which is not surprising based on the lack of effect of pertussis toxin on the receptor- and G-protein-regulated phospholipase C activity of turkey erythrocytes. Thus, stimulatory activity seems to reside in the

[4] J. L. Boyer, G. L. Waldo, A. J. Morris, and T. K. Harden, unpublished observations.

43-kDa protein(s) alone. This protein detected by Ag staining always coeluted with reconstituting activity, and removal of this protein from the purified fraction by immunoprecipitation resulted in loss of reconstituting activity. We have not identified this protein, but it seems safe to conclude that it is a member of the G_q class of proteins, one of which has been purified by Sternweis and colleagues (12), and several of which have been cloned from a mouse brain cDNA library by Strathmann *et al.* (13, 22). The report by Smrcka *et al.* (14) indicates that G_q and probably G_{11} will stimulate the activity of partially purified phospholipase C from bovine brain. The strong immunoreactivity of the 43-kDa protein purified from turkey erythrocytes with antisera against a 12-amino acid sequence found at the carboxyl terminus of each member of this class of G-proteins supports this idea; it is further strongly supported by the aforementioned immunoprecipitation results. It is not clear whether the 43-kDa species identified by Ag staining and immunoreactivity consists of a single protein or is made-up of more than one of the G_q class of proteins. These proteins share greater than 90% sequence homology, so internal amino acid sequence may not necessarily help to confirm the identity of the turkey G-protein, especially in light of possible species differences. Molecular cloning of the cDNA for the turkey protein seems necessary to fully answer this question. The relationship of the purified turkey erythrocyte phospholipase C-regulating protein to the one purified from liver membranes by Taylor *et al.* (10) has not been established, although based on similarity of size and properties it seems likely that the brain and liver proteins are similar if not the same.

Finally, work described here was directed toward identification/purification of a phospholipase C-activating protein, and has not produced insight into the mechanism by which the purified protein confers AlF_4^- sensitivity to phospholipase C. We believe the data indicate that combination of two proteins, a G-protein α-subunit and a purified phospholipase C, with substrate containing unilamellar phospholipid vesicles is necessary and sufficient to observe G-protein-regulated phospholipase C activity. The relatively low extent of guanine nucleotide regulation under these conditions potentially infers the necessity of a third protein for promotion of guanine nucleotide exchange, although there seems little compelling reason to propose that this component need be anything but an agonist-activated hormone receptor, *e.g.* the P_{2Y}-purinergic receptor. Further work will be required to define the nature of interaction of G-protein, phospholipase C, and the polyphosphoinositide substrate-containing unilamellar phospholipid vesicles, and to define the mechanism of activation. The capability of purifying a G-protein as well as the effector phospholipase C that it regulates supports the idea that the turkey erythrocyte will be a useful homogeneous cell model in which to study inositol lipid signalling at the protein level.

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SUPPLEMENTARY MATERIAL TO
PURIFICATION OF AN AlF_4^-- AND G-PROTEIN $\beta\gamma$-SUBUNIT-REGULATED PHOSPHOLIPASE C-ACTIVATING PROTEIN.

Gary L. Waldo, Jose L. Boyer, Andrew J. Morris
and T. Kendall Harden

EXPERIMENTAL PROCEDURES

MATERIALS. Q-Sepharose Fast Flow, Sephacryl S-300 HR, heparin-Sepharose, octyl-Sepharose, protein A-Sepharose, Sephadex G-50F, and Mono-Q HR 5/5 were purchased from Pharmacia (Piscataway, NJ). BioGel HTP hydroxylapatite and the HPHT column were from BioRad Labs (Richmond, CA). Bovine brain phosphatidylserine and phosphatidylethanolamine were from Avanti Polar Lipids, Birmingham, AL. [^{3}H]Inositol was from American Radiolabeled Chemicals (St. Louis, MO). Sodium cholate (Sigma, St. Louis, MO) was purified by the method of Ross and Schatz (21). CHAPS was from Pierce (Rockford, IL). Dr. Paul Sternweis generously provided antisera X-384 raised against the carboxyl terminal sequence ILQLNLKEYNLV of α_o/ α_{11} and WO82 raised against the internal (115-133) amino acid sequence EVDVERVSAFENPYVDAIK of α_q (see ref. 12 and 22). Dr. Allen Spiegel generously provided antibody EC^{4}raised against the carboxyl terminal sequence KNNLKECGLY of α_{i_3} (see ref. 23) and antibody AS raised against the carboxyl terminal sequence KENLKDCGLF of α_t but that recognizes α_{i1} and α_{i2} (see ref. 24). Dr. Tony Evans generously provided an antibody designated β-8 raised against G-protein β-subunit purified from human placenta. [^{3}H]PtdIns(4,5)P_2 and [^{3}H]PtdIns4P (specific radioactivity 0.8-1.0 Ci/mmol) were prepared from lipid extracts of [^{3}H]inositol labeled turkey erythrocytes and unlabeled PtdIns(4,5)P_2 and PtdIns4P were purified from lipid extracts of bovine brain as previously described (19).

PREPARATION OF TURKEY ERYTHROCYTE MEMBRANES AND CYTOSOL. Washed turkey erythrocytes were prepared from 40 l of whole blood and disrupted by nitrogen cavitation in a Parr cell disruption bomb as described previously (19). The resultant cell lysate was centrifuged (3250 x g) for 10 min in a Beckman J-6 centrifuge and the pellet, predominately consisting of nuclei, was discarded. The supernatant was collected and centrifuged at 36,000 x g for 20 min (Beckman J2-21 centrifuge, JA-14 rotor, brake setting 5). The supernatant from this step constituted a cytosolic fraction that was used for the purification of a 150 kDa phospholipase C (see below). The loose pellet from the 36,000 x g centrifugation step was carefully collected for preparation of plasma membranes. The pooled pellet fraction was diluted to 12 l with 5 mM Tris, pH 7.4, 1 mM EGTA, 5 mM $MgCl_2$, 0.1 mM benzamidine and 0.1 mM PMSF and centrifuged at 36,000 x g as described above. The loose pellet was collected again, resuspended to 6 l in the dilution buffer described above, and centrifuged as before. The easily resuspended pellets from this step were collected, discarding the tight pellet, and resuspended with 6 l of 20 mM Tris, pH 7.4, 5 mM EDTA, 0.1 mM benzamidine and 0.1 mM PMSF (Tris/EDTA). The samples were centrifuged as before and the pellets collected and resuspended to 3 l with Tris/EDTA and centrifuged. The pellets from this step were resuspended to 750 ml with Tris/EDTA and centrifuged at 40,000 x g for 15 min in a Beckman JA-17 rotor. The white upper portion of these pellets was carefully collected and diluted to 250 ml with Tris/EDTA. The samples were centrifuged again and the white upper layer of membranes was removed and resuspended to 200 ml with Tris/EDTA and stored frozen at -70°C. The yield of white membranes from 40 l of whole blood was 2-3 g.

PHOSPHOLIPASE C PURIFICATION. A G-protein regulated 150 kDa phospholipase C was purified from turkey erythrocyte cytosol as described previously in detail (19). Briefly, the phospholipase C was precipitated with ammonium sulfate, which was followed by sequential chromatography of the resuspended precipitate through columns of Q-Sepharose, hydroxylapatite, heparin-Sepharose, Sephacryl S-300 HR, fplc Mono-Q and BioGel HPHT.

RECONSTITUTION ASSAY. Phosphatidylserine, phosphatidylethanolamine, and [^{3}H]PtdIns(4,5)P_2 or [^{3}H]PtdIns4P were combined in chloroform in a molar ratio of 10:10:1. The phospholipids were dried under a stream of nitrogen and dispersed by sonication in buffer A (20 mM Hepes, pH 7.4, 2 mM $MgCl_2$, 1 mM DTT, 100 mM NaCl, 0.1 mM benzamidine, 0.1 mM PMSF and 1.2 % Na cholate) to final concentrations of 2 mM phosphatidylserine, 2 mM phosphatidylethanolamine, and 0.2 mM [^{3}H]PtdIns(4,5)P_2 or [^{3}H]PtdIns4P. Indicated volumes of extracts or column fractions to be tested for reconstitution were diluted to 150 ul with buffer A and combined with 150 ul of dispersed lipid solution. Phospholipid vesicles then were prepared by gel filtration through 5 ml columns of Sephadex G-50F equilibrated with buffer B (buffer A without Na cholate). The columns were washed with 1 ml of buffer B and phospholipid vesicles were eluted in the next 750 ul of wash at the column volume. Electron microscopy of these preparations after negative staining with uranyl acetate revealed relatively homogeneous apparently unilamellar vesicles with mean diameter of approximately 50 nm. Reconstitution assays (100 ul final volume) consisted of 50 ul lipid vesicles/G-protein, 25 ul drug and 25 ul of 4x assay buffer (480 mM KCl, 40 mM NaCl, 8 mM EGTA, 40 mM Hepes, pH 7.4, 16 mM $MgSO_4$ and added $CaCl_2$ to give 1 uM free Ca^{++} in the assay) containing 35 ng purified phospholipase C/assay unless otherwise indicated. The final concentration (moles of lipid/liter of assay solution) of [^{3}H]PtdIns(4,5)P_2 or [^{3}H]PtdIns4P in the assay was 15-20 uM containing 10,000-20,000 cpm/assay. Assays were for 10 min at 30°C and were terminated by the addition of 0.375 ml of $CHCl_3$:MeOH:HCl (20:40:1) followed by 0.125 ml $CHCl_3$ and 0.125 ml of 0.1 M HCl. Radioactivity released into the upper aqueous phase was quantitated by liquid scintillation spectrometry. For some experiments purified G-protein bc-subunits were combined with the purified 43 kDa phospholipase C-regulatory fraction and diluted to 150 ul with buffer B containing 0.8% Na cholate and 0.1% BSA. The samples were then mixed with 150 ul of dispersed lipids and passed over G-50 Sephadex columns as described above. Where indicated, protein-containing samples were heat inactivated by incubation for 15 min in boiling water prior to combination with lipids.

PURIFICATION OF G-PROTEIN $\beta\gamma$ SUBUNITS. G-protein $\beta\gamma$-subunit was purified from turkey erythrocytes as we have previously described (9).

PURIFICATION OF THE 43 kDa PHOSPHOLIPASE C-REGULATING PROTEIN.

Membrane extraction: A Na cholate extract was prepared by mixing 700 mg of purified turkey erythrocyte membranes in 420 ml of buffer C (20 mM Tris, pH 7.4, 1 mM DTT, 50 uM $AlCl_3$, 10 mM $MgCl_2$, 10 mM NaF, 0.1 mM benzamidine and 0.1 mM PMSF) containing 1.2% Na cholate. The solution was stirred at 4° for 60 min and centrifuged for 45 min at 140,000 x g in a Beckman Type 35 rotor. The resultant supernatant was collected as the soluble extract (390 ml, 100 mg protein).

Q-Sepharose chromatography: The Na cholate extract was applied to a 1.6 x 18 cm (36 ml) column of Q-Sepharose equilibrated at a flow rate of 2.5 ml/min with buffer C containing 0.8% Na cholate. The column was washed with 80 ml of equilibration buffer and eluted with a 400 ml linear gradient from 0-600 mM NaCl. The column then was washed with 50 ml of 1 M NaCl. The eluate was collected in 10 ml fractions. Fractions 17-24 were pooled (78 ml, 21 mg protein) and concentrated to 15 ml in an Amicon stirred cell concentrator using a PM-10 membrane.

Sephacryl S-300 HR chromatography: The concentrated pool from the Q-sepharose column was applied to a 2.5 x 92 cm (450 ml) column of Sephacryl S-300 HR. The column was eluted with buffer C containing 100 mM NaCl and 0.8% Na cholate at 1.5 ml/min. The eluate was collected in 5.6 ml fractions. Fractions 46-53 were pooled (41 ml, 1.64 mg protein).

Octyl-Sepharose chromatography: The Sephacryl S-300 pool was diluted to 217 ml with buffer C containing 592 mM NaCl. The final concentrations of Na cholate and NaCl were 0.15% and 500 mM, respectively. The diluted pool was applied to a 1.6 x 18 cm (36 ml) column of octyl-Sepharose equilibrated with buffer C containing 0.15% Na cholate and 500 mM NaCl at a flow rate of 1.85 ml/min. The column was washed with 40 ml of equilibration buffer and eluted at 0.75 ml/min with a 400 ml linear gradient from 0.15% Na cholate/500 mM NaCl to 1.2% Na cholate. The eluate was collected in 8 ml fractions. Fractions 43-73 were pooled (232 ml, 232 ug protein) and concentrated through an Amicon PM-10 membrane to 12.5 ml.

Hydroxylapatite chromatography: The concentrated pool from the octyl-Sepharose column was diluted to 62.5 ml with buffer D (20 mM Tris, pH 8.0, 1 mM DTT, 50 uM $AlCl_3$, 3 mM $MgCl_2$, 3 mM NaF, 0.1 mM benzamidine and 0.1 mM PMSF) containing 0.8% Na cholate and applied at 0.3 ml/min to a 7.8 x 100 (4.8 ml) BioGel HPHT column equilibrated with the same buffer. The column was washed with 6 ml of equilibration buffer followed by 10 ml buffer D containing 0.6% CHAPS. The column was eluted with a 50 ml linear gradient from 0-400 mM K_2HPO_4 in buffer D containing 0.6% CHAPS. The eluate was collected in 1 ml fractions. Fractions 35-36 were pooled (1.85 ml, 83 ug protein).

Mono-Q HR 5/5 chromatography: The HPHT pool was diluted to 18 ml with buffer C, pH 8.0, containing 0.6% CHAPS and applied to a 0.5 x 5 cm (1 ml) Mono-Q HR 5/5 column equilibrated in the same buffer. The column was washed with 4 ml of equilibration buffer and eluted with a 20 ml linear gradient from 0-400 mM NaCl. The eluate was collected in 0.5 ml fractions. Fractions 27-31 were pooled (2.25 ml, 18 ug protein).

IMMUNOPRECIPITATION. Approximately 200 ng of purified phospholipase C-regulating protein from the Mono-Q pool was diluted to 200 ul with buffer B containing 0.8% Na cholate and 0.1% BSA. Four ul of the indicated G-protein specific antisera were added and the solution was incubated at 4°C for 60 min with gentle rocking. Protein A-Sepharose beads (100 ul of a 10% suspension) were washed three times with buffer B containing 0.8% Na cholate and 0.1% BSA by centrifugation for 15 sec in a microfuge. The primary antisera/antigen solution was transferred to a tube containing the washed protein A-Sepharose beads and incubated at 4° for 60 min with gentle rocking. Sedimentable material was separated from supernatant by centrifugation, and the supernatant was collected for use in reconstitution assays and western blots.

GEL ELECTROPHORESIS AND IMMUNOBLOTS. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (25). G-protein containing samples were analyzed on gels of 8.5% acrylamide/0.5% bis-acrylamide. Phospholipase C samples were analyzed on 8.5% acrylamide/0.2% bis-acrylamide gels. Immunoblots were generated after electrophoretically transferring the proteins separated by SDS-PAGE to nitrocellulose sheets. The transblots were blocked with 3% bovine serum albumin, incubated with the indicated dilution of primary antisera for 2 hr, washed three times with 0.05% TWEEN-20, and incubated with an alkaline phosphatase-conjugated secondary antibody for 1 h. The immunoblots were washed three times with 0.05% TWEEN-20 and developed with a solution containing 3 mg/ml p-nitro blue tetrazolium, 1.5 mg/ml 5-bromo-4-chloro-3-indoyl phosphate, 1 mM $MgCl_2$, and 0.1 M $NaHCO_3$, pH 9.8.

PROTEIN ASSAY. Protein was determined by amido black according to Shaffner and Weissmann (27) using bovine serum albumin as standard.

DATA PRESENTATION. All data points are the mean of triplicate determinations the values of which varied by 15 percent or less. Data representative of results obtained in multiple experiments are presented.

RESULTS

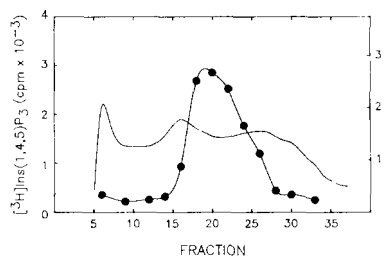

Figure 2. Q-Sepharose chromatography of PLC regulating activity. Cholate extract (100 mg protein) prepared from turkey erythrocyte plasma membranes was applied to a column of Q-Sepharose and eluted with a gradient of NaCl as described in Experimental Procedures. Fractions (1.7 ul/assay) were reconstituted in phospholipid vesicles with purified PLC (35 ng/assay), and PLC activity was measured in the presence of AlF_4^- (—●—) as described in Experimental Procedures. The elution of protein determined by absorbance at 280 nm also is illustrated (——).

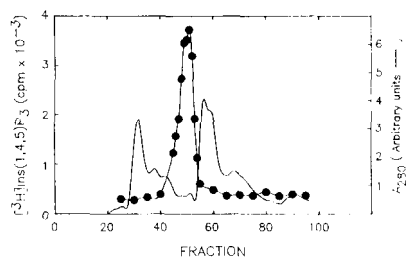

Figure 7. Purification of PLC-regulating activity by gel filtration chromatography on Sephacryl S-300 HR. Fractions containing PLC-regulatory activity from the Q-Sepharose column were pooled, concentrated and applied to a column of Sephacryl S-300 HR. The column was eluted and fractions (1.7 ul/assay) were reconstituted with purified PLC (35 ng/assay) and assayed in the presence of AlF_4^- (—●—) as described in Experimental Procedures. The elution of protein determined by absorbance at 280 nm also is illustrated.

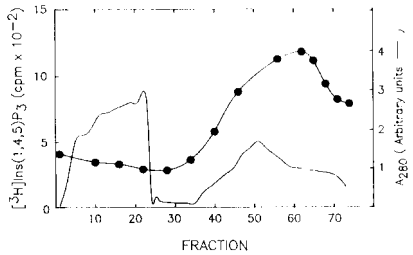

Figure 8. Purification of PLC-regulating activity by octyl-Sepharose chromatography. Fractions containing PLC-regulatory activity from the Sephacryl S-300 column were pooled, diluted and applied to a column of octyl-Sepharose. The column was eluted and fractions (6.7 ul/assay) were reconstituted with purified PLC (35 ng/assay) and assayed in the presence of AlF_4^- (-●-) as described in Experimental Procedures. The elution of protein determined by absorbance at 280 nm also is illustrated (——).

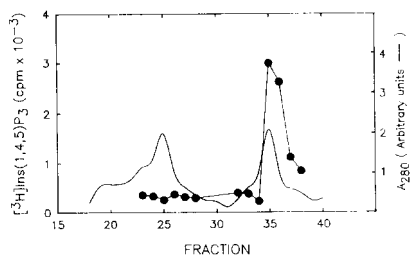

Figure 9. Purification of PLC-regulating activity by hydroxylapatite chromatography. Fractions containing PLC-regulatory activity from the octyl-Sepharose column were pooled and applied to a hydroxylapatite column. The column was eluted and fractions (1.7 ul/assay) were reconstituted with purified PLC (35 ng/assay) and assayed in the presence of AlF_4^- (-●-) as described in Experimental Procedures. The elution of protein determined by absorbance at 280 nm also is illustrated.

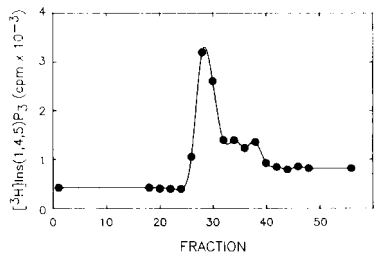

Figure 10. Purification of PLC-regulating activity by Mono-Q anion exchange chromatography. Fractions containing PLC-regulatory activity from the hydroxylapatite column were pooled and applied to a Mono-Q anion exchange fplc column. The column was eluted and fractions (1.7 ul/assay) were reconstituted with purified PLC (35 ng/assay) and assayed in the presence of AlF_4^- (-●-) as described in Experimental Procedures.