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Release of Glycosylphosphatidylinositol-Anchored Carboxypeptidase M by Phosphatidylinositol-Specific Phospholipase C Upregulates Enzyme Synthesis

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Carboxypeptidase M (CPM), a glycosylphosphatidylinositol (GPI)-anchored membrane protein, remained at a constant level in confluent Madin Darby canine kidney (MDCK) cells but was continually released into the medium in soluble form. The released CPM contained ethanolamine, indicating liberation by a phospholipase. Treatment of MDCK cells with 0.01 U/ml phosphatidylinositol-specific phospholipase C for 6 h led to a 5.5-fold increase in soluble CPM, yet the activity in cells remained constant, resulting in a 30% increase in total activity. The increase was due to new protein synthesis as evidenced by inhibition with 0.2 μM cycloheximide and a 63% increase in [^{35}S]methionine incorporation into newly synthesized CPM. MDCK cells treated with 1-alkyl-2-acyl-glycerol, the diglyceride component of mammalian glycosylphosphatidylinositol anchors, exhibited a 36% increase in CPM activity, but diacylglycerols or phorbol esters were ineffective. Thus, release of GPI-anchored CPM can generate a diglyceride signal to replenish and maintain constant levels on the cell surface.

Plasma membrane-bound proteins are commonly found in soluble form in biological fluids, but the mechanism of endogenous release has not been clearly elucidated (1, 2). Type I and type II membrane proteins, whose hydrophobic transmembrane domains span the lipid bilayer once, are released constitutively and in response to certain stimuli by a class of endogenous membrane-bound proteases termed "secretases" that have not been well characterized (1, 2). Another class of membrane proteins is anchored via glycosylphosphatidylinositol (GPI), which tethers proteins to the plasma membrane by a hydrophobic diglyceride moiety that is incorporated into the outer leaflet of the lipid bilayer (3, 4). The GPI anchor confers a number of unique properties on membrane proteins, among which is the ability to not only be released by proteolytic cleavage but also by phosphatidylinositol-specific phospholipases C (PI-PLC) or D (PI-PLD) (2, 4).

Carboxypeptidase M (CPM) is a widely distributed GPI-anchored membrane glycoprotein that is also present in biological fluids (5-8). It specifically cleaves C-terminal Arg or Lys from the C-terminus of peptides and proteins such as bradykinin, epidermal growth factor, Arg- or Lys-enkephalins, dynorphin and hemoglobin (5-8). Because of its distribution and subcellular localization, CPM may participate in a variety of processes, such as control of peptide hormone activity at the cell surface and in fluids such as urine, amniotic fluid and seminal plasma (5, 7, 8). CPM is readily released from cell membranes by treatment with exogenous proteases or bacterial PI-PLC, but the mechanism of its constitutive release from cells has not been determined. In the course of the present studies, we explored the mechanism of the endogenous release of CPM from Madin Darby canine kidney (MDCK) cells and discovered a novel signal by which constant levels of this important enzyme can be maintained on the cell surface.

MATERIALS AND METHODS

Materials. Tissue culture medium and additives were from GIBCO. Insoluble protein A (cell suspension), phorbol 12-myristate-13-acetate (PMA), 1-oleoyl-2-acetyl-rac-glycerol (OAG), 1,2-dimyris-

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Abbreviations used: GPI, glycosylphosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; PI-PLD, phosphatidylinositol-specific phospholipase D; PC-PLC, phosphatidylcholine-specific phospholipase C; CPM, carboxypeptidase M; MDCK, Madin Darby canine kidney; PMA, phorbol 12-myristate 13-acetate; OAG, 1-oleoyl-2-acetyl-rac-glycerol; PKC, protein kinase C; Dns-Ala-Arg, 5-dimethylaminonaphthalene-1-sulfonyl-L-alanyl-L-arginine; AAG, 1-alkyl-2-acyl-*sn*-glycerol; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline.

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toyl-glycerol, phorbol-12,13-dibutyrate, cycloheximide, phosphatidylcholine-specific phospholipase C (PC-PLC), reagents and molecular weight standards for electrophoresis, 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphocholine, 1-monopalmitoyl-rac glycerol and Triton X-114 were from Sigma Chemical Co. [^{35}S]L-methionine and [^{3}H]ethanolamine were from Amersham. Fluoro-Hance was from Research Products International and PI-PLC was from ICN. DL-2-Mercaptomethyl-3-guanidinoethylthiopropanoic acid (MGTA) was from Calbiochem. 5-Dimethylaminonaphthalene-1-sulfonyl-L-alanyl-L-arginine (Dns-Ala-Arg) was synthesized and purified as described (9, 10). 1-Alkyl-2-acyl-*sn*-glycerol (AAG; 1-alkyl = primarily C 16:0, 2-acyl = acetyl) was generated by digesting 2 mg of platelet activating factor (1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphocholine) with 10 units/ml of PC-PLC at 37°C for 20 h in 500 μl 0.1 M Tris-HCl, pH 8.0. The AAG was extracted with chloroform/methanol (2:1, v/v) and after evaporation of the solvent, was resuspended in 0.1 M Tris buffer, pH 8.0 with 1% chloroform. The purity of the generated AAG was checked on thin layer plates (silica gel 60) developed twice with ether:hexane: acetic acid (70:30:1) (11).

Cell culture. MDCK cells (obtained from Dr. Carlos Rabito, Harvard Medical school, Boston, MA) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM L-glutamine, 10% (v/v) heat-inactivated fetal bovine serum, 25 units/ml penicillin, and 25 μg/ml streptomycin. Cells were grown at 37°C in a humid atmosphere of 95% air/5% CO_2. Confluent cells were used for most enzyme studies. Cell monolayers were washed twice with phosphate buffered saline (PBS) and exposed to various agents in serum-free DMEM containing Ham's Nutrient Mixture F-12 for the indicated lengths of time. The conditioned medium was collected and centrifuged at 100,000 x g for 1 h and the supernatant fraction used for enzyme assays. The cells were washed twice with ice-cold PBS and harvested with a cell scraper.

Homogenization and fractionation. Harvested cells were suspended in PBS and centrifuged (800 x g, 10 min). The washed cells were resuspended in homogenization buffer (20 mM Tris-HCl, 250 mM sucrose, pH 7.4 at 4°C) and disrupted by sonication (two 15 s pulses at a power setting of 60%) using a Sonic Dismembranator (Artek Systems) with a microprobe. CPM activity was routinely measured in the cell lysates to determine total cellular activity. In some experiments, as indicated, subcellular fractions were obtained by sequential centrifugation at 800 x g for 10 min, 10,000 x g for 25 min, and 100,000 x g for one hour. The final pellet (P_3) was retained as the membrane enriched fraction and was resuspended in homogenization buffer.

Solubilization. The P_3 membrane fraction was incubated in homogenization buffer containing either 0.8% CHAPS or 1% Triton X-100 for one h at 4°C. After incubation, the suspension was centrifuged at 100,000 x g for 1 h and the supernatant fraction (solubilized membrane fraction) was used.

Enzyme assays. Carboxypeptidase activity was measured with Dns-Ala-Arg in a Perkin-Elmer LS-5 spectrofluorometer at 340 nm excitation and 495 nm emission as described (9, 10).

The activity of neutral endopeptidase 24.11 was measured with glutaryl-Ala-Ala-Phe-4-Methoxy-2 naphthylamide (Enzyme Systems Products) in a two step assay in the presence and absence of the specific inhibitor, phosphoramidon (12, 13).

Electrophoresis. SDS-PAGE was done in 9% gels (14) with a Mighty Small gel unit (Hoefer Scientific Instruments). Samples were boiled for 5 min in SDS-PAGE sample buffer containing 300 μg dithiothreitol as reducing agent.

Immunoprecipitation. Polyclonal antiserum to purified human placental CPM was raised in rabbits (6). The IgG fraction was obtained by chromatography on DEAE-Affi-Gel Blue (Bio-Rad) as described by the manufacturer. Conditioned medium (500 μl) was incubated with either 3 μl of anti-CPM serum (or equivalent amount of IgG) or 3 μl of non-immune rabbit serum (or equivalent amount of IgG) for 2 h at room temperature with constant mixing. At the end of the 2 h incubation, 50 μl of insoluble protein A suspension was added to the reaction mixture and incubated for an additional 30 min. The suspension was then centrifuged at 14,000 x g for 5 min and the carboxypeptidase activity in the supernatant fraction was measured.

Triton X-114 partitioning. Partitioning into Triton X-114 was carried out essentially as described (15) with slight modifications (16).

[^{35}S]methionine labeling of CPM. MDCK cells were grown to confluence, the monolayer was washed twice with cold PBS and the medium replaced with serum-free, methionine- deficient medium for 4 h. At the end of the incubation, the medium was replaced with fresh serum-free methionine-deficient medium containing 50 μCi of [^{35}S]methionine, and cells were incubated at 37°C overnight. The solubilized membrane fraction was obtained as above and was immunoprecipitated with anti-CPM antiserum (3 μl) and insoluble protein A (16, 17). The radioactivity (precipitated CPM) was released from the precipitated pellet by solubilization in SDS-PAGE sample buffer with dithiothreitol and subjected to electrophoresis. The gel was dried under vacuum and subjected to autoradiography. For quantification, the band corresponding to CPM (at 56 kDa) was excised, solubilized in H_2O_2:NH_4OH (19:1 v/v) overnight at 37°C and the radioactivity was measured in a scintillation counter. The total radioactivity incorporated into proteins in the solubilized P_3 membrane fraction was obtained by precipitation with 10% trichloroacetic acid. The counts incorporated into CPM were divided by total counts precipitated with trichloroacetic acid to correct for any variability in overall protein labeling.

[^{3}H]ethanolamine labeling. Cells were grown to 70% confluence in DMEM with 10% fetal bovine serum, and 50 μCi of [^{3}H]ethanolamine was added to the medium of each culture dish. Cells were allowed to grow to confluence (2-3 days), the medium was removed, the cells were washed with PBS and then maintained in serum-free medium for an additional three days. The medium containing released CPM was removed, centrifuged at 100,000 x g for 1 h, and the supernatant fraction concentrated with a YM-10 membrane in an Amicon stirred cell concentrator. The cells were harvested, fractionated as described above and the P_3 membrane fraction was treated with either 1% Triton X-100 at 4°C or 0.01% trypsin at 37°C for 2 h. The concentrated medium and solubilized membrane fractions were immunoprecipitated with anti-CPM IgG and then subjected to SDS-PAGE. The gel was fixed, immersed in Fluoro-Hance solution for 30 min, dried under vacuum with heat for 2 h and then exposed to X-ray film at −70°C for one month.

Protein and DNA assays. Protein concentrations were routinely determined by the method of Bradford (18) with bovine serum albumin as standard.

Because it was necessary to scrape cells from the culture dish [trypsin treatment removes CPM from the cell membrane (16)], it was difficult to accurately determine cell numbers because of clumping. Cell counts were thus determined by measuring DNA content using the diphenylamine method with salmon sperm DNA as standard (19). A conversion factor of 6.1 μg DNA per million cells was used as determined experimentally by counting control cultures of MDCK cells in a hemacytometer and determining their DNA content.

RESULTS

Release of CPM from MDCK cells. CPM is the only neutral pH-optimum Arg/Lys-specific (or "B-type") carboxypeptidase expressed on the plasma membrane of MDCK cells, where it is membrane-bound via a GPI anchor (16). CPM activity in subconfluent MDCK cells (4 days after plating) increased until the cells became confluent (7 days) and then remained stable (Fig. 1).

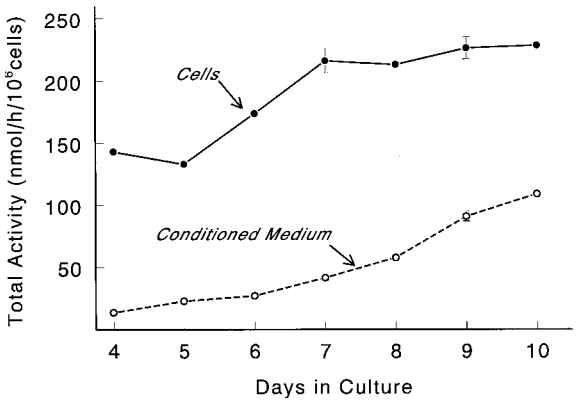

FIG. 1. CPM activity in MDCK cells and conditioned medium. MDCK cells were plated and cultured as described. From 4–10 days after plating, cells were harvested and conditioned medium was collected on the indicated day and the carboxypeptidase activity was measured with Dns-Ala-Arg as described under Materials and Methods. Total activity was calculated per 10^6 cells or, for conditioned medium, the total CPM activity released per 10^6 cells. The results shown are the mean ± S.E.M. of three separate cultures for each day (error bars smaller than the symbols are not shown).

Carboxypeptidase activity was also released into the conditioned medium and continued to increase after the cells became confluent (Fig. 1). The carboxypeptidase activity in the medium was completely inhibited by 0.1 mM MGTA, a specific inhibitor of B-type carboxypeptidases (10, 20). Because carboxypeptidases D, E and N can cleave the Dns-Ala-Arg substrate and may be secreted into the medium of other types of cultured cells (21-23), it was necessary to prove that the enzyme released into the medium of MDCK cells was authentic CPM. When carboxypeptidase activity in the medium was measured with Dns-Ala-Arg at pH values ranging from 3 to 9, the maximum activity was obtained at pH 7.0, consistent with the properties of CPM (not shown). At pH 6, the optimal pH for carboxypeptidase D (22, 24, 25), the carboxypeptidase activity in the medium had 54% of the pH 7.0 activity and at pH 5.5, the optimal pH for carboxypeptidase E (25, 26), it retained only 30% of its activity. In addition, 0.1 mM p-chloromercuriphenylsulfonate, a concentration which completely inhibits carboxypeptidase E (26) and inhibits carboxypeptidase D by about 50% (24, 25), did not inhibit the carboxypeptidase activity in the medium (not shown). To distinguish the medium carboxypeptidase from carboxypeptidase N IgG specific for CPM (16) was used for immunoprecipitation and Western blotting. In these experiments, 84.1 ± 0.9% (± S.E.M.; n = 4) of the carboxypeptidase activity in the medium was precipitated with anti-CPM IgG while only 6.3 ± 2.8% of the activity was precipitated by nonimmune rabbit IgG. Furthermore, when antiserum to CPM was used in Western blotting of the carboxypeptidase released into the medium, a single crossreacting band was seen at 56 kDa (not shown), corresponding to the molecular weight established for MDCK cell CPM (16).

To rule out the possibility that CPM in the medium is the result of "shedding" of small membrane particles, we employed Triton X-114 partitioning (15, 16). CPM solubilized with detergent from the P_3 membrane fraction of MDCK cells partitioned 91.0 ± 0.3 % (n = 4) into the Triton X-114 detergent phase. In contrast, 80.0 ± 0.5% of the CPM in the medium partitioned into the aqueous phase, as did 88.5 ± 0.2% of the CPM released from the membrane by exogenous PI-PLC, showing that the hydrophobic anchor had been removed. These results indicate that the release of CPM from MDCK cells is carried out by an endogenous enzyme or enzymes.

Although heat-inactivated serum was routinely used in these studies, to rule out the possibility that a heat-resistant enzyme in serum was responsible for the release of CPM, confluent MDCK cells were washed with and maintained in serum-free medium for an additional period of 24 h. In this case, 10-15% of the total CPM activity was found in the medium, similar to the amount of CPM released from over a 24 h period from cells grown in serum-containing medium (Fig. 1).

GPI anchored proteins, such as CPM, can be released by either proteases or phospholipases (3, 16). Addition of the protease inhibitors leupeptin (0.1 mM) or p-chloromercuriphenylsulfonate (0.1 mM) failed to block the release of CPM from MDCK cells (data not shown). Direct evidence against the role for a protease(s) in CPM release was obtained by [^{3}H]ethanolamine labeling. Ethanolamine is found in all GPI anchors (3, 4) and is an integral component of the GPI anchor of CPM in MDCK cells (16). Whereas a GPI-anchored protein released by PI-specific PLC or PLD would retain the ethanolamine label, release by a protease would remove it (3, 4). MDCK cells were labeled with [^{3}H]ethanolamine in serum free medium for 3 days and then CPM in the medium or solubilized from the P_3 membrane fraction with either detergent or trypsin were immunoprecipitated with specific anti-CPM antibody followed by SDS-PAGE and fluorography. Both CPM in the medium and detergent-solubilized membrane-bound CPM yielded a single radioactive band upon autoradiography while no radioactive band could be detected in CPM solubilized by trypsin (Fig. 2). The lack of labeling in the trypsin-treated sample was not due to general degradation of CPM because we previously showed that identical treatment of MDCK cell membranes released CPM into solution, but did not alter its size in SDS-PAGE or its activity (16). The slightly lower apparent molecular weight of the membrane-bound enzyme is likely due to binding of SDS to the hydrophobic diglyceride tail, causing the protein to migrate faster than its true molecular weight, as noted with other GPI-anchored proteins. These data, combined with the Triton X-114

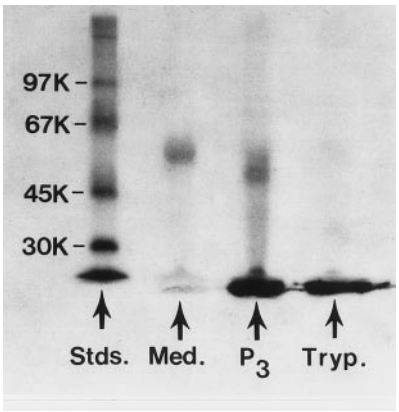

FIG. 2. [^{3}H]Ethanolamine labeling of CPM in MDCK cells and conditioned medium. MDCK cells were radiolabeled with [^{3}H]ethanolamine and the medium and cells were fractionated, solubilized, immunoprecipitated with anti-CPM antiserum, and analyzed by SDS-PAGE and autoradiography as described under Materials and Methods. Stds. = radiolabeled molecular weight markers; Med. = conditioned medium; P_3 = membrane fraction solubilized with 1% Triton X-100 for 2 h at 4°C; Tryp. = supernatant from P_3 membrane fraction treated with 0.01% trypsin for 2 h at 37° C.

partitioning experiments, indicate that the released form of CPM retains part of the GPI anchor but lacks the hydrophobic, diglyceride portion, consistent with its release by an endogenous phospholipase.

Effect of exogenous PI-PLC on CPM levels in MDCK cells. To determine the effect of enhanced release on cellular CPM levels, cells were treated with 0.01 U/ml of PI-PLC for 6 h and the CPM activity in the medium and cell lysates was measured. As shown in Table I, CPM activity in the medium increased 5.5-fold over control cells, yet the activity in the cells remained constant, leading to a 30% increase in total CPM activity (cells + medium). Treatment of the cells with 0.001% trypsin for 6 h also released CPM, leading to a 3.4-fold increase in activity in the medium, but also decreased cellular CPM activity, yielding no change in total CPM activity (Table I). The concentration of trypsin used did not cause detachment of cells from the dish, and in control experiments, treatment of CPM in serum-free medium for 6 h with 0.001% trypsin did not result in any loss of activity (data not shown).

We previously reported that PC-PLC does not release CPM from MDCK cell membrane fractions (16). In the current study, treatment of intact MDCK cells with 0.05 U/ml PC-PLC for 6 h did not release CPM into the medium (2.1 ± 0.3% vs. 1.8 ± 0.1% for control cells; n = 3) nor did it increase total CPM activity (Fig. 3).

To rule out the possibility that PI-PLC treatment caused a general increase in synthesis of membrane proteins, we measured the activity of neutral endopeptidase, a type II plasma-membrane enzyme (27) that is also found in MDCK cells (16). PI-PLC did not release neutral endopeptidase from the cell surface and total enzyme activity was not affected by PI-PLC treatment (Table I).

Effects of diglycerides and phorbol esters on CPM activity in MDCK cells. A potential signaling molecule that is released upon PI-PLC cleavage of CPM is the diglyceride moiety of the GPI anchor. To investigate this possibility, MDCK cells were treated for 6 h with either OAG, a synthetic diacylglycerol or PMA. OAG or PMA did not substantially increase the total CPM activity under conditions in which PI-PLC treatment increased the enzyme activity by 34% (Fig. 3). A more prolonged treatment (24 h) caused a larger increase in CPM activity in response to PI-PLC (54%) and 10^{-7} M PMA increased total CPM activity by 12.8% whereas OAG was still ineffective (Fig. 3). Although the effect of PMA was small, it was dose dependent as 10^{-8} M PMA increased activity 8.7 ± 1.3% and 10^{-6} M PMA increased activity 17.3 ± 2.0% (not shown). A second diacylglycerol, 1,2-dimyristoyl-glycerol, and phorbol-12,13-dibutyrate, another phorbol ester, also did not substantially increase CPM activity after 24 h

TABLE I

Effect of Phosphatidylinositol-Specific Phospholipase C on the Activity of CPM and Neutral Endopeptidase in MDCK Cells

	Enzyme activity (nmol/h/10^6 cells)					
	CPM			NEP		
Treatment	Cells	Medium	Total	Cells	Medium	Total
Control	43.5 ± 4.3	3.6 ± 0.8	47.1 ± 4.5	8.48 ± 0.16	0.14 ± 0.05	8.62 ± 0.16
PI-PLC	41.5 ± 3.7	19.8 ± 5.9	61.3 ± 9.7	8.77 ± 0.37	0.16 ± 0.06	8.93 ± 0.32
Trypsin	37.4 ± 2.2	12.1 ± 0.6	49.5 ± 2.2	n.d.	n.d.	n.d.

Note. Cells were incubated in the presence or absence of either 0.01 U/mL phosphatidylinositol-specific phospholipase (PI-PLC) or 0.001% trypsin for 6 h at 37°C. CPM activity was assayed with Dns-Ala-Arg and neutral endopeptidase (NEP) activity was assayed with glutaryl-Ala-Ala-Phe-4-methoxy-2-naphthylamide. Activity = mean ± S.E.M. (n = 4 for CPM; n = 3 for NEP). n.d. = not determined.

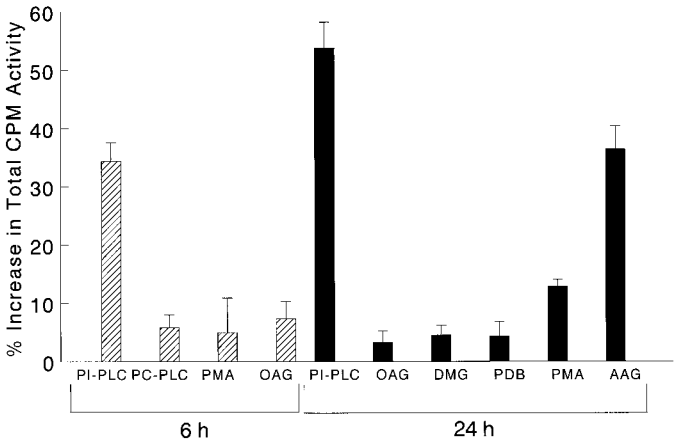

FIG. 3. Effect of PI-PLC, diglycerides, and phorbol esters on CPM activity in MDCK cells. Confluent MDCK cells in serum-free medium were incubated for 6 or 24 h at 37°C with the indicated agents. Cells were harvested, the medium was collected, and CPM activity of both cells and medium was assayed with Dns-Ala-Arg substrate as described under Materials and Methods. The % increase in total CPM activity (cells + medium) over control cells (untreated) is plotted as the mean ± S.E.M. of three separate experiments for each treatment. The following agents were used: PI-PLC = 0.05 U/ml phosphatidylinositol-specific phospholipase C; PC-PLC = 0.05 U/ml phosphatidylcholine-specific phospholipase C; PMA = 100 nM phorbol 12-myristate 13-acetate; OAG = 200 μg/ml 1-oleoyl-2-acetyl-rac-glycerol; DMG = 100 μg/ml 1,2-dimyristoyl-glycerol; PDB = 100 nM phorbol 12,13-dibutyrate; AAG = 50 μg/ml 1-alkyl-2-acetyl-glycerol.

(Fig. 3). Although the lipid moiety of GPI protein anchors in some cases is a diacylglycerol, most of the mammalian proteins that have been analyzed contain an AAG moiety (4). We therefore treated cells with AAG and found that it was more effective than diacylglycerols or phorbol esters, increasing CPM activity by 36% (Fig. 3).

To investigate whether the increase in CPM activity by PI-PLC treatment results from stimulation of new protein synthesis, MDCK cells were treated with PI-PLC for 24 h in the presence or absence of 0.2 μM cycloheximide. Cycloheximide blocked the upregulation of CPM activity by 77.6 ± 5.4% (n = 3), indicating that new protein synthesis is required. Additional proof for increased synthesis of CPM was obtained by radiolabeling newly synthesized CPM in MDCK cells with [^{35}S]methionine followed by immunoprecipitation with antiserum to CPM and SDS-PAGE. The results of 4 separate experiments revealed an increase in [^{35}S]methionine incorporation into CPM of 62.9 ± 11.3%.

DISCUSSION

The release of a membrane protein into extracellular fluids may allow it to act more systemically, or may be a way of regulating the protein's activity at the cell surface (1, 2). However, the mechanisms by which proteins are released and the signals involved in maintaining constant levels on the cell membrane in the face of this release remain unclear.

In this study, CPM was released into the medium of MDCK cells in a constitutive fashion via a cellular phospholipase. The endogenous PI-specific PLC(s) or PLD(s) that might be involved in the spontaneous release of GPI-anchored proteins are unknown. The PLCs and PLDs involved in phosphoinositide or phosphatidylcholine hydrolysis do not cleave GPI-glycans (28). A PI-PLC has been purified and characterized that can cleave non-protein anchor GPI-glycans that lack ethanolamine and are oriented intracellularly, with the diglyceride in the inner leaflet (29). The release of a diglyceride from these novel signaling molecules activates a PKC and mediates some of the cellular responses to insulin and nerve growth factor (28, 29). However, this PI-PLC is located on the inner leaflet of the bilayer and is incapable of releasing plasma membrane GPI-anchored proteins (29). Two well-characterized enzymes that can release GPI-anchored proteins are the bacterial PI-PLCs and a mammalian serum PI-PLD (28, 30). However, serum PI-PLD is not able to release GPI-anchored proteins in their native forms from cells without addition of detergent (31). To date, no mammalian cellular phospholipase capable of releasing GPI-anchored proteins from the plasma membrane of intact cells has been isolated, although this study and others (4) provide evidence for the existence of such an enzyme. For example, soluble 5′ nucleotidase from bovine cortex or *torpedo marmorata* contains a common 1,2-cyclic monophosphate antigenic epitope found in GPI-anchored proteins solubilized with PI-PLC (32). In addition, many GPI-anchored proteins can be found in soluble form in biological fluids, indicating that an endogenous release mechanism must exist (2, 4).

To maintain constant levels of a GPI-anchored protein on the cell surface, there must be a balance between its rate of synthesis and release. The regulatory mechanisms involved in this process are not known, however the present study suggests that the liberation of a diglyceride second messenger is involved. Although the released product would be in the outer leaflet of the bilayer, diglycerides can readily flip-flop into the inner leaflet (33) which could then provoke an intracellular signal. Diglyceride second messengers can vary structurally both with regard to the nature of the lipid moiety and the linkage to the glycerol backbone, which can either be an ester (acyl) or ether (alkyl) bond (34-37). Interestingly, in the mammalian proteins that have been analyzed, the diglyceride component of the GPI anchor is predominantly AAG (4). Thus, release of a protein such as CPM would likely release AAG, consistent with our finding that AAG treatment increases CPM activity in MDCK cells whereas diacylglycerols or PMA are ineffective.

Of the diglycerides generated during cellular signaling, diacylglycerols have received the most attention, however AAGs also have biological activity (38-41). The alkyl vs. acyl nature of the diglyceride affects the ability of the molecule to activate PKCs, although the structural features involved are not fully understood. Whereas diacylglycerols are endogenous activators of the conventional and novel PKCs, the AAGs have varying effects (35, 36). For example, AAGs stimulated rat brain PKC β (42), had no effect on mouse brain PKC (43) and inhibited diacylglycerol stimulation of PKC δ and ϵ activity in mesangial cells (44). In addition, interleukin-1, which stimulates the production of only ether-linked diglycerides (45), activates PKC ζ in mesangial cells (46). The target of a diglyceride released from CPM after PI-PLC release is unknown, but could be one of the PKC isozymes, likely an "atypical" PKC in view of the ineffectiveness of diacylglycerols and PMA. In support of this possibility, treatment of MDCK cells with PI-PLC or AAG increased calcium-independent membrane-associated PKC activity and a general PKC inhibitor blocked the upregulation of CPM activity caused by either PI-PLC or AAG.[2]

The upregulation of CPM synthesis by PI-PLC release could also be relevant to the regulation of the levels of other GPI-anchored proteins. For example, co-transfection of COS cells with a GPI-specific PLD and GPI-anchored alkaline phosphatase resulted in greatly increased release of alkaline phosphatase into the medium and an overall 3 to 4-fold greater expression than in cells transfected with alkaline phosphatase alone (47). The reason for the higher expression of alkaline phosphatase was not investigated. N-CAM120, a neural cell adhesion molecule with a GPI anchor, is spontaneously released into culture medium from a C6 rat astrocytoma cell line, and treatment with exogenous PI-PLC increased its biosynthetic rate by about 33% (48). Finally, treatment of the parasite *Schistosoma mansoni* with exogenous PI-PLC released GPI-anchored acetylcholinesterase and increased the total amount of enzyme (in the parasite + medium) by 40–50% and increased its rate of synthesis (49). The response could be mimicked by diacylglycerols and it was theorized that stimulation of a PKC was involved (49). Taken together, these data and our results indicate that release of GPI-anchored proteins from the cell surface upregulates their synthesis. This response is likely mediated via a diglyceride signaling molecule released from the membrane anchor by a phospholipase. Although the stimulation is moderate, this level of upregulation would be optimally suited to maintain constant levels of a GPI-anchored protein on the cell surface in the face of constant, low level constitutive release found with most of these membrane proteins.

[2] Li, X.-Y., and Skidgel, R.A., unpublished.

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