Regulation of Phosphatidic Acid Phosphohydrolase by Epidermal Growth Factor

REDUCED ASSOCIATION WITH THE EGF RECEPTOR FOLLOWED BY INCREASED ASSOCIATION WITH PROTEIN KINASE Ce

(Received for publication, August 22, 1996, and in revised form, September 19, 1996)

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An important component of receptor-mediated intracellular signal transduction is the generation of lipid second messengers. Lipid second messenger production is a complex process involving a variety of regulatory enzymes that control the intracellular response to the extracellular signal. Phosphatidic acid (PA) is generated in response to phospholipase D and can be converted to other lipid second messengers including diacylglycerol (DG) and lysophosphatidic acid. PA is converted to DG by PA phosphohydrolase (PAP). We report here that PAP activity can be detected in epidermal growth factor (EGF) receptor immunoprecipitates. Following treatment with EGF, there is a substantial reduction in the PAP activity that co-precipitates with the EGF receptor. The loss of EGF receptor-associated PAP activity occurs with a concomitant increase in PAP activity associated with the ε isof orm of protein kinase C (PKC). The PAP activity associated with PKCe was dependent upon the PKC co-factors phosphatidylserine and DG but was independent of the kinase activity of PKCe. These data suggest a novel signaling mechanism for the regulation of lipid second messenger production and implicate PAP as an important regulatory component for lipid second messenger production in receptor-mediated intracellular signaling.

One of the earliest responses to extracellular signals is the metabolic conversion of membrane phospholipids into intracellular second messengers (1, 2). The best studied second messenger-generating system is the activation of phospholipase Cγ (PLCγ), which catalyzes the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP2) to diglyceride (DG) and inositol 1,4,5-trisphosphate (3). Another mechanism for generating DG is through the activation of type D phospholipases (PLD) that catalyze the hydrolysis of phosphatidylcholine (PC) to phosphatic acid (PA) and choline (4). PA can be converted to DG by a PA phosphohydrolase (PAP). Regulation of PAP is likely to be important because PA can be converted to biologically active molecules other than DG. Lyso-PA can be generated from PA and has been shown to mediate several biological activities (5–7).

We previously demonstrated that EGF induces an increase in DG that was generated from PC-derived PA (17), suggesting a role for PAP. In this report, we describe a novel mechanism for regulation of lipid second messenger production in response to EGF.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture Conditions—A431 cells, obtained from the American Type Culture Collection were maintained in Dulbecco’s modified Eagle medium supplemented with 10% bovine calf serum (Life Technologies, Inc.). Cells were routinely placed in serum-free medium prior to treatment with EGF to reduce background PAP activity. Materials—[3H]PA was obtained from New England Nuclear. PIP2, PC, phosphatidylserine (PS), DG were obtained from Sigma. The anti-EGF receptor antibody was obtained from Oncogene Science; antibodies specific for the α, ε, and γ PKC isozymes were obtained from Transduction Laboratories.

Assay of PAP Activity—A431 cells were grown to confluence in 150-mm culture dishes and then placed in serum-free medium overnight. Cells were then washed with cold isotonic buffer, scraped from the plates, and suspended in 2 ml of hypotonic buffer A (20 mM Tris-HCl, pH 7.3; 5 mM NaCl; 5 mM Na2HPO4; 1 mM EDTA; 1 mM EGTA; 0.2 mM phenylmethylsulfonyl fluoride; 1 μg/ml leupeptin; 1 μg/ml aprotinin; 400 μM Na3VO4). The suspended cells were then broken by Dounce homogenization (15 strokes with type B pestle). Disrupted cells were centrifuged at 500 × g for 10 min to clear nuclei and unbroken cells. The post-nuclear homogenate was used as the whole cell lysate. The post-nuclear homogenate can be separated into membrane and cytosolic fractions by centrifugation at 100,000 × g for 60 min. The supernatant is saved as the cytosolic fraction. The membrane fraction is recovered from the pellet by resuspending in buffer A. Liposomes are made by mixing lipids in chloroform, drying under a stream of nitrogen, and resuspending in assay buffer (20 mM Tris-HCl, pH 7.3, 5 mM Na2HPO4, 140 mM NaCl, 0.1 mM EGTA, 2 mM MgCl2, 0.5 mM CaCl2) with vortexing and then sonicing for 3 min. Unless otherwise indicated, the liposome suspension was 6 μg/ml dipalmitoyl-PA (~0.1 mol fraction), 10 μg/ml PC (from egg yolk), 10 μg/ml PIP2, 30 μg/ml PS, 3 μg/ml DG (Sigma), and 20,000 cpm [3H]dipalmitoyl-PA (100 Ci/mmol). The sonicated solution is kept at room temperature for 2 h to equilibrate. The PAP assay is initiated with substrate.
by the addition of 20 μl of homogenate (40 μg of protein) to prewarmed (37°C) liposome suspension. Where appropriate, fatty acid-free bovine serum albumin was added to maintain constant protein concentration (1 mg/ml) in the reaction mixture. The final volume of the reaction mixture was 140 μl. The mixture is incubated at 37°C for 15 min unless otherwise indicated. The reaction consumed less than 20% of the [14C]PA. The reaction is terminated by the addition of 800 μl of chloroform/methanol (6:2 v/v). DG is resolved by thin layer chromatography as described previously (18).

**Immunoprecipitations**—membrane fractions were treated with buffer A containing 1% Triton X-100 and 140 mM NaCl. Insoluble material was cleared by centrifugation (12,000 × g, 30 min). The 1 ml of supernatant (1 mg of protein) was then incubated with the indicated antibody (2 μg) overnight at 4°C with shaking. The antibody-antigen complexes were then recovered with 20 μl of either protein A-Sepharose or protein G-agarose suspensions (Santa Cruz Biotechnology) (2 h, 4°C with shaking) by microcentrifugation and washing four times in buffer A containing 1% Triton X-100 and 140 mM NaCl. Liposome suspensions were then added to the immunoprecipitates, and PAP activity was determined as described above.

**RESULTS**

**PAP Activity in A431 Cells**—There are currently no direct assays for PAP activity in intact cells. However, PAP activity can be measured in vitro by adding cell extracts or lysates containing PAP to liposomes containing radiolabeled PA and examining the conversion of PA to DG. An in vitro PAP assay based on strategies developed by Brindley and co-workers (19, 20) is used to investigate PAP activity in A431 cells. In A431 cells PAP activity could be detected in both cytosolic and membrane fractions (Table I). It has been reported that in several different cell types, two distinct PAP activities exist that can be distinguished on the basis of a differential sensitivity to N-ethylmaleimide (NEM) and Mg2+ (19–21). In A431 cells, we found that virtually all of the PAP activity, both membrane and cytosolic, was sensitive to NEM (Table I). This difference was not likely due to differences in experimental protocol, because we also find that in 3Y1 rat fibroblasts 80% of the PAP activity was resistant to NEM treatment (data not shown). NEM-resistant PAP was reported to be insensitive to Mg2+ (19), whereas the NEM-sensitive PAP activity was stimulated by Mg2+ (19). As shown in Table I, the NEM-sensitive PAP activity from both membranes and cytosol was dependent upon Mg2+. Thus, we were unable to detect significant biochemical differences between the membrane and cytosolic PAP activities in A431 cells. These properties are characteristic of the PAP previously described.

**TABLE I**

| Liposomes composition | Membrane | Cytosol |
|-----------------------|----------|---------|
|                        | Cpm/mg protein × 10^-7 |         |
| Liposomes only         | 0.05 ± 0.01 | 0.03 ± 0.01 |
| Complete               | 2.78 ± 0.09 (1.00) | 0.84 ± 0.12 (1.00) |
| – Mg2+                 | 0.10 ± 0.01 (0.04) | 0.13 ± 0.09 (0.15) |
| – Ca2+                 | 2.74 ± 0.13 (0.99) | 0.96 ± 0.07 (1.14) |
| + NEM                  | 0.05 ± 0.01 (0.02) | 0.03 ± 0.01 (0.04) |
| Heat                   | 0.23 ± 0.08 (0.08) | 0.14 ± 0.04 (0.16) |
| + Propranolol          | 0.51 ± 0.23 (0.18) | ND       |
| + Sphingosine          | 0.05 ± 0.01 (0.02) | ND       |
| + Chlorpromazine       | 0.11 ± 0.02 (0.04) | ND       |

The effect of phospholipid composition and EGF (100 nM, 5 min) on PAP activity in A431 cells that had been placed in serum-free media for 24 h was investigated as shown. The ratio of included phospholipids in the liposome reaction mix was as follows: [PA (1:0):PC (2:0):PIP2 (1:4):PS (6:0):DG (8:0)]. The relative effects on PAP activity are shown in parentheses after normalizing to the PAP activity found using liposomes containing PA only in the absence of EGF. The PAP assay was performed as in Table I using a complete cell lysate as described under "Experimental Procedures." The data represent the average of duplicates (± range) from a representative experiment that was repeated twice.

**TABLE II**

| Liposome composition | PAP activity [14C]DG | PAP activity [14C]DG |
|----------------------|---------------------|---------------------|
|                      | Membrane            | Cytosol             |
| Liposome composition | Cpm/mg protein × 10^-7 | Cpm/mg protein × 10^-7 |
| PA only              | 1.07 ± 0.17 (1.0) | 1.59 ± 0.10 (1.5) |
| PA + PC              | 1.18 ± 0.10 (1.1) | 1.72 ± 0.12 (1.6) |
| PA + PC + PIP2       | 3.40 ± 0.15 (3.2) | 5.62 ± 0.24 (5.3) |
| PA + PC + PIP2 + DG  | 2.13 ± 0.14 (2.0) | 5.76 ± 0.33 (5.4) |
| PA + PC + PIP2 + PS  | 4.13 ± 0.30 (3.9) | 6.15 ± 0.46 (5.8) |
| PA + PC + PIP2 + PS + DG | 5.28 ± 0.36 (4.9) | 6.57 ± 0.40 (6.1) |

The effect of phospholipid composition and EGF (100 nM, 5 min) on PAP activity in A431 cells that had been placed in serum-free media for 24 h was investigated as shown. The ratio of included phospholipids in the liposome reaction mix was as follows: [PA (1:0):PC (2:0):PIP2 (1:4):PS (6:0):DG (8:0)]. The relative effects on PAP activity are shown in parentheses after normalizing to the PAP activity found using liposomes containing PA only in the absence of EGF. The PAP assay was performed as in Table I using a complete cell lysate as described under "Experimental Procedures." The data represent the average of duplicates (± range) from a representative experiment that was repeated twice.

ignated PAP1 (19). As expected, the PAP activity was sensitive to the amphiphilic cations propranolol, chlorpromazine, and sphingosine, which have been shown previously to inhibit PAP activity (19).

**Liposome composition can also affect in vitro PAP activity** (19). We therefore examined the effect of the phospholipid composition of the liposomes used in the PAP assay. As shown in Table II, PAP activity was stimulated by the inclusion of PIP2, although PAP activity was not dependent upon PIP2 as demonstrated for PLD (9, 16). PIP2 is a substrate for PLCγ, and it has been reported that PA can stimulate PLCγ activity (22). The PA stimulation of PLCγ activity was dependent upon Ca2+ (22); however, as shown in Table I, Ca2+ had no effect upon the PAP activity in the presence of PIP2. As shown in Table II, the addition of DG (the product of PLCγ) to the liposomes actually inhibited the PAP activity in the presence of PIP2. Thus, the effect of PIP2 is not likely due to an effect of PLCγ on PAP activity. The addition of PS and DG was also stimulatory for PAP activity (Table II). The effect of PS and DG, which are co-factors for PKC activity, suggested the possible PKC involvement in PAP activity. The effect of EGF on the PAP activity from A431 cells was examined, and although EGF reproducibly elevated PAP activity, the effect varied with the composition of the liposomes (Table II).

**PAP Activity Co-immunoprecipitates with the EGF Receptor and Is Reduced upon Treatment with EGF**—The EGF receptor has previously been demonstrated to be in complexes with molecules that transduce intracellular signals (1, 23, 24). We therefore investigated whether PAP activity could be detected in EGF receptor immunoprecipitates. Membranes were isolated from EGF-treated A431 cells and untreated controls. Detergent lysates of the membrane fractions were treated with antibodies raised against the EGF receptor. Immunoprecipitates were recovered, and PAP activity was examined. As shown in Fig. 1A, PAP activity could be easily detected in the EGF receptor immunoprecipitates. However, in contrast to expectations, activity was lost upon EGF treatment (Fig. 1A). The loss of PAP activity associated with the EGF receptor in response to EGF was both time- (Fig. 1A) and dose-dependent (Fig. 1B) with the loss in activity being detectable within 1 min and at 1 nM EGF. This reduction in PAP activity was not due to a reduced ability of the antibody to precipitate EGF-treated receptor, because there were no differences in the amount of EGF receptor precipitated from the EGF-treated and untreated cells (Fig. 1C). This suggested that either the PAP activity was
reduced or that PAP protein was released from the receptor in response to EGF. Because we could detect overall increases in total PAP activity in response to EGF, we considered the first possibility unlikely. Thus, the data in Fig. 1 suggest that the PAP associated with the EGF receptor is released in response to EGF treatment.

PAP Activity Associates with PKCε in Response to EGF—In Table II it was shown that PAP activity was enhanced by the presence of PS and DG in the liposomes. This suggested an involvement of PKC. Jaken and co-workers have characterized a number of proteins that interact directly with PKC isoforms (25). We therefore investigated the possibility that PAP activity could be associated with PKC isoforms present in A431 cells. The predominant PKC isoforms present in A431 cells are the α, ε, and ζ isoforms.2 PAP activity in PKC isoform immunoprecipitates from lysates of A431 cells that had been either treated or untreated with EGF was determined. As shown in Fig. 2A, a very strong EGF-dependent PAP activity was detected in PKCε but not PKCα or ζ immunoprecipitates. The lack of PAP in the PKCα and ζ immunoprecipitates was not due to an inability to precipitate PKCα and ζ because these isoforms could be detected at levels comparable with the ε isoform in the immunoprecipitates (data not shown). A PKCε peptide against which the antibody was raised prevented precipitation of PAP with the PKCε antibody (Fig. 2A). The kinetics of association of PAP activity with PKCε after EGF treatment was determined as shown.

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FIG. 1. PAP activity in EGF receptor immunoprecipitates. A, the membrane fraction from A431 cells that were either untreated or treated with EGF (100 nM) for the indicated times were harvested as in Table I and then lysed with 1% Triton. The lysate was then incubated with an anti-EGF receptor antibody (12 h, 4 °C). Antigen-antibody complexes were recovered with protein G-agarose and added directly to the complete liposome mixture described in Table II, and PAP activity in the immunoprecipitates was determined. The data presented are from a representative experiment that was repeated three times. The dose response (B) for the effect seen in A was determined as shown. C, the amount of EGF receptor immunoprecipitated in EGF-treated (100 nM, 5 min) and untreated A431 cells was determined by Western blot analysis as described previously (29). The PAP activity in these anti-EGF receptor immunoprecipitates is also presented.

FIG. 2. PAP activity can be co-immunoprecipitated with PKCε after EGF treatment. A, the membrane fraction from A431 cells that were either untreated or treated with EGF (100 nM) for 5 min were harvested and then lysed with 1% Triton as in Fig. 1. The lysate was then incubated with antibodies specific for the α, ε, and ζ PKC isoforms (12 h, 4 °C). The antigen-antibody complexes were recovered with protein A-Sepharose for PKCα and ε and protein G-agarose for PKCζ, and PAP activity was determined as in Fig. 1. To establish that the effect was specific for PKCε, a PKCε-specific peptide against which the antibody was raised was included in the immunoprecipitation and the ability to immunoprecipitate PAP activity was determined. B, the time course for association of PAP activity with PKCε after EGF treatment was determined as shown.
PKC-associated PAP activity is dependent upon DG and PS but independent of ATP. The PAP activity associated with PKCe was examined using liposomes containing and lacking the PKC co-factors DG and PS as shown. The effect of ATP (0.1 μM), ATPγS (0.1 μM), and staurosporine (70 nM) on the PKCe-associated PAP activity is also presented.

PAP Activity Associated with PKCe Is Dependent upon the Presence of DG and PS in Liposomes, but Not PKC Kinase Activity—The in vitro PAP activity from A431 cells was significantly enhanced when PS and DG were included in the liposomes used in the PAP assay (Table II). We therefore examined the effect of DG and PS in the liposomes when the PKCe-associated PAP activity was determined. In the absence of DG and PS, we were unable to detect any PAP activity in the PKCe immunoprecipitates in response to EGF (Fig. 3). The DG and PS requirement suggested that PKCe activity is important in the in vitro liposome assay. However, as shown in Fig. 3, neither ATP nor ATPγS had any effect on the PAP activity associated with PKCe, suggesting that the kinase activity is not required. Consistent with this result, staurosporine, which inhibits PKC by competing for ATP binding, also had no effect upon the in vitro PAP activity in the PKCe immunoprecipitates (Fig. 3B). These surprising results suggest that although the PKC co-factors DG and PS are required to observe the PAP activity associated with PKCe, the kinase activity of PKCe is apparently not required for the in vitro PAP activity.

DISCUSSION

In this report, data have been presented suggesting a novel signaling mechanism in which PAP associated with the EGF receptor becomes associated with PKCe. Upon EGF treatment, PLCγ is activated (26). The generation of DG by PLCγ could lead to the observed increase in membrane localization of PKCe in response to EGF. PAP could then become associated with membrane-bound PKCe. Although the data presented here do not demonstrate that PAP activity is actually elevated in response to EGF, the kinetics of the EGF-induced association of PAP with PKCe correlate well with the EGF-induced increase in PC-derivatized DG reported previously (17). It is possible that there are no significant changes in the specific activity of PAP in response to EGF and that regulation is accomplished by the induced change in the association of PAP from the EGF receptor to an association PKCe. We were unable to detect PAP activity in anti-phosphotyrosine immunoprecipitates, suggesting that the PAP is not a direct substrate of the EGF receptor. Thus, a molecular mechanism for the putative dissociation of PAP from the EGF receptor and association with PKCe remains to be determined.

The requirement for PS and DG in order to see the PKCe-associated PAP activity suggested a requirement for PKC activity. However, because ATP, ATPγS, nor the ATP analog staurosporine had any effect on the PKCe-associated PAP activity, it is possible that the PS and DG are functioning to localize PKCe and the associated PAP to the liposomes where the PA substrate is present. In this regard, it is of interest that PKC has been shown to stimulate PLD activity via an ATP-independent mechanism (27, 28). These observations, along with data presented here, suggest that PKC(s) may have kinase-independent roles in the regulation of intracellular signals. It is possible that PKC may serve to either allosterically modify PAP or to localize PAP to appropriate membrane locations where PA substrate is localized. PKC could be important for positioning PAP in a molecular complex where the enzyme functions as a component of a signaling complex that generates DG, which activates the associated PKC, which would then phosphorylate other substrates.

Acknowledgment—We thank Richard Kolesnick for comments on the manuscript.

REFERENCES

1. Foster, D. A. (1993) Cell. Signalling 5, 389–399
2. Spiegel, S., Foster, D. A., and Kolesnick, R. (1996) Curr. Opin. Cell Biol. 8, 159–167
3. Exton, J. H. (1994) Biochim. Biophys. Acta 1212, 26–42
4. Berriege, M. J. (1987) Annu. Rev. Biochem. 56, 159–184
5. Moolenaar, W. H. (1995) J. Biol. Chem. 270, 12949–12952
6. van Corven, E. J., Groenink, A., Jalink, K., Eichholz, T., and Moolenaar, W. H. (1989) Cell 59, 45–54
7. Vane, D. E., and Vance, J. (eds) (1985) Biochemistry of Lipids and Membranes, Benjamin/Cummings, Menlo Park, CA
8. Randazzo, P. A., and Kahn, R. A. (1994) J. Biol. Chem. 269, 10758–10763
9. Brown, H. A., Gutscher, S., Moomaw, C. R., Slaughter, C., and Sternweis, P. C. (1993) Cell 75, 1137–1144
10. Cockcroft, S., Thomas, G. M. H., Fanssone, A., Genyi, B., Cunningham, E., Gout, I., Hiles, I., Totty, V., Truong, O., and Huaan, J. J. (1994) Science 263, 525–526
11. Yu, C.-L., Tsai, M. H., and Stacey, D. W. (1990) Mol. Cell. Biol. 10, 6683–6689
12. Tsai, M. H., Yu, C.-L., and Stacey, D. W. (1990) Science 250, 982–985
13. Cavenago, A., Cuadrado, A., del Peso, L., and Lacal, J. C. (1994) Oncogene 9, 1387–1395
14. Jiang, H., Lu, Z., Luo, J. Q., Wolfman, A., and Foster, D. A. (1995) J. Biol. Chem. 270, 6006–6009
15. Moritz, A., Graan, P. N. E., Gispen, W. H., and Wirtz, K. W. A. (1992) J. Biol. Chem. 267, 7427–7430
16. Lisovskiy, M., and Cantley, L. C. (1995) Cell 81, 659–662
17. Singh, J., Jiang, X.-W., and Foster, D. A. (1994) Cell Growth Differ. 5, 79–85
18. Jiang, Y.-W., Song, J., Zang, Q., and Foster, D. A. (1996) Biochem. Biophys. Res. Commun. 230, 1195–1203
19. Jamal, Z., Martin, A., Gomez-Munoz, A., and Brindley, D. N. (1991) J. Biol. Chem. 266, 2988–2996
20. Martin, A., Gomez-Munoz, A., Wagggoner, D. W., Stone, J. C., and Brindley, D. N. (1993) J. Biol. Chem. 268, 23932–23932
21. Jadmar, S. C., and Cao, W. F. (1994) Biochem. J. 301, 793–799
22. Jones, G. A., and Carpenter, G. (1993) J. Biol. Chem. 268, 20845–20850
23. Ulrich, A., and Schlesinger, J. (1990) Cell 61, 203–212
24. Cantley, L., Auger, K. R., Carpenter, C., Dukewich, B., Graziani, A., Kapeller, R., and Soltoff, S. (1991) Cell 64, 281–302
25. Jaken, S. (1996) Curr. Opin. Cell Biol. 8, 168–173
26. Margolis, B., Rhee, S. G., Felder, S., Mervic, M., Lyall, R., Levitzki, A., Ulrich, A., Zilberstein, A., and Schlesinger, J. (1989) Cell 77, 1101–1107
27. Conricode, K. M., Brewer, K. A., and Exton, J. H. (1992) J. Biol. Chem. 267, 7199–7202
28. Singer, W. D., Brown, H. A., Jiang, X., and Sternweis, P. C. (1996) J. Biol. Chem. 271, 4504–4510
29. Jiang, H., Lu, J.-Q., Urano, T., Lu, Z., Foster, D. A., and Feig, L. (1995) Nature 378, 409–412