Activation and Translocation of Rho (and ADP Ribosylation Factor) by Insulin in Rat Adipocytes

APPARENT INVOLVEMENT OF PHOSPHATIDYLINOSITOL 3-KINASE*

(Received for publication, August 12, 1996, and in revised form, December 17, 1996)

Purushotham Karnam, Mary L. Standaert, Lamar Galloway, and Robert V. Farese‡

From the J. A. Haley Veterans’ Hospital Research Service and the Departments of Internal Medicine and Biochemistry/Molecular Biology, University of South Florida, Tampa, Florida 33612

Insulin reportedly (Standaert, M. L., Avignon, A., Yamada, K., Bandyopadhyay, G., and Farese, R. V. (1996) Biochem. J. 313, 1039–1046) activates phospholipase D (PLD)-dependent hydrolysis of phosphatidylcholine (PC) in plasma membranes of rat adipocytes by a mechanism that may involve wortmannin-sensitive phosphatidylinositol (PI) 3-kinase. Because Rho and ADP ribosylation factor (ARF) activate PC-PLD, we questioned whether these small G-proteins are regulated by insulin and PI 3-kinase. We found that insulin provoked a rapid translocation of both Rho and ARF to the plasma membrane and increased GTP loading of Rho. Wortmannin and LY294002 inhibited Rho translocation in intact adipocytes, and the polyphosphoinositide, PI 4,5-(PO₄)₂, stimulated Rho translocation in adipocyte homogenates. On the other hand, wortmannin did not block GTP loading of Rho. Guanosine 5'-3-O- (thio)triphosphate stimulated both Rho and ARF translocation and activated PC-PLD in homogenates. C3 transferase, which inhibits and depletes Rho, inhibited PC-PLD activation by insulin in intact adipocytes. C3 transferase also inhibited insulin stimulation of [³²P]inositol phosphate (IP₃) and 32P-labeled adenosine diphosphate ribosylation (ARF) translocation and activated PC-PLD in homogenates. Our findings suggest that: (a) insulin translocates Rho by a PI 3-kinase-dependent mechanism, but another factor is responsible for GTP loading of Rho; (b) both Rho and ARF may contribute to PC-PLD activation during insulin action; and (c) Rho may be required for insulin stimulation of glucose transport.

* The abbreviations used are: PLD, phospholipase D; PC, phosphatidylcholine; PI, phosphatidylinositol; KRP, Krebs Ringer phosphate buffer; BSA, bovine serum albumin; FAGE, polyacrylamide gel electrophoresis; PIP₂, phosphatidylinositol-4,5-(PO₄)₂; IRS-1, insulin receptor substrate-1; PKC, protein kinase C; PKR1, protein kinase C-related kinase-1; PKN, protein kinase N; GTPγS, guanosine 5'-3-O-(thio)triphosphate; 2-DOG, [³²H]2-deoxyglucose; ARF, ADP ribosylation factor.

† To whom correspondence should be addressed: Research Service (VAR 151), VA Haley VA Hospital, 13000 Bruce Downs Blvd., Tampa, FL 33612. Tel.: 813-972-7662; Fax: 813-972-7623.

‡ The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Phospholipase D (PLD)-mediated¹ hydrolysis of phosphatidylcholine (PC) is a major signaling system for agonists that activate tyrosine kinases. Insulin activates PC-PLD in rat adipocytes (1) and other cells (2–4), and this may be important for activation of signaling and targeting processes in the plasma membrane (1, 4, 5). In adipocytes, insulin-induced activation of PC-PLD is inhibited by wortmannin (1), an inhibitor of phosphatidylinositol (PI) 3-kinase, which is activated through its SH₂ domains by specific phosphotyrosine motifs in proteins, such as insulin receptor substrate-1 (IRS-1) (6); thus, PI 3-kinase may be required for PC-PLD activation. Accordingly, polyphosphoinositides, which are increased by insulin (7–9) through PI 3-kinase action (9) and which may be required for PC-PLD activation (10–13), may contribute directly to the stimulation of PLD by insulin. However, PC-PLD is also activated by Rho and ARF (10–15), and we presently questioned whether these small G-proteins are regulated by insulin and PI 3-kinase. In addition, because Rho and PI 3-kinase are thought to be involved in vesicle trafficking and because PI 3-kinase appears to play an important role in insulin-stimulated glucose transport (6), we questioned whether Rho may also be required for the latter process.

EXPERIMENTAL PROCEDURES

Adipocytes were prepared from epididymal fat pads (see Ref. 1), equilibrated in glucose-free Krebs Ringer phosphate (KRP) buffer containing 1% bovine serum albumin (BSA), and treated with wortmannin (Sigma), LY294002 (BioMol), and/or insulin (Eliance) as described in the text.

To study Rho/ARF translocation in intact cells, after incubation, the cells were chilled and sonicated in Buffer 1, which contained 250 mM sucrose, 20 mM Tris-HCl (pH 7.5), 1.2 mM EGTA, 20 μg/ml aprotonin, 20 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 20 mM β-mercaptoethanol. Homogenates were centrifuged first at 500 × g for 10 min to remove nuclei, debris, and the fat cake and then for 30 min at 100,000 × g to obtain membrane and cytosol fractions. Membranes were suspended in Buffer 1 supplemented with 5 mM EGTA, 2 mM EDTA, and 1% Triton X-100, and insoluble (cytoskeleton) substances were removed by centrifugation. Plasma membranes, microsomes, and mixed nuclear/mitochondrial fractions were obtained as described (1). Routinely, 60 μg of cytosolic protein and 80 μg of membrane protein were used for immunoblotting (note that cytosolic protein is three or four times more abundant than membrane protein).

To study Rho/ARF translocation in vitro, post-nuclear homogenates were prepared in Buffer 1 containing 1 mM EDTA and incubated first for 20 min at room temperature to release GDP and then for 20 min at 37 °C after adding 10 mM MgCl₂ with or without GTPγS (Sigma) or PI 4,5-(PO₄)₂ (PIP₂; Fluka). Subcellular fractions were then obtained as described above.

Subcellular fractions were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and immunoblotted, using chemiluminescence (ECL, Amersham Corp.) as described (1). Immunoblots were quantified with a Bio-Rad Molecular Analyst technology, Inc.

To obtain membrane and cytosol fractions. Membranes were prepared in Buffer I supplemented with 1 mM EDTA and incubated for four times more abundant than membrane protein). Routinely, 60 μg of cytosolic protein and 80 μg of membrane protein were used for immunoblotting (note that cytosolic protein is three or four times more abundant than membrane protein).
treated with insulin, and Rho was quantitatively immunoprecipitated from total cell lysates in buffer containing 20 mM Tris-HCl (pH 7.4), 250 mM sucrose, 150 mM NaCl, 2 mM EDTA, 10 mM MgCl$_2$, 1 mM Na$_3$PO$_4$, 1 mM NaF, 1 mM Na$_3$VO$_4$, 1% Triton X-100, 0.5% Nonidet, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 20 μg/ml apro tinin. Precipitates were collected on protein AG-Sepharose beads, and then heated for 20 min at 68°C in buffer containing 5 mM EDTA, 2 mM diethiolethiol, 0.2% SDS, 0.5 mM GTP, and 0.5 mM GDP. GDP and GTP were separated on polyethyleneimine-cellulose plates (Merck) developed with 1 mM KH$_2$PO$_4$ (pH 3.4). Plates were scanned and quantified in the Bio-Rad $^{32}$P Imaging System.

PLD activation was measured both in situ in intact adipocytes and in vitro in post-nuclear homogenates of adipocytes after overnight labeling with $[^{3}H]$oleic acid as described (1). For in situ assays, cells were electroporated as described (16) in Dulbecco’s modified Eagle’s medium with or without 1 μg/ml Clostridium botulinum C3 transference (List), a selective inhibitor of Rho (17). After overnight labeling (with or without C3 transference), cells were equilibrated in KR buffer containing 50 mM NBS and 2% ethanol, and treated for 0–20 min with 10 nM insulin (see Ref. 1). For in vitro assays, labeled cells were sonicated in Buffer I containing 25 mM HEPES (pH 7.4), 1 mM EDTA, 100 mM KCl, and 3 mM NaCl. After equilibration of homogenates for 20 min at room temperature to reach GDP, 1 μM CaCl$_2$, 5 mM MgCl$_2$, 2.5% ethanol, and GTPγS were added, and incubation at 37°C was continued for 30 min. $[^{3}H]$Phosphatidylethanol was isolated by TLC as described (1).

Glucose transport was assayed by measurement of $[^{3}H]$2-deoxyglucose (2-DOG) uptake during a 1-min period, following treatment of adipocytes with vehicle (controls) or 10 nM insulin for 30 min as described (1). Where indicated, cells were electroporated in the absence or presence of C3 transference and incubated for designated times prior to assessment of basal and insulin-stimulated 2-DOG uptake.

RESULTS

Rho Translocation in Intact Cells—21-kDa ARF was localized almost exclusively (>95%) in the cytosolic fraction in control adipocytes. Like Rho, ARF rapidly translocated to the plasma membrane, but not to microsomes, in response to 300 nM insulin treatment (Fig. 1); cytosolic ARF levels did not decrease significantly, probably reflecting a smaller fraction undergoing translocation (Fig. 1). Increases in membrane ARF levels were also observed at 1–20 min of treatment with 10 nM insulin, but as in the case of Rho, these increases were approximately 50% less than those seen with 300 nM insulin (not shown).

Effects of Wortmannin and LY294002 on Rho Translocation—Because wortmannin inhibits insulin effects on PLD (1), we questioned whether Rho translocation was also sensitive to PI 3-kinase inhibitors. As shown in Fig. 2, both wortmannin and LY294002 inhibited insulin-induced increases in Rho translocation but did not alter the subcellular distribution of Rho in control cells.

Effects of 2-DOG Uptake—Because of the possibility that GTP loading and/or polyphosphoinositides, e.g. as resulting from PI 3-kinase activation, may be important for Rho/ARF translocation. Accordingly, both Rho and ARF (not Cdc42Hs or PI 3-kinase) translocated from the cytosol to total, plasma and microsomal membrane fractions when GTPγS was added to post-nuclear homogenates; PIP$_2$, on the other hand, stimulated Rho, but not ARF, translocation to plasma and microsomal membranes (Fig. 4 and Table I). The translocation of Rho and/or ARF to microsomal as well as plasma membranes in response to GTPγS and/or PIP$_2$ suggested that insulin treatment in intact cells must also activate a process that targets Rho and ARF to the plasma membrane.

Effects of C3 Transference on 2-DOG Uptake—Because of the apparent requirement for PI 3-kinase in the activation of Rho translocation and glucose transport by insulin, we questioned whether Rho may be required for insulin stimulation of glu-
normal activation of IRS-1-dependent PI 3-kinase by insulin
glucose and fatty acids into all major classes of lipids and
metabolically intact, as evidenced by incorporation of labeled
adipocytes were morphologically (as per light microscopy) and
by others (19). It may also be noted that C3 transferase-treated
overnight treatment with C3 resulted in a marked diminution
effects on 2-DOG uptake (Fig. 5)
treated cells more than 3 h (Fig. 5B); and (c) electroporation/overnight treatment with C3 resulted in a marked diminution in immunoreactive Rho (Fig. 5A, inset), similar to that reported by others (19). It may also be noted that C3 transferase-treated adipocytes were morphologically (as per light microscopy) and metabolically intact, as evidenced by incorporation of labeled glucose and fatty acids into all major classes of lipids and normal activation of IRS-1-dependent PI 3-kinase by insulin (not shown).

PLD Activation—In conjunction with Rho and ARF translocation, GTPyS stimulated PLD activity in post-nuclear homogenates (Fig. 4). In intact adipocytes, C3 transferase pretreatment largely inhibited insulin-induced activation of PLD (Fig. 4).

DISCUSSION

The present findings suggested that Rho and ARF may participate in the activation of PC-PLD by insulin in rat adipocytes. Both Rho and ARF translocated to plasma membranes sufficiently rapidly to contribute to the rapid activation of plasma membrane PC-PLD by insulin (see Ref. 1), and both G-proteins have been reported to activate PC-PLD (10–15). In addition, GTPyS have been reported to activate PC-PLD (10–15). In addition, GTPyS stimulated Rho and ARF translocation to the plasma membrane, as well as PLD activity, in adipocyte homogenates, and insulin increased GTP loading of Rho in intact cells. Moreover, insulin effects on PLD were inhibited by C3 transferase, which markedly depleted Rho.

The present findings also suggested that PI 3-kinase activation, perhaps via polyphosphoinositides, may contribute to Rho translocation. Accordingly, both wortmannin and LY294002 inhibited the translocation of Rho in intact adipocytes, and PI3P stimulated Rho translocation in vitro. In addition, polyphosphoinositides may be required for PC-PLD activation (10–13), and wortmannin inhibits PC-PLD activation in both insulin-treated rat adipocytes (1) and f-Met-Leu-Phe-stimulated human neutrophils (20). It is therefore possible that insulin-induced activation of PI 3-kinase leads to production of polyphosphoinositides in the plasma membrane, followed by translocation of Rho, GTP loading, and PLD activation. Along these lines, PI3P increases GDP dissociation from ARF (11) and Cdc42Hs and Rho (see Ref. 22); however, this dissociation does not necessarily result in GTP loading (22), and, as shown presently, GTP loading of Rho appeared to be due to a factor distinct from PI 3-kinase.

Recently, there has been keen interest in mechanisms whereby small G-proteins activate PC-PLD on the one hand and, on the other hand, regulate vesicle formation and traffick-
Samples as the percentage of one or several controls from the same experiment analyzed on the same immunoblot.

HPRl prior to measurement of 2-DOG uptake in the presence or the absence of 10 nM insulin. In C, all cells were electroporated in the presence of or the absence of 5 μg/ml C3 transferase and then incubated for 3 h prior to measurement of 2-DOG uptake in the presence or the absence of 10 nM insulin. C3 transferase was used with or without electroprotein (Ep), and cells were incubated for 20 h prior to measurement of 2-DOG uptake in the presence or the absence of 10 nM insulin.

and in a variety of cytoskeletal events. Of Rho family members (Rho, Rac, and Cdc42Hs subtypes), Rho A is important in PC-PLD activation (10-14) and formation of actin stress fibers and focal adhesions (23). Rho has also been suggested to operate upstream of PI 3-kinase in human platelets (24, 25), although in insulin-stimulated adipocytes, PI-3-kinase appears to be primarily activated by IRS-1 or other phosphotyrosine-containing proteins (6). Rac1 is important in membrane ruffling (23) and seems to operate downstream of PI 3-kinase in insulin-induced membrane ruffling (26). Rac1 and Cdc42Hs (but not Rho A) activate a 62–65-kDa protein kinase that regulates stress-activated protein kinase/c-Jun NH2-terminal kinase (27-30). GTPγS-containing forms of Cdc42Hs and Rac1 also bind to and activate PI 3-kinase, and although this suggested that PI 3-kinase may be a downstream effector, it was surmised from other evidence that PI 3-kinase operates upstream of Cdc42Hs and Rac1 (31). Of further interest, PI 3-kinase and these small G-proteins in the activation of PI 3-kinase (9), may activate PKN (PRK1), which is activated by polyphosphoinositides. Thus, insulin-induced increases in polyphosphoinositides (7-9), which occur through the activation of PI 3-kinase (9), may activate PKN (PRK1), both directly via polyphosphoinositides and indirectly through Rho. The co-activation of Rho and PKN (PRK1) by polyphosphoinositides may facilitate their co-localization and may also co-ordinate the activation of PKN (PRK1) with other PKCs that are activated by PI 3-kinase through its lipid products or PC-PLD. With respect to PKC, it should be noted that we have not observed significant activation of PC-PLD or translocation of Rho during phorbol ester treatment in rat adipocytes.

Finally, it was of interest to find that insulin-stimulated glucose transport was inhibited in cells depleted of Rho by C3 transferase treatment. This apparent requirement for Rho, coupled with the fact that Rho is translocated and activated by insulin, suggests that Rho may have a role in insulin stimulation of glucose transport. Clearly, more studies are needed to test this possibility and further define the role of Rho.

In summary, insulin provoked rapid increases in Rho and ARF translocation to the plasma membrane and GTP loading of Rho in rat adipocytes. In addition, wortmannin and LY294002 inhibited insulin effects on Rho translocation in intact adipocytes, but wortmannin did not inhibit GTP loading of Rho. Of further note, PIP2 and GTPγS stimulated Rho translocation in adipocyte homogenates, and C3 transferase inhibited PLD activation in intact adipocytes. Collectively, these findings suggest that insulin translocates Rho by a PI 3-kinase-dependent mechanism but stimulates GTP loading of Rho by a PI 3-kinase-independent mechanism, and both Rho and ARF may participate in the activation of PLD. Further studies will be required to define: (a) the precise mechanisms for activation and translocation of Rho and ARF and (b) the interrelated roles of PI 3-kinase and these small G-proteins in the activation of PC-PLD, various lipid-regulated protein kinases, vesicle trafficking, cytoskeletal events, and other cellular processes.

REFERENCES

1. Standart, M. L., Avignon, A., Yamada, K., Bandyopadhyay, G., and Farese, R. V. (1996) Biochem. J. 313, 1039–1046
2. Baldini, P. M., Zannetti, A., Donchenko, V., Dini, L., and Luly, P. (1992) Biochem. Biophys. Acta 1170, 208–214
3. Donchenko, V., Zannetti, A., and Baldini, P. M. (1994) Biochem. Biophys. Acta.
Rho and PI 3-kinase in Insulin Action

4. Standaert, M. L., Musunuru, K., Yamada, K., Cooper, D. R., and Farese, R. V. (1994) Cell Signalling 6, 707–716

5. Hoffman, J. M., Standaert, M. L., Nair, G. P., and Farese, R. V. (1991) Biochemistry 30, 3315–3322

6. White, M. F., and Kahn, C. R. (1994) J. Biol. Chem. 269, 1–4

7. Farese, R. V., Larson, R. E., and Sabir, M. A. (1982) J. Biol. Chem. 257, 4042–4045

8. Farese, R. V., Davis, J. S., Barnes, D. E., Standaert, M. L., Babischkin, J. S., Hock, R., Rosic, N. K., and Pallet, R. J. (1985) Biochem. J. 231, 269–278

9. Ruderman, N. B., Kapeller, R., White, M. F., and Cantley, L. C. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1411–1415

10. Brown, H. A., Gutowski, S., Kahan, R. A., and Sternweis, P. C. (1995) J. Biol. Chem. 270, 14935–14943

11. Terui, T., Kahn, R. A., and Randazzo, P. A. (1994) J. Biol. Chem. 269, 28130–28135

12. Pertile, P., Liscovitch, M., Chalifa-V., and Cantley, L. C. (1995) J. Biol. Chem. 270, 5130–5135

13. Siddiqi, A. R., Smith, J. L., Ross, A. H., Qiu, R. G., Symons, M., and Exton, J. H. (1995) J. Biol. Chem. 270, 8466–8473

14. Cockcroft, S., Thomas G. M., Fensome, A., Geny, B., Cunningham, E., Gout, I., Hiles, I., Totty, N. F., Truong, O., and Hsuan, J. J. (1994) Science 263, 523–526

15. Bowman, E. P., Uhlinger, D. J., and Lambeth, J. D. (1995) J. Biol. Chem. 268, 21509–21512

16. Cooper, D. R., Watson, J. E., Hernandez, H., Yu, B., Standaert, M. L., Ways, D. K., Arnold, T., Ishizuka, T., and Farese, R. V. (1992) Biochem. Biophys. Res. Commun. 188, 142–148

17. Aktories, K., and Just, I. (1995) Methods Enzymol. 256, 184–195

18. Kelly, K. L., Ruderman, N. B., and Chen, K. S. (1992) J. Biol. Chem. 267, 3423–3428

19. Malcolm, K. C., Elliott, C. M., and Exton, J. H. (1996) J. Biol. Chem. 271, 13135–13139

20. Reinhold, S. L., Prescott, S. M., Zimmerman, G. A., and McIntyre, T. M. (1990) FASEB J. 4, 208–214

21. Amano, M., Mukai, H., Ono, Y., Chihara, K., Matsui, T., Hamajima, Y., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996) Science 271, 648–650

22. Zheng, Y., Glaven, J. A., Wu, W. J., and Cerione, R. A. (1996) J. Biol. Chem. 271, 23815–23819

23. Ridley, A. J., Paterson, H. F., Johnston, C. L., Dickmann, D., Hall, A. (1992) Cell 70, 401–410

24. Zhang, J., King, W. G., Dillon, S., Hall, A., Feig, L., and Rittenhouse, S. E. (1993) J. Biol. Chem. 268, 22251–22254

25. Kunagai, N., Morii, N., Fujisawa, K., Nemoto, Y., and Narumiya, S. (1993) J. Biol. Chem. 268, 24535–24538

26. Katani, K., Hara, K., Katani, K., Yonezawa, K., and Kasuga, M. (1995) Biochem. Biophys. Res. Commun. 208, 985–990

27. Bagrodia, S., Taylor, S. J., Creasy, C. L., Chernoff, J., and Cerione, R. A. (1995) J. Biol. Chem. 270, 22731–22737

28. Zhang, S., Han, J., Sells, M. A., Chernoff, J., Knaus, U. G., Ulevitch, R. J., and Bokoch, G. M. (1995) J. Biol. Chem. 270, 23934–23936

29. Teo, M., Manser, E., and Lim, L. (1995) J. Biol. Chem. 270, 26690–26697

30. Polverino, A., Frost, J., Yang, P., Hatchison, M., Neiman, A. M., Cobb, M. H., and Marcus, S. (1995) J. Biol. Chem. 270, 26067–26070

31. Tolias, K. F., Cantley, L. C., and Carpenter, C. L. (1995) J. Biol. Chem. 270, 17656–17659

32. Liscovitch, M., and Cantely, L. C. (1995) Cell 81, 659–662

33. Nakaniishi, H., Brewer, K. A., and Exton, J. H. (1993) J. Biol. Chem. 268, 13–16

34. Palmer, R. H., Dekker, L. V., Woscholski, R., LeGood, J. A., Gigg, R., and Parker, P. J. (1995) J. Biol. Chem. 270, 22412–22416

35. Houle, M. G., Kahn, R. A., Naccache, P. H., and Bourgoin, S. (1995) J. Biol. Chem. 270, 22795–22800

36. Laflamme, C., Campbell, J. J., and Butcher, E. C. (1996) Science 271, 981–983

37. Watanabe, G., Saito, Y., Madaule, P., Ishizaka, T., Fujisawa, K., Morii, N., Mukai, H., Ono, Y., Kakizuka, A., and Narumiya, S. (1996) Science 271, 645–648