In this study, we investigated the influence of the protein kinase C (PKC)-dependent system upon the ability of insulin to stimulate p21rasGTP loading in 3T3-L1 adipocytes. Activation of PKC by 12-0-tetradecanoylphorbol-13-acetate (TPA) did not affect the basal amount of p21rasGTP but significantly reduced insulin-induced increases in p21rasGTP. This reduction was due to inhibition of the insulin's ability to stimulate guanine nucleotide exchange activity of Sos in cells incubated with 100 nM TPA for either 30 min or 3 h. TPA had no effect on basal activity of Sos.

Depletion of PKC by an 18-h incubation with TPA or inhibition by bisindolylmaleimide resulted in profound inhibition of the insulin-induced p21rasGTP loading. In contrast to PKC activation, removal of PKC did not influence Sos activity but resulted in a 2-fold stimulation of GTPase activating protein (GAP). This effect of PKC depletion is unique to 3T3-L1 adipocytes and was not observed in either 3T3-L1 fibroblasts or Rat-1 fibroblasts. Thus, it appears that in 3T3-L1 adipocytes, PKC has a constitutive inhibitory effect on GAP that permits insulin to activate Sos and p21ras. Removal of this inhibitory influence activates GAP and reduces insulin-stimulated p21ras-GTP loading.

Complexity of the insulin signaling network is accentuated by the interaction of this network with the signaling input generated by other second messenger systems. Reciprocal influence of the insulin and PKC-dependent systems upon cellular responses to insulin has been a subject of numerous investigations (1–2). Activation of the PKC-dependent system has been previously shown to mimic some insulin effects in the absence of insulin (3–5) and at the same time significantly inhibit insulin-stimulated cellular responses (6–8). The mechanism of PKC interference with insulin action may involve serine phosphorylation of the C-terminal domain of the insulin receptor with a subsequent reduction of its tyrosine kinase activity (9–12). The latter is accompanied by a decrease in the magnitude of insulin signaling, thus interfering with insulin action.

Many aspects of insulin action involve activation of p21ras, which plays a pivotal role in propagating insulin signaling downstream (13–15). Insulin stimulates the guanine nucleotide exchange activity of Sos that facilitates an exchange of GDP for GTP on p21ras proteins, promoting p21ras-GTP loading (16–17), a process that activates p21ras. GTPase activating protein (GAP) is then hydrolyzed and reverts to GDP by p21ras and itself and GTPase activating proteins (GAP), thus returning p21ras to its inactive conformation (p21ras,GDP) (18–19). Since the activation of PKC has been shown to influence activities of several signaling intermediates (20–22), we examined potential interactions between PKC and insulin as they might relate to insulin ability to stimulate Sos and p21ras in the insulin-responsive 3T3-L1 adipocytes.

**Experimental Procedures**

Materials—Cell culture media and supplies were from Life Technologies, Inc. and Gemini Bioproducts (Calabasas, CA); radiotopes were from DuPont NEN. All standard chemicals and 12-0-tetradecanoylphorbol-13-acetate (TPA) were from Sigma. Insulin was kindly provided by Lilly, and Ha-Ras was a gift from Dr. A. Wolfman, Cleveland Clinic Foundation (Cleveland, OH). Anti-Ras antibody Y13–259 was from Oncogene Science (Cambridge, MA) and bisindolylmaleimide was purchased from Calbiochem.

Cell Culture—Rat-1 fibroblasts transfected with wild type human insulin receptors (HIRc with approximately 3 × 10⁵ insulin receptors/cell) were grown to confluence in Dulbecco’s modified Eagle’s medium/Ham’s F12 with 10% fetal calf serum (FCS), 50 μg/ml gentamicin, and 50 μM methotrexate. 3T3-L1 fibroblasts were grown to confluence in fibroblast growth media (Dulbecco’s modified Eagle’s medium, 25 μM glucose with 10% FCS, 50 μg/ml gentamicin, 0.5 mM glutamine). Differentiation of 3T3-L1 fibroblasts into adipocytes was induced via the following protocol. When fibroblasts were 80% confluent, they were reseeded into fibroblast growth media. Two days later, cells were fed differentiation media (Dulbecco’s modified Eagle’s medium, 25 μM glucose with 10% FCS, 50 μg/ml gentamicin, 0.5 mM glutamine plus differentiation mix: 2.5 μl of 10 × PBS, 0.055 g of isobutylmethylxanthene, 20 μl of deionized water, 250 μl of 49 μM dexamethasone, and 2.5 μg of insulin). On day 4, cells were fed adipocyte growth media (Dulbecco’s modified Eagle’s medium containing 25 μM glucose with 10% FCS, 50 μg/ml gentamicin, 0.5 mM glutamine) and 1 μg/ml insulin. Cells were refed adipocyte growth media without insulin every 2 days and used on days 10–12.

Insulin-mediated p21ras-GTP Formation—Confluent HIRc, 3T3-L1 fibroblasts, and adipocytes were serum- and phosphate-starved for 24 h and labeled with [125I]Orthophosphate (0.25 μCi/ml) overnight. Cells were incubated with TPA (100 nM) for 30 min, 3 h, or 18 h, followed by incubation with 100 nM insulin for 10 min. The increment in percent p21ras was determined as described previously (16) with minor modifications. Briefly, pre-cleared lysates were immunoprecipitated with anti-Ras antibody Y13–259, and the nucleotides were separated by thin layer chromatography. GTP and GDP were visualized by autoradiography and, using acid molybdate reagent, were cut and quantified by liquid scintillation counting.

GAP Activity—Determinations of GAP activity were performed as described previously with minor modifications (16). In brief, cell lysates were prepared from 3T3-L1 adipocytes either untreated or pretreated with 100 nM TPA for 30 min, 3 h, or 18 h and then incubated with or without 100 nM insulin for 10 min. To measure GAP activity, c-Ha-Ras (367 ng) was incubated with 6.7 μM (γ-32P)GTP (200 μCi/ml) and 745

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1 The abbreviations used are: PKC, protein kinase C; TPA, 12-0-tetradecanoylphorbol-13-acetate; HIRc, human insulin receptor; FCS, fetal calf serum; DTT, dithiothreitol; GAP, GTPase activating protein.
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Fig. 1. Effect of PKC activation on the insulin-stimulated p21
toGTP loading. 3T3-L1 adipocytes were incubated with 100 nM TPA for 30 min or 3 h before being challenged with insulin (100 nM for 10 min). Percent p21
toGTP was determined as described under “Experimental Procedures.” Results represent the mean ± S.E. of 14 independent experiments. **p < 0.01 versus basal levels; ***p < 0.05 versus insulin-stimulated levels in the absence of TPA.

Fig. 2. Effect of PKC activation on guanine nucleotide exchange activity of Sos in 3T3-L1 adipocytes. The cells were pretreated with 100 nM TPA for 30 min or 3 h before being challenged with insulin (100 nM). TPA did not affect basal (open bars) activity of Sos but significantly reduced insulin-stimulated (hatched bars) Sos activity. Results are expressed as mean ± S.E. of three experiments, each performed in duplicate.

Fig. 3. Effect of PKC depletion on insulin-stimulated p21
toGTP loading in 3T3-L1 adipocytes, 3T3-L1 fibroblasts, and HIRc cells. The cells were incubated with 100 nM TPA for 18 h before being challenged with insulin (100 nM). Results represent the mean ± S.E. of 14 experiments in 3T3-L1 adipocytes and 4 experiments in HIRc and 3T3-L1 fibroblasts, respectively. **p < 0.01 versus basal levels, ***p < 0.01 versus insulin-stimulated levels in the absence of TPA.

Fig. 4. Effect of PKC depletion on GAP activity in 3T3-L1 adipocytes. The cells were pretreated with 100 nM TPA for 18 h and then challenged with insulin (100 nM for 10 min). Lysates from control (open bars) and insulin-treated (hatched bars) cells were then used in the GAP assay as described under “Experimental Procedures.” Results are expressed as mean ± S.E. of three independent experiments, each performed in duplicate. Exposure of cells to TPA for 18 h significantly (p < 0.01) increased GAP activity in the absence and in the presence of insulin.

μg/ml chain-A insulin in low Mg2+ binding buffer (25 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 400 μg/ml bovine serum albumin) at 30°C for 15 min. The GTP loading reaction was stopped with addition of excess free Mg2+ (10 mM). Ras-GTP complex (250 μl, 50 ng) was added to 50 μl of lysate (50 μg of protein) or lysis buffer and incubated at 23°C. At intervals (0 and 15 min), aliquots were removed and filtered through 0.45-μm Millipore nitrocellulose filters and washed three times with 1 ml of ice-cold buffer. The GAP activity of cell lysates was determined by measuring the loss of protein-bound radioactivity by liquid scintillation counting. The results are expressed as percent hydrolyzed GTP. A value of 100% remaining represents 20,051 ± 1,069 cpm bound. Background counts/min were less than 2% of total bound [32P]GTP.

Guanine Nucleotide Exchange Activity of Sos—Determination of nucleotide exchange activity of Sos was performed as described previously (16). In brief, cell lysates were prepared from 3T3-L1 adipocytes either untreated or pretreated with 100 nM TPA for 30 min, 3 h, or 18 h and then incubated with or without 100 nM insulin for 10 min. To measure Sos activity, c-Ha-Ras (376 ng) was incubated with 3 μM [3H]GDP (32 Ci/mmol) in 50 μl of binding buffer (25 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 400 μg/ml bovine serum albumin) for 15 min at 30°C. The complex was stabilized by addition of 750 μl of incubation buffer (25 mM Tris, pH 7.5, 100 mM NaCl, 10.7 mM MgCl2, 1 mM DTT, 400 μg/ml bovine serum albumin). Unlabeled cultured cells were stimulated with 100 nM insulin for 10 min at 37°C, and lysates were prepared as before. Ras-GDP complex (250 μl, 60 ng) was added to 50 μl of lysate (50 μg of protein) or lysis buffer and incubated at 23°C. At intervals, 50-μl aliquots were removed and filtered through 0.45-μm Millipore nitrocellulose filters which were washed twice with 1 ml of ice-cold buffer containing 25 mM Tris, pH 7.5, 100 mM NaCl, 10 mM MgCl2, 1 mM DTT. The radioactivity of [3H]GDP bound to p21
was quantitated by scintillation counting. A value of 0% release represents 15,365 ± 658 cpm bound. Background counts/min were less than 1% of the total bound [3H]GDP. [3H]GDP binding to p21 was confirmed by immunoprecipitation.

PKC Activity and Immunoblotting—Protein kinase C activity (α, β, and γ isoforms) was determined using the PKC assay system supplied by Life Technologies, Inc. 3T3-L1 adipocytes were treated with or without TPA (100 nM) for 30 min or 18 h. The cells were then harvested in extraction buffer and homogenized with 15 strokes in an ice-cold Dounce homogenizer. Cell debris and nuclei were removed by centrifugation at 750 × g for 3 min. The supernatant was fractionated into cytosolic and plasma membrane fractions by centrifugation at 100,000 × g for 30 min in a Beckman TL-100 ultracentrifuge. Each fraction was partially purified by passing over Whatman DE52 cellulose columns. The eluants were assayed for PKC activity using Ac-myelin basic protein peptide as a substrate. Cytosolic and plasma membrane fractions were also immunoprecipitated using anti-Pan Protein Kinase C antibody (Upstate Biotechnology Inc., Lake Placid, NY) and utilizing Protein G-Plus/Protein A-agarose to capture the immunocomplex. The agarose beads were collected by microcentrifugation, resuspended in 2×...
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RESULTS AND DISCUSSION

PKC Activation—In our initial experiments, we examined the influence of PKC activation upon basal and insulin-stimulated p21\textsuperscript{ras}-GTP loading. In agreement with previous observations in fibroblasts (23), activation of PKC in 3T3-L1 adipocytes with 100 nM TPA for 30 min or 3 h did not influence the basal level of p21\textsuperscript{ras}-GTP. However, when the 3T3-L1 adipocytes pretreated with TPA were challenged with insulin, the amount of p21\textsuperscript{ras}-GTP was significantly reduced as compared with control insulin-treated cells (Fig. 1). This inhibition of insulin-stimulated p21\textsuperscript{ras}-GTP loading is consistent with the overall diminution of the magnitude of insulin signaling in cells with activated PKC (9–10, 24–27). We then examined the effect of PKC activation on Sos and GAP, the two upstream intermediates that influence the balance between p21\textsuperscript{ras}-GTP and p21\textsuperscript{ras}-GDP (16–19). Basal guanine nucleotide exchange activity of Sos was not influenced by TPA, but insulin-stimulated Sos activity was significantly inhibited by pretreatment of 3T3-L1 adipocytes with TPA for 30 min and 3 h (Fig. 2). Neither insulin alone nor pretreatment of adipocytes with TPA with or without subsequent stimulation with insulin had any effect on GAP activity in these cells (not shown). Thus, these results are consistent with the notion that PKC activation interferes with insulin-generated signaling (9–12, 24–27), reducing the activity of Sos, and lowering the magnitude of the insulin-stimulated p21\textsuperscript{ras}-GTP loading.

PKC Depletion and Inhibition—If PKC activation interferes with the insulin-generated signaling, then PKC depletion or inhibition would be expected to result in either normal or exaggerated insulin action. However, the next series of experiments with PKC depletion and inhibition yielded unexpected results. Whereas depletion of PKC with an 18-h pretreatment of adipocytes with TPA did not significantly influence the unstimulated (basal) levels of p21\textsuperscript{ras}-GTP, exposure of these cells to insulin resulted in profound inhibition of p21\textsuperscript{ras}-GTP loading (Fig. 3). This effect was specific for 3T3-L1 adipocytes and was not present in either Rat-1 fibroblasts transfected with H1Rc or 3T3-L1 fibroblasts (Fig. 3). We have previously observed similar tissue-specific regulation of insulin-induced p21\textsuperscript{ras}-GTP loading in response to inhibition of the phosphatidylinositol 3-kinase activity (28). Inhibition of phosphatidylinositol 3-kinase with wortmannin or LY294002 or immunodepletion of phosphatidylinositol 3-kinase resulted in significant activation of GAP and reduction in p21\textsuperscript{ras}-GTP in adipocytes but not in fibroblasts or Chinese hamster ovary cells. We wish to speculate that metabolically active adipocytes and mitogenically active fibroblasts display distinct regulations of their signal transduction machinery in response to insulin.

Two opposing forces regulate the balance between p21\textsuperscript{ras}-GDP and p21\textsuperscript{ras}-GTP (19). Guanine nucleotide exchange activity of Sos is responsible for GTP loading as it stimulates dissociation of GDP from p21\textsuperscript{ras}-GTP (16, 29). GAP facilitates conversion of p21\textsuperscript{ras} into its inactive form by hydrolyzing GTP into GDP (18–19). Thus, either inhibition of Sos activity or enhanced GAP activity in the PKC-depleted cells may be responsible for low p21\textsuperscript{ras}-GTP loading in response to insulin in these cells. We examined both possibilities. PKC depletion had no effect on guanine nucleotide exchange activity of Sos (not shown) but resulted in a significant increase in GAP activity (Fig. 4). Not only was basal GAP activity significantly enhanced by PKC depletion, but we also observed that insulin began exerting an additional effect on GAP (albeit nonsignificant), an effect never seen in control cells. Inhibition of PKC activity with its specific inhibitor, bisindolylmaleimide, also resulted in a dose-dependent increase in the basal GAP activity in these cells (Fig. 5). Addition of insulin (which does not influence GAP activity in control cells) had a consistent, albeit nonsignificant additional stimulatory effect on GAP.

**TABLE I**

|              | Plasma membrane | Cytosol |
|--------------|-----------------|---------|
|              | (n=3)           | (n=3)   |
| TPA 0 nM     | 2.9 ± 0.3       | 10.6 ± 1.5 |
| TPA 1 nM     | 13.3 ± 0.7      | 5.6 ± 0.4  |
| TPA 10 nM    | 0.9 ± 0.2       | 2.2 ± 0.4  |
| TPA 100 nM   |                 |         |

Laemmli sample buffer, boiled for 5 min, and resolved on SDS-polyacrylamide gel electrophoresis. Subsequent immunoblot analysis was performed using the enhanced chemiluminescence Western blotting protocol of Amersham Life Sciences, Inc.

Statistics—All statistics were analyzed by Student's t test with a p value < 0.05 considered significant. Results are expressed as mean ± S.E. in comparison to control groups.

**Fig. 5.** Effect of PKC inhibition with bisindolylmaleimide on GAP activity in 3T3-L1 adipocytes. The cells were treated with increasing concentrations of bisindolylmaleimide and then challenged with insulin (100 nM). Lysates from control (open bars) and insulin-treated (hatched bars) cells were used in the GAP assay as described under experimental procedures. Results are expressed as mean ± S.E. of three independent experiments, each performed in duplicate. Basal GAP activity was significantly increased by bisindolylmaleimide in a dose-dependent manner.

**Fig. 6.** Activation and depletion of PKC by TPA in 3T3-L1 adipocytes. Adipocytes were treated with 100 nM TPA for either 30 min or 18 h. After cell debris and nuclei were removed by centrifugation; the supernatants were fractionated into cytosolic and plasma membrane (PM) fractions as described under experimental procedures. The fractions were then immunoprecipitated using the anti-pPKC antibody resolved on SDS-polyacrylamide gel electrophoresis and immunoblotted with the same antibody. Lane 1, control cells; lane 2, 30-min exposure; and lane 3, 18-h exposure to TPA.
Two important control experiments have been performed to support our conclusions that these findings are related to the depletion of PKC. First, we confirmed that TPA increased PKC activity and translocated this activity to the plasma membrane at 30 min and depleted cellular PKC activity by 70–80% at 18 h of incubation (Table I). The depletion of PKC from both membranous and cytosolic fractions was further substantiated by immunoblotting (Fig. 6). Second, the exposure of the adipocytes to TPA for either 30 min, 3 h, or 18 h did not alter the amount of GAP protein in these cells, as determined by Western blotting (Fig. 6). Thus, it appears that in 3T3-L1 adipocytes, PKC has a constitutive inhibitory effect on GAP that is not potentiated by either stimulation with TPA or by insulin. Previous studies have indicated that PKC, arachidonic acid with its lipoxygenase inhibitory effect on GAP that is not potentiated by PKC influence on GAP remains unknown, removal of this inhibitory influence activates GAP and reduces insulin-stimulated p21ras-GTP loading.

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REFERENCES
1. Farese, R. V., Barnes, D. E., Davis, J. S., Standaert, M. L., and Pollet, R. J. (1987) J. Biol. Chem. 259, 7094–7100
2. Farese, R. V. (1988) Am. J. Med. 85, Suppl 5A, 36–43
3. Cherqui, G., Caron, M., Wicke, D., Lascols, O., Capeau, J., and Picard, J. (1986) Endocrinology 118, 1759–1769
4. Messina, J. L., Hamlin, J., and Larner, J. (1987) J. Biol. Chem. 262, 5699–5704
5. Muñlbacher, C., Karnieli, E., Schaff, P., Obermaier, B., Mushack, J., Rattenhuber, E., and Häring, H. U. (1988) Biochem. J. 249, 865–870
6. van de Werve, G., Proteglio, J., and Jeanrenaud, B. (1985) Biochem. J. 225, 523–527
7. Kirsch, D., Obermaier, B., and Häring, H. U. (1989) Biochem. Biophys. Res. Commun. 128, 824–832
8. Standaert, M. L., Mojsilovic, L., Farese, R. V., and Pollet, R. J. (1987) Endocrinology 121, 941–947
9. Bollag, G. E., Roth, R. A., Beaudon, J., Mochly-Rosen, D., and Koshland, D. E., Jr. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 5822–5824
10. Takayama, S., White, M. F., and Kahn, C. R. (1988) J. Biol. Chem. 263, 3440–3447
11. Coghlan, M. P., Pillay, T. S., Tavare, J. M., and Siddique, K. (1994) Biochem. J. 303, 893–899
12. Lewis, R. E., Volle, D. J., and Sanderson, S. D. (1994) J. Biol. Chem. 269, 26259–26266
13. Jhun, B. H., Meinkoth, J. L., Leitner, J. W., Draznin, B., and Olefsky, J. M. (1994) J. Biol. Chem. 269, 5699–5704
14. Medema, R. H., DeVries-Smits, A. M. M., van der Zon, G. C. M., Maassen, J. A., and Bos, J. L. (1993) Mol. Cell. Biol. 13, 155–162
15. Skolnik, E. Y., Batzer, A., Lee, C. H., Lowenstein, E., Mohammadi, M., Margolis, B., and Schlessinger, J. (1993) Science 260, 1953–1955
16. Draznin, B., Chang, L., Leitner, J. W., Takata, Y., and Olefsky, J. M. (1993) J. Biol. Chem. 268, 19998–20001
17. Langlois, J., Leitner, J. W., Medh, J., Sasaoka, T., Olefsky, J. M., and Draznin, B. (1995) Endocrine 3, 475–479
18. Bourne, H. R., Sanders, D. A., and McCormick, F. (1990) Nature 348, 125–132
19. Polakis, P., and McCormick, F. (1993) J. Biol. Chem. 268, 9157–9160
20. Chao, T.-S. O., Foster, D. A., Rapp, U. R., and Rosner, M. R. (1994) J. Biol. Chem. 269, 7337–7341
21. Hii, C. S. T., Ferrante, A., Edwards, Y. S., Huang, Z. H., Hartfield, P. J., Rathjen, D. A., Poulo, L., and Murray, A. W. (1995) J. Biol. Chem. 270, 4201–4204
22. Danielsen, A. G., Liu, F., Hosomi, Y., Shi, K., and Roth, R. A. (1995) J. Biol. Chem. 270, 21600–21605
23. Medema, R. H., Burgering, B. M. Th., and Bos, J. L. (1991) J. Biol. Chem. 266, 21186–21189
24. Berti, L., Mostha, L., Kroder, G., Keller, M., Tippmer, S., Mushack, J., Seefer, E., Seedorf, G., and Häring, H. (1994) J. Biol. Chem. 269, 3381–3386
25. Lewis, R. E., Cas, L., Perrequa, D., and Czech, M. P. (1990) Biochemistry 29, 1807–1813
26. Chin, J. E., Dickens, M., Tavare, J. M., and Roth, R. A. (1993) J. Biol. Chem. 268, 6338–6347
27. Lin, F., and Roth, R. A. (1994) FEBS Lett. 352, 389–392
28. DePaolo, R., Riehs, J. E. B., Carter, K., Bhuripanyo, P., Leitner, J. W., and Draznin, B. (1996) Mol. Cell. Biol. 16, 1450–1457
29. Satoh, T., Nakafuku, M., and Kozori, Y. (1992) J. Biol. Chem. 267, 24149–24152
30. Downward, J., Graves, J. D., Warne, P. H., Rayter, S., and Cantrell, D. A. (1994) Nature 364, 719–723
31. Resnick, R. J., and Tomška, L. (1994) J. Biol. Chem. 269, 32336–32341
32. Yu, C.-L., Tsai, M.-H., and Stacey, D. W. (1990) J. Biol. Chem. 265, 6683–6689
33. Golubic, M., Tanaka, K., Dobrowolski, S., Wood, D., Tsai, M. H., Marshall, M., Tamanoi, F., and Stacey, D. W. (1991) EMBO J. 10, 2897–2903
34. Tsai, M.-H., Yu, C.-L., Wei, F.-S., and Stacey, D. W. (1989) Science 243, 522–525
Interactions of Protein Kinase C with Insulin Signaling: INFLUENCE ON GAP AND SOS ACTIVITIES
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