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IL-3 and IL-4 Activate Cyclic Nucleotide Phosphodiesterases 3 (PDE3) and 4 (PDE4) by Different Mechanisms in FDCP2 Myeloid Cells

Faiyaz Ahmad,* Guang Gao,* Ling Mei Wang,† Tova Rahn Landstrom,‡ Eva Degerman,‡ Jacalyn H. Pierce,† and Vincent C. Manganiello1*

In FDCP2 myeloid cells, IL-3, IL-4 activated cyclic nucleotide phosphodiesterases PDE3 and PDE4, whereas IL-3, granulocyte-macrophage CSF (GM-CSF), and phorbol ester (PMA) selectively activated PDE4. IL-4 (not IL-3 or GM-CSF) induced tyrosine phosphorylation of insulin-receptor substrate-2 (IRS-2) and its association with phosphatidylinositol 3-kinase (PI3-K). TNF-α, AG-490 (Janus kinase inhibitor), and wortmannin (PI3-K inhibitor) inhibited activation of PDE3 and PDE4 by IL-4. TNF-α also blocked IL-4-induced tyrosine phosphorylation of IRS-2, but not of STAT6. AG-490 and wortmannin, not TNF-α, inhibited activation of PDE4 by IL-3. These results suggested that IL-4-induced activation of PDE3 and PDE4 was downstream of IRS-2/PI3-K, not STAT6, and that inhibition of tyrosine phosphorylation of IRS molecules might be one mechanism whereby TNF-α could selectively regulate activities of cytokines that utilized IRS proteins as signal transducers. RO31-7549 (protein kinase C (PKC) inhibitor) inhibited activation of PDE4 by PMA. IL-4, IL-3, and GM-CSF activated mitogen-activated protein (MAP) kinase and protein kinase B via PI3-K signals; PMA activated only MAP kinase via PKC signals. The MAP kinase (MEK-1) inhibitor PD98059 inhibited IL-4-, IL-3-, and PMA-induced activation of MAP kinase and PDE4, but not IL-4-induced activation of PDE3. In FDCP2 cells transfected with constitutively activated MEK, MAP kinase and PDE4, not PDE3, were activated. Thus, in FDCP2 cells, PDE4 can be activated by overlapping MAP kinase-dependent pathways involving PI3-K (IL-3, IL-4, GM-CSF) or PKC (PMA), but selective activation of PDE3 by IL-4 is MAP kinase independent (but perhaps IRS-2/PI3-K dependent). The Journal of Immunology, 1999, 162: 4864–4875.
is important in IL-4-induced mitogenic signals (11, 17–19). Some IL-4/IRS-2 signals are similar to those generated by insulin and IGF-1 receptors (12, 17). IL-3 also induces tyrosine phosphorylation of cellular proteins, including PI3-K (20, 21) and Src (22), and activates Ras, Raf, MAP kinase, and PI3-K (23) and PKB (24) in transducing mitogenic signals (25). In FDCP2 cells (11), IL-4 and IL-3 regulate different phosphorylation cascades, and thus, presumably, different biologic effects. Little is known of the effects of IL-4 and IL-3 or other cytokines on distinct and overlapping signaling pathways that regulate different PDEs in the same cell.

In adipocytes, insulin-induced activation of PDE3B is important in the antipolysaccharide action of insulin (2). Binding of insulin to its receptor leads to activation of the intrinsic tyrosine kinase activity of the receptor and tyrosine phosphorylation of IRS proteins (26, 27), resulting in activation of PI3-K (26, 27) and initiation of downstream signaling events, including activation of a PDE3B kinase (2, 28). The PI3-K inhibitor wortmannin blocked insulin-induced activation of PDE3B kinase and phosphorylation/activation of PDE3B as well as the antipolysaccharide action of insulin (28). Insulin-induced activation of several serine/threonine protein kinases, including MAP kinases (29, 30), p70S6 kinase (31, 32), and PKB (33–37), known as Ras or Akt, can also be mediated by PI3-K–generated signals. In intact rat adipocytes, insulin-induced activation of PDE3B is independent of MAP kinase and p70S6 kinase, but may involve PKB (37).

In this study, we demonstrate, with the use of TNF-α and various kinase inhibitors, that in FDCP2 cells both PDE3 and PDE4 are activated by IL-4 via IRS-2 and PI3-K–dependent signals. Downstream of PI3-K, however, the signals diverge, resulting in MAP kinase-dependent and -independent pathways for activation of PDE4 and PDE3, respectively. Furthermore, PDE4 is selectively activated by IL-3 and GM-CSF via PI3-K (not IRS-2) and MAP kinase signals, and by PMA via PKC and MAP kinase signals. MAP kinase and PDE4, not PDE3, are activated in FDCP2 cells transfected with constitutively active MEK or in FDCP2 cells transfected with wild-type MEK and treated with IL-3. The MEK inhibitor PD98059 blocks activation of MAP kinase and PDE4 in both. Thus, PDE4 can be regulated by MAP kinase-dependent signals involving PI3-K (IL-4, IL-3, GM-CSF) or PKC (PMA). PKB is activated by IL-4 as well as by IL-3 and GM-CSF, not PMA. TNF-α, which inhibits tyrosine phosphorylation of IRS-2 and its association with PI3-K, blocks effects of IL-4 on PDE3 and PDE4, but not effects of IL-3 or PMA on PDE4. In FDCP2 cells, IL-4-induced activation of IRS-2 and PI3-K may initiate a specific subset of signaling events that target PDE3 in a manner similar to activation of PDE3B by insulin in adipocytes.

Materials and Methods
Recombinant murine IL-4, IL-3, and GM-CSF were purchased from Peprotech (Rocky Hill, NJ); transferrin and selenium from Collaborative Research (Boston, MA); and PKB from Tranduction Laboratories (Lexington, KY); polyclonal anti-rat PI3-K, anti-mouse IRS2, polyclonal anti-erk1–CT, and anti-phosphotyrosine Abs from UBI (Lake Placid, NY); goat polyclonal anti-PKB-CT, goat polyclonal anti-STAT6, anti-JAK 1,2,3, and anti-MEK Abs from Upstate (Lake Placid, NY); goat polyclonal anti-rat PI3-K, anti-mouse IRS2, polyclonal anti- 

Cell culture and incubations
The murine IL-3-dependent hematopoietic cell line, FDCP2, was propagated as described in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% FBS and 5% WEHI-3B (American Type Culture Collection, Manassas, VA)–conditioned medium and 2 mM glutamine (11). Cells were maintained in RPMI 1640 medium supplemented with 15% FBS and 2 mM glutamine for 4 days before conditioned medium was collected and used to supplement growth medium for FDCP2 cells. For most experiments, exponentially growing FDCP2 cells (2–5 × 106 cells/ml) were collected, centrifuged (5 min, 1200 × g), washed twice, and cultured overnight in RPMI 1640/10% FBS without conditioned medium. Immediately before experiments, cells were washed twice; suspended in serum-free RPMI 1640 medium containing transferrin (5 μg/ml), selenium (10 μM), and BSA (1 mg/ml); and incubated (3 ml cells/well, 7–10×106 cells) in six-well Costar plates (11) with cAMP buffer containing PMA, etc., as were added as indicated. Finally, cells were harvested by centrifugation (1200 × g, 5 min), suspended, homogenized (10–15 strokes in a Dounce homogenizer [Kontes Instruments, Vineland, NJ]) in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM MgCl2, 1% Nonidet P-40 detergent, 1 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate, 1 mM Na3VO4, 1 mM PMFS (Sigma, St. Louis, MO), and 20 μg/ml each of aprotinin and leupeptin (Boehringer Mannheim, Indianapolis, IN), and kept for 30 min at 4°C. Protein was measured using the Bradford assay with BSA as standard.

cAMP PDE assay
Portions of lysates (usually 100 μl) were assayed for 10 min at 30°C in a total volume of 0.3 ml containing 50 mM HEPES, pH 7.5, 8.3 mM MgCl2, 0.1 mM EDTA, and 0.1 μM [3H]cAMP (25–35,000 cpm) as substrate (4). After dephosphorylation of 5-AMP to adenosine with Crotalus atrox venom (Sigma), the product was separated from substrate using ion exchange chromatography (QAE-Septadex; Pharmacia, Piscataway, NJ) and quantified by scintillation counting. Lysates were diluted so that hydrolysis of substrate was usually less than 20%. Total PDE, PDE3, and PDE4 activities were measured. PDE3 activity is that inhibited by 0.3 μM cilostamide, a specific PDE3 inhibitor; PDE4 activity, that inhibited by 0.5 μM rolipram, a specific PDE4 inhibitor (38). Inhibitor vehicle (DMSO), added in equal quantities to samples without inhibitor, did not influence PDE activities.

Immunoprecipitation and immunoblotting
For most experiments, portions of FDCP2 lysates (2–3 mg protein) were precleared by incubation with 1 μg normal or preimmune mouse, rat, or rabbit IgG, as appropriate, for 1 h at room temperature before addition of 40 μl of monoclonal protein A (Pierce, Rockford, IL), protein G-Sepharose (Pharmacia Biotech, Piscataway, NJ), or mouse IgG-agarose (Sigma) for 30 min before centrifugation (2800 × g, 4°C, 5 min). Precleared cell lysates were incubated with Abs for 2 h at room temperature, followed by incubation with fresh trisacryl protein A, protein G-Sepharose, or mouse IgG-agarose for 30 min before centrifugation (2800 × g, 4°C, 5 min). Immunoprecipitates were washed five times (10 min each) with lysis buffer containing 0.1% Nonidet P-40, boiled in Laemmli buffer, and subjected to SDS-PAGE (Novex, San Diego, CA) (39). Proteins were transferred to nitrocellulose membranes in Tris-glycine buffer (25 mM Tris-base and 192 mM glycine at pH 8.3), containing 20% methanol. Membranes were incubated in blotting buffer (150 mM NaCl, 0.05% (v/v) Nonidet P-40, 0.01% NaN3, and 10 mM Tris, pH 7.4), containing BSA (50 mg/ml) and OVA (10 mg/ml), for 1 h at room temperature with rocking and then for an additional 2 h with the appropriate Abs (40). Membranes were washed three times (10 min each) in blotting buffer without Abs, followed by incubation with 125I– 

MAP kinase activation and activity assays
Activation of MAP kinase was assessed by measuring 1) the change in electrophoretic mobility of MAP kinase on SDS-PAGE/Western immunoblots using anti-erk1 Ab, 2) tyrosine phosphorylation of MAP kinase detected with anti-phosphotyrosine Ab, or 3) phosphorylation of MBP or...
MBP peptide by MAP kinase immunoprecipitates. 1) Portions of cell lysates (~20 μg) were boiled in Laemmli buffer (Bio-Rad), subjected to SDS-PAGE (10% gel, 120 V for 90 min), and transferred to nitrocellulose (NC) filters; MAP kinase was detected with anti-erbK1a Abs using ECL reagents. 2) Samples of precleared FDCP2 cell lysates (~2 mg protein) were precleared with anti-erbK1-CT Ab (21 μl/10 μg IgG; UBI) for 2 h at room temperature and precipitated with 40 μl protein G-Sepharose; proteins were subjected to SDS-PAGE, transferred to nitrocellulose filters, immunoblotted with polyclonal anti-phosphotyrosine Ab (UBI), followed by 125I-labeled antibody A and quantified by PhosphorImager analysis. 3) Portions of immunoprecipitates, prepared as in 2, were incubated at 30°C for 90 min in 50 mM Tris (pH 7.5), 0.4 mM EDTA, 0.4 mM Na2VO4, 25 mM MgCl2, 150 μM ATP, 10 μCi [γ-32P]ATP (10 Ci/mmol), 5 μg cAMP protein kinase inhibitor (Calbiochem, San Diego, CA), and substrate, either 5 μM MBP (18 kDa) (UBI) or 5 μM MBP peptide (APRTPGRRR (UBI)), in final volume of 50 μl. With MBP as substrate, reactions were terminated by addition of 30 μl of 2× SDS sample buffer. Proteins were separated by SDS-PAGE (0.75 mm, 12% gel), according to the method of Laemmli (39). Gels were then treated as described by Kameshita and Fujisawa (41) with modifications (42). After incubation at room temperature for 1 h, the gels were washed five times with 5% (w/v) TCA and 1% (w/v) sodium pyrophosphate. 32P incorporated into MBP was visualized and quantified by PhosphorImager analysis of dried gels.

When MBP peptide was substrate, reactions were terminated by addition of 10 μl 1% BSA, 1 mM ATP, pH 3, and 5 μl 30% TCA. For assay reaction blanks, the stopping solution was added to immunoprecipitated samples before assay buffer. Samples were centrifuged, and 25 μl of supernatant was applied to phosphocellulose (P81, Whatmann) paper (2 × 2-cm squares), which were washed three times (5 min each) with phosphoric acid (7.5 mg/ml), and once with acetic (5 min) before radioassay of 32P incorporated into substrate.

PKB assays with histone 2B, Crossstide, and K9 peptide substrates

Samples of precleared FDCP2 cell lysates (~2 mg protein) were incubated with anti-PKB-CT Ab (50 μl/10 μg IgG) for 2 h at room temperature and precipitated with 40 μl protein G-Sepharose beads. Immunoprecipitates were washed three times with lysis buffer (containing 0.1% Nonidet P-40), suspended in 20–30 μl of reaction buffer (20 mM HEPES, pH 7.4, 1 mM DTT, 10 mM MnCl2, 10 mM MgCl2, 5 μM ATP, 10 μCi [γ-32P]ATP, 5 μg cAMP protein kinase inhibitor) containing either 13 μg K9 peptide (KKRRRTLTK, 1 μg of Crossstide (GRPRRTSSFAEG), or 2.5 μg of histone-2B (H2B) (Boehringer Mannheim) as substrate (35–37), and incubated for 15 min at 30°C. For assays with K9 or Crossstide peptides, reactions were terminated and phosphocellulose squares were washed and analyzed as described for MAP kinase assays.

PKB activity was proportional to incubation time and amount of protein. K9 peptides, which have also been used to detect p70S6 kinase activity (43), and the Crossstide peptide, which closely resembles the sequence containing the site phosphorylated by PKB in glycygen synthase kinase-3 (44), have been used to determine PKB activity in both immunoprecipitates and partially purified preparations of PKB from rat adipocytes (35–37). In FDCP2 cell lysates, use of either Crossstide or K9 peptide gives very similar results. Treatment of FDCP2 cells with 20 mM rapamycin (p70S6 kinase inhibitor) had no significant effect on IL-4-induced activation of the kinase activity detected with K9 as substrate, suggesting that p70S6 kinase does not contribute to the IL-4-induced K9-kinase activity in PKB immunoprecipitates.

With histone 2B as substrate for PKB, reactions were terminated by addition of Laemmli buffer and boiling. Proteins were separated by SDS-PAGE, the gel was dried, and 32P-labeled histone 2B was detected and quantified by PhosphorImager analysis of dried gels.

Expression of MEK (MAP kinase kinase) in FDCP2 cells

Several plasmid vector constructs (provided by J. S. Gutkind, NIDR, National Institute of Health) were transfected into Escherichia coli, amplified, and purified. pcDNA3 (F/V), which generates high levels of expression, was used as parent control vector for subsequent constructions and transfections into FDCP2 cells. Wild-type (WT) MEK (F/M) (45) was cloned into the pcDNA3 vector as a BamHI-Hind fragment. Constitutively active (CA) MEK (F/M*) was generated by replacing Ser218 and Ser222 by glutamic acid (46). Kinase-inactive MEK (F/M-), which generated Ser218 and Ser222 by alanine (46).

Purified DNA (~20 μg) was introduced into FDCP2 cells by electroporation; transfectants (F/V, F/M, F/M*, and F/M-) were selected by growing cells in 24-well culture plates in medium with G418 (750 mg/L), as previously described (18, 47). Six different colonies of each construct were isolated. FDCP2 cells expressing similar amounts of wild-type (F/M), constitutively active (F/M*), and kinase-inactive (F/M-) MEK were selected by analyzing lysates (30–40 μg protein) from equivalent numbers of cells by SDS-PAGE (Novex), immunoblotting with anti-MEK Abs, and assaying MAP kinase activity.

Results

Activation of PDE3 and PDE4 by IL-4, IL-3, GM-CSF, and PMA in FDCP2 cells

Both PDE3 and PDE4 enzymes exhibit high affinity for cAMP (1–3). PDE3 activity represents cAMP-hydrolyzing activity inhibited by cilostamide, a specific PDE3 inhibitor; PDE4 activity, activity inhibited by rolipram, a specific PDE4 inhibitor (38). As shown in Table I, PDE3 and PDE4 activities account for most of the cAMP hydrolytic activity (with 0.1 mM 3H-cAMP as substrate) in FDCP2 cell lysates. As seen in Fig. 1A, incubation of FDCP2 cells with IL-4 increased both PDE3 and 4 activities (by ~twofold), whereas IL-3, GM-CSF (GM-CSF and IL-3 receptors share a common β subunit and activate similar signaling pathways (13)), and PMA selectively increased PDE4 activity.

To define pathways utilized by cytokines and PMA to activate PDE3 and PDE4, we first evaluated their effects on different candidate signaling components, especially those apparently involved in insulin-induced activation of PDE3B in adipocytes (2), which is dependent on PI3-K and, perhaps, PKB signaling, but independent of MAP kinase (28, 37). To further identify these signaling pathways, we used the proinflammatory cytokine TNF-α and a panel of protein kinase inhibitors, including AG-490 (which inhibits JAKs, but not other tyrosine kinases (48)), PD98059 (a selective MEK-1 protein kinase inhibitors, including AG-490 (which inhibits JAKs, but not other tyrosine kinases (48)), PD98059 (a selective MEK-1

Effects of IL-4, IL-3, GM-CSF, and PMA on tyrosine phosphorylation of IRS-2, and activation of MAP kinase and PKB

As reported previously (11, IL-4, not IL-3, increased tyrosine phosphorylation of IRS-2 (Fig. 1B). GM-CSF and PMA also induced little or no tyrosine phosphorylation of IRS-2. On the other hand, as shown in Fig. 1C, IL-4, IL-3, GM-CSF, and PMA all activated MAP kinase. IL-4, which has been reported to activate
MAP kinase in some (54, 55), but not all, cells (56), increased MAP kinase activity to a lesser extent than IL-3, GM-CSF, and PMA, assessed by phosphorylation of MBP (Fig. 1C) or MBP peptide (not shown). IL-4 also activated PKB to a lesser extent than did IL-3 or GM-CSF, assessed by phosphorylation of histone (Fig. 1D) or Crosstide (not shown). As reported in adipocytes (36, 37) and lymphocytes (57), PMA did not activate PKB in FDCP2 cells (Fig. 1D).

**Time course of effects of IL-4 on PDE activity, tyrosine phosphorylation of IRS-2, translocation of PI3-K, and activation of MAP kinase and PKB in FDCP2 cells**

As shown in Fig. 2A, incubation with IL-4 resulted in activation of total PDE, which was increased by twofold within 10 min and declined to control values within 25–30 min. This transient activation of PDE (which was dependent on IL-4 concentration, with EC_{50} ~ 10 ng/ml IL-4 (data not shown)) was associated with a transient increase in tyrosine phosphorylation of IRS-2 (Fig. 2A) and its association with the p85α subunit of PI3-K (Fig. 2B). As seen in Fig. 2B, when lysates from IL-4-stimulated FDCP2 cells were immunoprecipitated with anti-IRS-2 Ab and immunoblotted with anti-phosphotyrosine Abs, phosphorylated IRS-2 was detected (data not shown). As shown in Fig. 2B, inhibitors, including STAT proteins, is thought to be mediated by JAK kinases (1 and 3) (11, 12, 58). TNF-α has been shown to reduce insulin-induced tyrosine phosphorylation of IRS-1 in Fao hepatoma cells (59, 60). As shown in Fig. 3, TNF-α, in a concentration (Fig. 3, A and B)- and time-dependent (Fig. 3C) fashion, and the JAK inhibitor AG-490 (Fig. 3D) reduced IL-4-induced tyrosine phosphorylation of IRS-2 (*, p < 0.001; +, p = NS).

**FIGURE 1.** Effects of IL-4, IL-3, GM-CSF, and PMA on activation of PDE3, PDE4, MAP kinase, and PKB, and on tyrosine phosphorylation of IRS-2. FDCP2 cells were incubated with IL-4 (7.4 nM for 10 min), IL-3 (3 nM for 10 min), GM-CSF (3 nM for 10 min), or PMA (50 nM for 30 min). A, Cell lysates were assayed for total PDE activity ( ), PDE3 ( ), PDE4 ( ), and PDE1 ( ). B, After incubation of FDCP2 cells with IL-4, IL-3, GM-CSF, and PMA for 10 min, cell lysates were immunoprecipitated with anti-IRS-2 Ab and immunoblotted with anti-phosphotyrosine Abs and anti-IRS-2 Abs. C, MAP kinase activity was assessed by phosphorylation of MBP protein by MAP kinase immunoprecipitates. D, PKB activity was assessed by phosphorylation of histone 2B by PKB immunoprecipitates. Results in B–D are representative of two or more individual experiments. Similar results were obtained if MAP kinase and PKB activities were assessed by phosphorylation of MBP peptide or Crosstide, respectively (data not shown).

**FIGURE 2.** Time course of IL-4-induced tyrosine phosphorylation of IRS-2 and activation of PDE, MAP kinase, and PKB. FDCP2 cells were incubated for the indicated times with 7.4 nM IL-4. A, Cell lysates from control and IL-4-treated cells were assayed for total PDE activity (control, □; IL-4 treated, ■) or immunoprecipitated with anti-IRS-2 Ab (○). After immunoblotting with anti-phosphotyrosine Abs, phosphorylated IRS-2 bands (○) were quantitated by PhosphorImager analysis and expressed as arbitrary PhosphorImager units. B, IRS-2 immunoprecipitates were immunoblotted with anti-PI3-K and anti-IRS-2 Abs. C, MAP kinase activity was assessed by phosphorylation of MBP peptide by MAP kinase immunoprecipitates. D, PKB activity was assessed by phosphorylation of Crosstide by PKB immunoprecipitates. Results represent mean values ± SEM from three or more individual experiments. Where not present, SEs are too small to be presented graphically. IL-4, IL-3, GM-CSF, and PMA activated PDE4, MAP kinase, and PKB, and on tyrosine phosphorylation of IRS-2. FDCP2 cells were incubated with IL-4 (7.4 nM for 10 min), IL-3 (3 nM for 10 min), IL-4 (7.4 nM for 10 min), IL-3 (3 nM for 10 min), GM-CSF (3 nM for 10 min), or PMA (50 nM for 30 min).

**Effects of TNF-α, AG-490, and wortmannin on IL-4-induced tyrosine phosphorylation of IRS-2 and STAT6 and on translocation of PI3-K**

The IL-4R lacks intrinsic protein tyrosine kinase activity. IL-4-induced tyrosine phosphorylation of IRS-2 and other cellular targets, including STAT proteins, is thought to be mediated by JAK kinases (1 and 3) (11, 12, 58). TNF-α has been shown to reduce insulin-induced tyrosine phosphorylation of IRS-1 in Fao hepatoma cells (59, 60). As shown in Fig. 3, TNF-α, in a concentration (Fig. 3, A and B)- and time-dependent (Fig. 3C) fashion, and the JAK inhibitor AG-490 (Fig. 3D) reduced IL-4-induced tyrosine phosphorylation of IRS-2 (*, p < 0.001; +, p = NS).
phosphorylation of IRS-2, and its association with PI3-K (Fig. 3, D, E, and F) in FDCP2 cells. Wortmannin, an inhibitor of PI3-K, also blocked association of PI3-K and IRS-2 (Fig. 3, E and F), but did not inhibit IL-4-induced phosphorylation of IRS-2 (Fig. 3D). Similar effects of TNF-α, wortmannin, and AG-490 in blocking association of PI3-K and IRS-2 were observed after either immunoprecipitation of IRS-2 and immunoblotting with anti-p85 (Fig. 3E) or immunoprecipitation of PI3-K and immunoblotting with anti-IRS-2 (Fig. 3F). The detailed mechanisms whereby TNF-α reduces tyrosine phosphorylation of IRS-2, and wortmannin blocks the association of IRS-2 with PI3-K, are unknown. Others have suggested that TNF-α stimulation of serine phosphorylation of IRS-1 was associated with inhibition of tyrosine phosphorylation (60).

As shown in Fig. 3G, the JAK kinase inhibitor AG-490 inhibited phosphorylation of STAT6 as well as of IRS-2 (Fig. 3D). In contrast to its effect on IRS-2 phosphorylation, however, TNF-α did not block IL-4-induced phosphorylation of STAT6 (Fig. 3G).

**Downstream of IRS-2 and PI3-K, IL-4 activates PDE3 by MAP kinase-independent and PDE4 by MAP kinase-dependent signals**

Incubation of FDCP2 cells with 5 nM TNF-α for 2 h, or with 5 μM AG-490, 100 nM wortmannin, or 50 μM LY294002 for 30 min before addition of IL-4 completely blocked IL-4-induced activation of MAP kinase and total PDE activity (Fig. 4A). Since TNF-α did not block tyrosine phosphorylation of STAT6 (Fig. 3G), these results suggest that activation of PDE by IL-4 is downstream of IRS-2/PI3-K signals, not STAT signals. The MEK-1 inhibitor PD98059 (10 μM), which completely blocked IL-4-induced activation of MAP kinase (Fig. 4A), only partially blocked activation of total PDE activity (Fig. 4, A and B). This partial inhibition of PD98059 on total PDE reflected almost complete inhibition of IL-4-induced activation of PDE4 with little effect on IL-4-induced activation of PDE3 (Fig. 4B). Neither rapamycin (20 nM) nor RO31-7549 (2 μM) interfered with IL-4-induced activation of MAP kinase or PDE (Fig. 4A). Thus, downstream of PI3-K, IL-4-induced signals diverge, with PDE4 regulated by MAP kinase-dependent and PDE3 by MAP kinase-independent mechanisms.

**IL-3 and PMA activate PDE4 by MAP kinase-dependent signals**

As seen in Fig. 5, IL-3-induced activation of MAP kinase and PDE4 (Fig. 5A) was transient, with a maximal increase within 10–20 min, and declining thereafter. PD98059 blocked IL-3-induced activation of MAP kinase and PDE4 in a concentration-dependent manner (Fig. 5B), with maximal effect on both at 10 μM PD98059. As seen in Fig. 6, IL-3-induced activation of MAP kinase (Fig. 6A) and PDE4 (Fig. 6B) was blocked by AG-490 and wortmannin and PD98059, but not by TNF-α, RO31-7549, or rapamycin, suggesting that JAK kinases participate in activating PI3-K, leading to activation of MAP kinase, PDE4, and PKB.
were assayed for total (7.4 nM IL-4 in the absence or presence of PD98059 for 10 min, cell lysates always in the same experiment. Results, expressed as percentage of control (C100%), represent mean values ± SEM from five or four (PD

PDE4 and inhibition by PD98059. A and B, FDCP2 cells were incubated without and with IL-3 (3 nM) for the indicated times (A) or with the indicated concentrations of PD98059 for 30 min and then IL-3 (3 nM) for 10 min (B). A and B, Cell lysates were assayed for PDE4 activity (●, i.e., activity inhibited by 0.5 μM rolipram); MAP kinase activity (●) was assessed by phosphorylation of MBP peptide in MAP kinase immunoprecipitates. Results in A and B represent mean values ± SEM from three or more individual experiments. Where not present, SEs were too small to be presented graphically. IL-4 increased MAP kinase and PDE4 in a time-dependent manner; PD98059, in a concentration-dependent manner, inhibited IL-3-induced activation of PDE4 and MAP kinase (+, p < 0.001; **, p < 0.01).

As seen in Fig. 7, the time course of PMA-induced activation of PDE4 and MAP kinase is different from that induced by IL-4 or IL-3. PMA-induced activation of PDE4 was dependent on PMA concentration (data not shown), was maximal within 25–30 min, and remained elevated for ~60 min (Fig. 7A). The time course of PDE4 activation correlated with that for MAP kinase activation (Fig. 7B). As shown in Fig. 6, PMA-induced activation of MAP kinase (Fig. 6C) and PDE4 (Fig. 6D) was inhibited by RO31-7549 and PD98059, but not TNF-α, wortmannin, AG-490, or rapamycin, suggesting that PMA activates MAP kinase by PKC-dependent signals, not via IRS-2, PI3-K, JAK, or p70S6 kinase-dependent signals.

Activation of PKB by IL-4 and IL-3

No specific inhibitors or pharmacological tools are available to examine the role of PKB in the activation of PDE3 and PDE4 by IL-4 and IL-3. As is the case for activation of PDE3, activation of PKB by IL-4 (Fig. 8A) was blocked by inhibitors of IRS-2 phosphorylation and PI3-K, i.e., AG-490, TNF-α, LY 294002, and wortmannin, findings consistent with the idea that PDE3 and PKB are regulated by PI3-K-dependent mechanisms. As seen in Fig. 8B, IL-3 activation of PKB was blocked by inhibitors acting upstream of, or directly on, PI3-K, including AG-490 and wortmannin. Activation of PKB (Fig. 8D) or MAP kinase and PDE4 (Fig. 6) by IL-3, which did not induce tyrosine phosphorylation of IRS-2 (Fig. 1), was not blocked by TNF-α. As seen in Fig. 8, A and B, neither PD98059 (which did block effects of IL-3 and IL-4 on PDE4) nor rapamycin inhibited PKB, although higher concentrations of PD98059 (50 μM) did partially block activation of PKB by IL-3 (unpublished). As also seen in Fig. 8, A and B, the PKC inhibitor (RO31-7549) did not block PKB activation, consistent with the finding that PMA did not activate PKB in FDCP2 cells (Fig. 1D). In FDCP2 cells that overexpress MEK

FIGURE 5. Time course of IL-3-induced activation of MAP kinase and PDE4 and inhibition by PD98059. A and B, FDCP2 cells were incubated without and with IL-3 (3 nM) for the indicated times (A) or with the indicated concentrations of PD98059 for 30 min and then IL-3 (3 nM) for 10 min (B). A and B, Cell lysates were assayed for PDE4 activity (●, i.e., activity inhibited by 0.5 μM rolipram); MAP kinase activity (●) was assessed by phosphorylation of MBP peptide in MAP kinase immunoprecipitates. Results in A and B represent mean values ± SEM from three or more individual experiments. Where not present, SEs were too small to be presented graphically. IL-4 increased MAP kinase and PDE4 in a time-dependent manner; PD98059, in a concentration-dependent manner, inhibited IL-3-induced activation of PDE4 and MAP kinase (+, p < 0.001; **, p < 0.01).

PKB by IL-4 (Fig. 8A) was blocked by inhibitors of IRS-2 phosphorylation and PI3-K, i.e., AG-490, TNF-α, LY 294002, and wortmannin, findings consistent with the idea that PDE3 and PKB are regulated by PI3-K-dependent mechanisms. As seen in Fig. 8B, IL-3 activation of PKB was blocked by inhibitors acting upstream of, or directly on, PI3-K, including AG-490 and wortmannin. Activation of PKB (Fig. 8D) or MAP kinase and PDE4 (Fig. 6) by IL-3, which did not induce tyrosine phosphorylation of IRS-2 (Fig. 1), was not blocked by TNF-α. As seen in Fig. 8, A and B, neither PD98059 (which did block effects of IL-3 and IL-4 on PDE4) nor rapamycin inhibited PKB, although higher concentrations of PD98059 (50 μM) did partially block activation of PKB by IL-3 (unpublished). As also seen in Fig. 8, A and B, the PKC inhibitor (RO31-7549) did not block PKB activation, consistent with the finding that PMA did not activate PKB in FDCP2 cells (Fig. 1D).

Activation of MAP kinase and PDE4 in FDCP2 cells that overexpress MEK

Several independently isolated cell lines were generated during transfection of FDCP2 cells with constructs that expressed vector alone (F/V) and wild-type (F/M), constitutively active (F/M¢), and kinase inactive (F/M¢) forms of MEK. Immune reactive MEK was much higher in cells transfected with MEK constructs than in uninfected cells (Fig. 9). In the absence of IL-3, MAP kinase activity was similar in F/V and F/M¢ cells, slightly elevated in F/M, and markedly increased in F/M¢ cells that overexpressed constitutively activated MEK, as evidenced by the mobility shift of MAP kinase during SDS-PAGE (Fig. 9B) or phosphorylation of MBP peptide in MAP kinase immunoprecipitates (Fig. 9C). PDE4, not PDE3,
was activated in F/M* cells (Fig. 9, Table I). Incubation with IL-3 activated MAP kinase in F/V, F/M, and F/M* 2 cells (Fig. 9, B and C). Incubation with IL-3 resulted in three- to fourfold activation of MAP kinase and twofold activation of PDE4 (not PDE3) in F/V and F/M* cells (Table I, Fig. 9), suggesting that kinase-inactive MEK was not functioning as a dominant negative with respect to activation of either MAP kinase or PDE4. Incubation of F/M cells with IL-3 increased both MAP kinase and PDE4 to levels comparable with those in F/M* cells (Fig. 9, Table I); IL-3 produced a much smaller increase in MAP kinase or PDE4 in F/M* cells than in F/M cells (Fig. 9, Table I). PKB was not increased in cells transfected with MEK constructs; IL-3 activated PKB to the same extent in all FDCP2 cells, indicating that even in cells overexpressing wild-type and constitutively activated MEK, PKB was regulated appropriately by IL-3 (Fig. 9). Although kinase-inactive MEK did not function as a dominant negative with respect to activation of either MAP kinase or PDE4. Incubation of F/M cells with IL-3 increased both MAP kinase and PDE4 to levels comparable with those in F/M* cells (Fig. 9, Table I); IL-3 produced a much smaller increase in MAP kinase or PDE4 in F/M* cells than in F/M cells (Fig. 9, Table I). PKB was not increased in cells transfected with MEK constructs; IL-3 activated PKB to the same extent in all FDCP2 cells, indicating that even in cells overexpressing wild-type and constitutively activated MEK, PKB was regulated appropriately by IL-3 (Fig. 9). Although kinase-inactive MEK did not function as a dominant negative with respect to activation of PDE4 (Fig. 9, Table I), in F/M* cells and IL-3-treated F/V and F/M cells, the MEK inhibitor PD98059 inhibited activation of MAP kinase and PDE4. These results are consistent with the idea that PDE4 is activated by MAP kinase-dependent signals (Fig. 10).

Discussion

Nine different PDE gene families have been identified (1–6). Each family contains closely related subfamilies that are products of different genes or arise from alternative transcription initiation sites on the same gene or by alternative splicing of mRNAs. Representatitives of at least two to three different families are found in most cells. Although they are highly regulated enzymes (1–3), little is known of the effects of cytokines on PDEs. Our results demonstrate that PMA and the cytokines, IL-4, IL-3, and GM-CSF, activate PDE3 and PDE4 by distinct signaling pathways in FDCP2...
cells. Activation of PDE3 and PDE4 also exhibits a common characteristic of cytokine-mediated events, namely overlapping (activation of PDE4 by IL-4, IL-3, GM-CSF) and specific (activation of PDE3 by IL-4) effects. PDEs may thus serve as one locus for cross-talk between cytokine and cyclic nucleotide signaling systems.

Whether activation of PDE3 and PDE4 by cytokines mediates specific effects of IL-4 and IL-3 is not known. Although we utilized pharmacologic agents and inhibitors (with their inherent uncertainties as to absolute specificity of action) to help identify signaling pathways (presented in a schematic fashion in Fig. 10) involved in activation of PDE3 by IL-4, IL-3, GM-CSF, and PMA, our conclusions are supported by their selective effects, i.e., TNF-α blocking IL-4, not IL-3, responses; PD98059 blocking activation of PDE4, not PDE3; AG-490 blocking cytokine, not PMA responses; PKC inhibitor RO31-7549 blocking PKC, not cytokine, responses, etc.

Effects of IL-4 and IL-3 on PDE3 and PDE4 were inhibited by JAK and PI3-K inhibitors, consistent with the idea that PI3-K signals are responsible for PDE activation and that receptor-mediated activation of JAKs is central to cytokine-induced tyrosine phosphorylation of specific intracellular proteins and activation of signaling pathways, including PI3-K-regulated signals (Fig. 10). Since IL-4, not IL-3, induces JAK-dependent tyrosine phosphorylation of IRS-2, its association with PI3-K and activation of PDE3 and PDE4, IRS-2/PI3-K signals may be critical in these effects of IL-4. This idea is supported by selective effects of TNF-α on IL-4 signals. Activation of PDE4 by IL-3, which does not induce tyrosine phosphorylation of IRS-2, is blocked by JAK and PI3-K inhibitors, but not TNF-α. TNF-α, which does not block IL-4-induced phosphorylation of STAT6, reduces IL-4-induced tyrosine phosphorylation of STAT6.
phosphorylation of IRS-2 and its association with PI3-K and inhibits IL-4-induced activation of MAP-kinase, PKB, PDE3, and PDE4. Thus, IL-4-induced activation of PDE3 and PDE4 may involve IRS-2/PI3-K signals, not STAT signals, and TNF-α may interfere with IRS-2/PI3-K signals. These observations also support the idea that different regions of the IL-4R regulate different functions, with the region between amino acids 437–557 as critical for IRS-2 interaction/phosphorylation and IL-4-induced proliferation, and the region between amino acids 557–657, for regulation of JAK phosphorylation/activation of STAT6 and induction of gene expression (19). Some effects of TNF-α on IL-4 signaling (as well as on signaling by insulin and other growth factors and cytokines) may be mediated via TNF-α inhibition of tyrosine phosphorylation of, and signaling via, IRS proteins. While all effects of TNF-α are not mediated via inhibition of IRS signaling, disruption of these signals may represent one mechanism whereby TNF-α can selectively regulate certain actions of specific cytokines, i.e., those that utilize IRS adapter proteins.

Whereas IL-4-induced translocation of PI3-K to IRS-2 represents a plausible mechanism for IL-4-induced activation of PI3-K, other mechanisms are responsible for activation of PI3-K by IL-3 and GM-CSF (61). The phosphatase SHPTP2 binds to the tyrosine-phosphorylated βc subunit of the IL-3R, where it is tyrosine phosphorylated (by JAK2 or src family kinases) and may function as an adapter molecule between activated IL-3 βc and Grb/SOS and PI3-K, thus regulating both Ras/Raf and PI3-K signaling pathways (15, 20, 21). Others have implicated a tyrosine-phosphorylated p40 protein in forming a Shc/Grb2/SOS complex and initiating downstream signals from the IL-3R (22).

Although IL-4 and IL-3 activated PDE3 and PDE4 via PI3-K, PMA activated PDE4 via PKC (based on sensitivity to RO31-7549). Downstream of PI3-K and PKC, IL-4 activated PDE3 by MAP kinase-independent signals, whereas IL-4, IL-3, and PMA activated PDE4 by MAP kinase-dependent signals (Fig. 10). In FDCP2 cells permanently transfected with and overexpressing wild-type MEK, IL-3 activated MAP kinase and PDE4 (not PDE3); in cells transfected with constitutively active MEK, MAP kinase and PDE4 (not PDE3) were activated in the absence of IL-3, and only slightly further increased by IL-3. The MEK inhibitor PD98059 blocked activation of MAP kinase and PDE4 in F/M* cells transfected with constitutively active MEK, or in control (F/V) cells or WT (F/M) cells incubated with IL-3. Whether MAP kinase is the proximate kinase that regulates PDE4 or is part of a kinase cascade is not known. PDE4 was phosphorylated by MAP kinase in vitro, but with no change in PDE activity (62). The mechanisms whereby PKC and PI3-K activate MAP kinase have not yet been completely defined. MAP kinases are phosphorylated and activated by MEKs that can be phosphorylated and activated by Raf-1 kinase (29), PMA has been reported to activate Raf and MAP kinases in intact cells (63, 64). Inhibition of PI3-K with wortmannin or expression of dominant-negative PI3-K blocked activation of MAP kinase in some (37, 65–67), but not all (68) cells. Wortmannin also blocked activation of Raf-1 by platelet-derived growth factor in Chinese hamster ovary cells (65), by insulin in 3T3-L1 adipocytes (30), and by IGF-1 in L6 cells (67). Overexpression of p110α PI3-K activated Raf-1 and MAP kinase in frog oocytes (69), but not other cells (70, 71). Others, however, have suggested that PI3-K is a direct target of ras (72).

The initial impetus for the studies in this work came from our interest in regulation of PDE3B in adipocytes (2, 28), from identifying PDE3B mRNAs in human lymphocytes (4), and in recognizing analogous signaling mechanisms for IL-4 in FDCP2 cells (11, 12, 17, 18) and insulin in adipocytes (2, 28) (Fig. 10). In some respects, the effects of insulin and IL-4 on PDE3 in adipocytes and FDCP2 cells are similar. Insulin stimulation of adipocytes induces association of tyrosine-phosphorylated IRS-1 with the p85 regulatory subunit of PI3-K, activation of PI3-K, and phosphorylation and activation of a microsomal PDE3B (28, 73). Wortmannin, not PD98059, blocked insulin- and IL-4–induced activation of PKB and PDE3 in adipocytes (28, 36, 37) and FDCP2 cells, respectively. In these cells, PDE3 is apparently activated by PI3-K-dependent/MAP kinase-independent pathways (Fig. 10). Wijkander et al. (37) demonstrated that during partial purification of activated PKB from insulin-stimulated adipocytes, insulin-sensitive kinase activity that phosphorylated PDE3B in vitro cofractionated with PKB. Further characterization of the role of PKB in regulation of PDE3 in intact cells will require pharmacological inhibitors of PKB, and transfection of wild-type and mutant PKB into FDCP2 cells. Initial studies in FDCP2 cells that overexpress rPKB do indicate that PDE3B (not PDE4) is a downstream target of activated PKB.3

Although PI3-K may mediate effects of both IL-4 and IL-3 on PDE3 and PDE4, IL-4 signals differ from those of IL-3. Effects of IL-4 are blocked by TNF-α and may depend on PI3-K/IRS-2 signals.


FIGURE 10. Effect of PD98059 on IL-3-induced activation of MAP kinase and PDE4 in MEK-transfected cells. FDCP2 cells transfected with vector alone (F/V) and wild-type (F/M) and constitutively active (F/M*) MEK constructs were incubated without or with PD98059 (10 μM) for 30 min and then without or with IL-3 for 10 min. A, MAP kinase activity was assessed by phosphorylation of MBP peptide in MAP kinase immunoprecipitates. B, Total PDE ( ), PDE3 ( ), and PDE4 ( ) activities were assayed in cell lysates. Results in A and B represent mean values ± SEM from three or more individual experiments. PD98059 significantly inhibited MAP kinase and total PDE and PDE4 activities in F/M*, F/V, and F/M cells treated with IL-3 (*, p < 0.001). Where not present, SEs were too small to be presented graphically.
by JAK-dependent signals that are independent of IRS-2 and of TNF-α. If activation of PDE3 in adipocytes by insulin (36, 37), or in FDCP2 cells by IL-4, does involve IRS-2/Pi3-K-mediated activation of PKB, mechanisms must exist whereby PKB activated by IL-4, not by IL-3, specifically targets PDE3. Perhaps IRS-2 (or the IL-4R) recruits and/or initiates a specific signaling complex (a specific physical or functional signaling subset utilizing PI3-K and downstream kinases, including PKB) that targets PDE3, since activation of PI3-K and PKB by IL-4 are not in themselves sufficient for activation of PDE3 (Fig. 10). Whether, for example, IL-3 and IL-4 induce formation of different signaling complexes (or similar complexes in different subcellular compartments) or utilize different isoforms of PI3-K, PKB, and/or other signaling molecules is not known. From this perspective, regulation of PKB and PDE3 in FDCP2 cells may be analogous to regulation of glucose transport in adipocytes, where both insulin and platelet-derived growth factor increase PI3-K activity, but only insulin stimulates glucose transport (74).

In adipocytes, insulin-induced activation of PDE3B (which decreases cAMP and AK-kinase and, consequently, hormone-stimulated triglyceride lipase activity) is important in the antilipolytic action of insulin (2, 28). In frog oocytes, insulin- or IGF-1-induced activation of PI3-K, PKB, and/or other signaling molecules is not yet proven. In FDCP2 cells, IL-4 also activates PDE3 by MAP kinase-independent signals, whereas IL-4, IL-3, GM-CSF, and PMA all activate PDE4 via MAP kinase signals. Whether MAP kinase and PKB directly phosphorylate/activate specific PDE4 and PDE3 isoforms, respectively, in FDCP2 cells is also not certain.

FIGURE 11. Hypothesis regarding signaling pathways involved in activation of PDE3 in adipocytes and of PDE3 and PDE4 in FDCP2 cells. In rat adipocytes, insulin activates PDE3B by MAP kinase- and p70S6 kinase-independent signals that may involve activation of PKB kinase (PDK-1) and PKB. Whether PKB is the kinase that phosphorylates PDE3B in intact adipocytes is not yet proven. In FDCP2 cells, IL-4 also activates PDE3 by MAP kinase-independent signals, whereas IL-4, IL-3, GM-CSF, and PMA all activate PDE4 via MAP kinase signals. Whether MAP kinase and PKB directly phosphorylate/activate specific PDE4 and PDE3 isoforms, respectively, in FDCP2 cells is also not certain.

References


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