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Dexamethasone Suppresses Antigen-Induced Activation of Phosphatidylinositol 3-Kinase and Downstream Responses in Mast Cells

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Dexamethasone and other glucocorticoids suppress FcεRI-mediated release of inflammatory mediators from mast cells. Suppression of cytokine production is attributed to repression of cytokine gene transcription but no mechanism has been described for the suppression of degranulation. We show that therapeutic concentrations of dexamethasone inhibit intermediate signaling events, in particular the activation of phosphatidylinositol (PI3)-kinase and downstream signaling events that lead to degranulation in rat basophilic leukemia 2H3 cells. This inhibitory action is mediated via the glucocorticoid receptor and is not apparent when cells are stimulated via Kit in a mouse bone marrow-derived mast cell line. The primary perturbation appears to be the failure of the regulatory p85 subunit of PI3-kinase to engage with the adaptor protein Grb2-associated binder 2 leading to suppression of phosphorylation of phospholipase Cγ2, the calcium signal, and degranulation. Suppression of PI3-kinase activation by dexamethasone may also contribute to reduced cytokine production because the PI3-kinase inhibitor LY294002, like dexamethasone, inhibits Ag-induced transcription of cytokine genes as well as degranulation.

pluripotent stem cells (32,33) and Cdc42 (26,27), whereas dephosphorylation of Tyr-688 by SHP-1 can reverse the activation process (28). As noted previously, activation of PI3-kinase via FcεRI in mast cells is dependent on LAT (20) or Gab2 (21), which presumably facilitate efficient communication between the PI3-kinase and activating molecules.

On re-investigating the mechanism by which dexamethasone suppresses PLC-dependent signals in mast cells, we discovered that activation of PI3-kinase and downstream signaling events are markedly suppressed in cells exposed to nanomolar concentrations of dexamethasone. The constellation of signaling events affected indicated that the calcium signal and degranulation were regulated primarily by PLCγ2 and not PLCγ1 in a PI3-kinase/Gab2-dependent manner. We then examined the functional relevance of the inhibition of PI3-kinase by comparing the effects of dexamethasone and the PI3-kinase inhibitor LY294002 on degranulation and cytokine production. This report describes these investigations.

Materials and Methods

Materials

Ag (DNP-BSA), and the mixture of bovine brain phosphoinositides from Sigma-Aldrich (St. Louis, MO); phosphatidylinositol 3,4,5-trisphosphate (PIP3) was from Matreya (Pleasant Gap, PA); LY294002 was from Calbiochem (La Jolla, CA); phospholipids were from Avanti Polar Lipids (Alaba ster, AL); recombinant murine p110α, β, and δ subunits of PI3-kinase, PLCγ1, SHP1, Fyn, and Lyn were from Santa Cruz Biotechnology (Santa Cruz, CA); polyclonal Abs against phospho-Src (Tyr-416), phospho-Akt (Ser-473), phospho-Akt (Thr-308), Akt, and phospho-PLCγ2 (Ser-241), phospho-Btk (Tyr-222), phospho-PLCγ2 (Tyr-217), PLCγ2 were from Cell Signaling (Beverly, MA); polyclonal Ab against phospho-PLCγ1 (Tyr-198) was from BioSource International (Camarillo, CA). Abs against the p85α and p85β subunits of PI3-kinase and Src, kinase substrates, and the activation kits for Rac and Cdc42 were from Upstate Biotechnology (Lake Placid, NY); the ECL kit was from Amersham Pharmacia Biotech (Little Chalfont, U.K.); radiolabeled products were from ICN (Santa Ana, CA); the MultiProbe RNase Protection Assay System was from BD Pharmingen (San Diego, CA); and fura 2-AM ester was from Molecular Probes (Eugene, OR). Dr. J. Rivera (National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health) kindly supplied DNP-specific monoclonal IgE and the corresponding IgG1 control. All other chemicals were from Sigma-Aldrich or the manufac turer.

Cell culture and stimulation

RBL-2H3 cells were plated in 12-well plate (1 × 106 cells/1 ml), 60 mm2 (2.5 × 106 cells/5 ml) or 145 mm2 (5 × 106 cells/10 ml) dishes in complete growth medium (MEM) supplemented with 15% FCS, glutamine, antibiotic, and antymycotic agents (31). BMCC1 were grown in suspension in 60-mm2 dishes (5 × 106 cells/5 ml) in DMEM (catalogue no. 9417; Irvine Scientific, Santa Ana, CA) supplemented with 10% FCS, 10% Wehi 3BD-conditioned medium, 2 mM glutamine, 100 μM nonessential amino acids, 10 μM 2-ME, and 1 mM sodium pyruvate. Cultures were incubated at 37°C for 18 h with 0.5 μg/ml DNPIgE to achieve 100% occupancy of FcεRI by IgE in the presence or absence of dexamethasone. For time course studies, dexamethasone was added at different times before the experiment. The cultures were then washed with a glucose saline/PIPES buffer (32) and stimulated with Ag (20 ng/ml DNP-BSA) in the same buffer for 15 min unless indicated otherwise. Cultures were placed on ice for subsequent assays.

Western blot analysis and immunoprecipitation

Whole cell lysates were prepared with ice-cold reagents. The lysis buffers included: buffer A (20 mM HEPES, pH 7.3, 1% Triton X-100, 10% glycerol, 12.5 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM PMSF, 30 μg/ml leupeptin, 30 μg/ml apro tinin, and 25 mM 3- nitrophenyl phosphate) or buffer B (25 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 12.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM PMSF, 25 μg/ml leupeptin, and 25 μg/ml apro tinin). Buffer B was clarified by centrifugation at 500 × g (10 min). Cells were washed first with PBS and then lysed (10 min) in either 0.3 ml of buffer A (for 12-well plates) for immunoblotting or 1.0 ml of buffer B (for 60 mm2 dishes) for immunoprecipitation. For immunoblotting, proteins were separated by SDS-PAGE. Blots were probed with the indicated primary Abs and peroxidase-labeled secondary Abs and visualized by chemiluminescence. For immunoprecipitation, samples of equivalent protein content were incubated for 30 min withagarose beads before overnight incubation with the appropriate Ab. Samples were then incubated for a further 2 h with protein A-agarose beads. The beads were washed four times with buffer B and dissolved in 80 μl of SDS buffer (2×).

Measurement of [32P]PIP3

Previously described procedures (33) were adapted as follows. RBL-2H3 cells were sensitized with IgE and treated with 100 nM dexamethasone as previously described and suspended in a glucose saline/PIPES-BSA buffer (32). Phosphoinositide [32P]phosphorylation was monitored by incubation of 90 min at 37°C, washed once with the glucose saline/PIPES-BSA buffer, and resuspended in the same buffer. Where indicated, LY294002 (20 μM) was added 30 min before addition of Ag. Cells were stimulated with Ag or not for 5 min later. [32P]PIP3 and other radiolabeled phospholipids were extracted and separated by thin layer chromatography for densitometric assay exactly as described (33).

Assay of immunoprecipitated PI3-kinase activity

For the assay of PI3-kinase we modified recommended procedures (cata log: Upstate Biotechnology) based on the use of an anti-pan p85 Ab that coimmunoprecipitates the p110 catalytic and p85 regulatory subunits. The immunoprecipitates were washed thrice with buffer B and twice with buffer C (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, and 0.1 mM sodium orthovanadate). The immunoprecipitates were resuspended in 70 μl of the reaction buffer that consisted of 50 μl of buffer C, 10 μl of the substrate phosphatidylinositol in suspension (2 mg/ml prepared by sonication in 10 mM Tris-HCl and 1 mM EGTA buffer, pH 7.4), and 10 μl of 100 μM MgCl2. The reaction was started by addition of 5 μl of 0.88 mM ATP that contained 30 μCi of [γ-32P]ATP and 20 mM MgCl2 and allowed to proceed for 30 min at room temperature. LY294002 (10 μM) was added to matched samples to verify specificity of the kinase assay. The reaction was terminated by addition of 20 μl of 6 N HCl. Lipids were extracted into the organic phase after addition of 200 μl of chloroform:methanol (1:1) that contained 5 μg of phosphatidylinositol as monophosphate as the carrier for the labeled product. The organic phase was washed with 100 μl of 0.1 N HCl and then dried by vacuum centrifugation. The residue was dissolved in 15 μl of chloroform:methanol (1:1) and spotted on olate-treated silica Gel G 60 thin-layer chromatographic plates in chloroform:methanol: NH4OH:H2O (60:47:2:11.5 by volume). Radionabeled phosphatidylinositol monophosphate was visualized by autoradiography and quantitated by densitometric scanning.

Assay of Src kinases, Rac1, Cdc42, and SHP1 activities

RBL-2H3 cells were incubated with or without 100 nM dexamethasone for 18 h, stimulated with Ag for 5 min, and then lysed with the lysis buffer. Src kinases (Lyn, Fyn, and Src) were immunoprecipitated with kinase-specific Abs as initially described. Immunoblots were prepared and probed with Abs against the indicated Src kinase as well as an anti-phospho-Src family Ab, which detects phospho-Src (Tyr-416) and cross-reacts with other Src kinases when phosphorylated at equivalent sites. The immunoprecipitates were assayed also for kinase activity by use of a colorimetric in vitro kinase assay kit (Tyrosine Kinase Assay kit; Upstate Biotechnology) according to the manufacturer’s instruction.

The levels of Rac-GTP were measured with a Rac Activation Assay kit by a previously described procedure (34) in which Rac-GTP in whole cell lysates was separated by use of affinity beads conjugated with the p21 binding domain of human p21-activated kinase-1. The isolated Rac-GTP was quantitated by immunoblotting with the supplied anti-Rac mAb. Cdc42-GTP was not detected in RBL-2H3 cells by use of the Cdc42 activation kit and no further assays were performed.
For the assay of SHP-1 tyrosine phosphatase activity, SHP-1 was immunoprecipitated from cell lysates with anti-SHP-1 Ab. The immunoprecipitates were washed twice with a phosphatase buffer (62.5 mM HEPES, pH 7, and 6.25 mM EDTA) and resuspended in 100 μl of the same buffer that contained 10 mM p-nitrophenyl phosphate and 5 mM DTT. The mixture was incubated (37°C) for 30 min and the reaction stopped by addition of 400 μl of NaOH (200 mM). The amount of product, DNP, was determined by measurement of absorbance at 405 nm (35).

**Measurement of cytosolic Ca**

Cells (5 × 10^6 cells/0.1 ml/well) were incubated with 2 μM fura 2-AM ester for 30 min in 96-well black culture plates (PerkinElmer Life Sciences, Boston MA). Fluorescence was monitored in a Wallac Victor 2 plate reader (PerkinElmer Life Sciences) by alternating excitation at 340 nm and 380 nm. Data were corrected for background fluorescence of cells that did not contain fura 2 and the ratio of fluorescence at 340 nm and 380 nm excitation determined. The calibration procedure of Petr and Wurster (36), which uses the equation in Grynkiewicz et al. (37), was used to estimate the concentration of free cytosolic Ca**

**Measurement of degranulation and cytokine/chemokine mRNA**

Degranulation was determined from the release of the granule marker, β-hexosaminidase, which was measured in 10-μl aliquots of medium and cell lysates in microtiter plates by a colorimetric assay as described previously (38). Values were expressed as the percentage of intracellular hexosaminidase that was released into the medium after correction for spontaneous release.

mRNA for the individual cytokines and chemokines were assayed simultaneously by use of multiprobe RNase protection assay kits. Multiple mouse (for BMMCt) or rat (for RBL-2H3 cells) radiolabeled RNA cytokine probes were prepared and used according to the manufacturer’s instructions. Protected probes were separated by gel electrophoresis and quantified by autoradiography and densitometric scanning of the X-ray films. Data were corrected for small variation in levels of housekeeping gene transcripts that are incorporated in the kits.

**Results**

**Dexamethasone suppresses production of [32P]PIP3 and PI3-kinase activity**

Labeling of RBL-2H3 cells with [32P]orthophosphate and separation of [32P]phospholipids by thin-layer chromatography revealed that the increase in levels of radiolabeled PIP3 following Ag stimulation was much reduced in cells previously exposed to 100 nM dexamethasone for 18 h or to the PI3-kinase inhibitor, LY294002, for 10 min (Fig. 1, A and B). The levels of other identifiable phospholipids namely, phosphatidylserine, phosphatidylinositol, phosphatidylinositol 4-monophosphate, and phosphatidylinositol 4,5-bisphosphate were not substantially altered by either dexamethasone or LY294002 (Fig. 1A).

Assay of immunoprecipitated PI3-kinase by the formation of [32P]-phosphatidylinositol monophosphate from phosphatidylinositol in the presence of [γ-32P]ATP in vitro showed that Ag stimulation elicited a 2- to 3-fold increase in PI3-kinase activity (data not shown) and this increase was suppressed in dexamethasone-treated cells in a dose-dependent manner (Fig. 1C). Treatment with as little as 5 nM dexamethasone significantly reduced PI3-kinase activity. This reduction was probably due to diminished intrinsic activity of the immunoprecipitated PI3-kinase because dexamethasone treatment did not substantially alter expression of the detectable isoforms of the p85 and p110 subunits of PI3-kinase in RBL-2H3 cells (Fig. 1D).

**FIGURE 1.** Dexamethasone inhibits Ag-stimulated production of PIP3 and activation of PI3-kinase. A and B, Cells were incubated with 100 nM dexamethasone (Dex) along with DNP-specific IgE for 18 h before labeling with [32P]orthophosphate. LY294002 (LY), 20 μM, was added 30 min before stimulation with Ag (Ag) or not for 5 min as described in Materials and Methods. 32P-Phospholipids were extracted, separated by thin layer chromatography, and visualized by autoradiography and densitometric scanning. The various phospholipids were identified by addition of unlabelled phosphatidylserine (PS), phosphatidylglycerol (PI), phosphatidylinositol 4-monophosphate (PIP), phosphatidylinositol 4,5-bisphosphate (PIP2), and PIP3 to the extracts before chromatography and then visualized by iodine staining. A representative autoradiogram (A) is shown and the relative amounts of [32P]PIP3 (B) (mean ± SEM) for four such experiments are represented. Asterisks indicate significant suppression in levels of [32P]PIP3 (*, >0.05 and **, >0.01). C, RBL-2H3 cells were incubated with the indicated concentrations of dexamethasone for 18 h along with IgE and then stimulated with Ag or not for 15 min before immunoprecipitation of both subunits of PI3-kinase with an anti-pan p85 Ab. Kinase activity was assayed in vitro by measurement of formation of [32P]-phosphatidylinositol 4-monophosphate from phosphatidylinositol in the presence of [γ-32P]ATP as described in Materials and Methods. Ag stimulation caused a 2- to 3-fold increase in activity and values are expressed as a percentage of stimulated activity in the absence of dexamethasone (mean ± SEM of values from six experiments). D, Immunoblots were also prepared from whole cell lysates for the detection of the various subunits with specific Abs. A typical blot from three experiments is shown.
Gab2 phosphorylation and interaction with p85 are impaired by dexamethasone

We next determined whether or not dexamethasone treatment impaired the association of the p85 subunit of PI3-kinase with the docking proteins Gab2 and LAT. Of the two isoforms of p85, only p85α was detected by immunoblotting in immunoprecipitates of Gab2 (data not shown). Ag stimulation resulted in an increase in the amount of p85α that coimmunoprecipitated with Gab2 (Fig. 2A). This apparent association of p85α with Gab2 was much reduced in dexamethasone-treated cells whether cells were not stimulated or stimulated with Ag. Decreased phosphorylation of Gab2 tyrosine was also observed although the retardation in Gab2 migration on SDS-PAGE that is observed after Ag stimulation (22) was still observed (Fig. 2B). In contrast to Gab2, the association of p85α with LAT was unaffected by treatment with dexamethasone in nonstimulated or stimulated cells (Fig. 2C). Treatment with dexamethasone at concentrations up to 100 nM had no effect on expression of Gab2 or LAT (Fig. 2D).

The decreased phosphorylation of Gab2 was not due to decreased expression of Fyn, the tyrosine kinase primarily responsible for Gab2 phosphorylation (22), or to an impediment in the tyrosine phosphorylation of the activating loop of Fyn (39). The levels of Fyn protein remained unchanged and the extent of basal tyrosine phosphorylation of Fyn, as detected by an anti-phospho-Src (Tyr416) Ab, actually increased after treatment with dexamethasone (Fig. 3). Ag stimulation induced a weak but visible increase in tyrosine phosphorylation of Fyn in both untreated and dexamethasone-treated cells. A similar pattern was observed for two other Src kinases, Lyn and Src (Fig. 3). Measurement of kinase activities of immunoprecipitated Fyn, Lyn, and Src indicated that the increased tyrosine phosphorylation after treatment with dexamethasone was accompanied by a 2- to 3-fold increase in basal kinase activity in three separate experiments (data not shown). However, we were unable to detect a significant increase in activity of any of these kinases upon Ag stimulation in either untreated or dexamethasone-treated cells. Other reports have noted a similar lack of activation of Fyn and Lyn in Ag-stimulated RBL-2H3 cells (J. Rivera, unpublished observations), a possible reflection of the relatively small proportion of available kinase in the cell that is recruited by FcεRI during stimulation (40, 41).

FIGURE 2. Dexamethasone inhibits the association of p85α with Gab2 and phosphorylation of Gab2. A and B, RBL-2H3 cells were incubated with vehicle or dexamethasone (Dex) for 18 h and then stimulated with Ag (Ag) for the indicated times. Immunoblots of immunoprecipitated (IP) Gab2 were probed with Abs against p85α (A), p85β (not detected), and phosphotyrosine (B). Densitometric data (mean ± SEM) for p85α (A, inset) from three experiments are shown. C, Similarly experiments were conducted with immunoprecipitated LAT that was probed for p85α. Densitometric data from three experiments is shown in the inset. D, Immunoblots were also prepared from whole cell lysates and probed with Abs against Gab2 and LAT to assess effects on expression of these proteins.

FIGURE 3. Expression and tyrosine phosphorylation of Fyn and other Src kinases are enhanced in dexamethasone-treated cells. RBL-2H3 cells were incubated with IgE and 100 nM dexamethasone (Dex) or vehicle for 18 h before stimulation with Ag (Ag) for 5 min. The indicated Src kinases were immunoprecipitated (IP) with kinase-specific Abs from whole cell lysates and then separated by SDS-PAGE. Immunoblots were probed with kinase-specific Abs and an anti-phospho-Src (Tyr416) Ab, which cross-reacts with other Src kinases that are phosphorylated at equivalent sites. A typical immunoblot from one of three experiments is shown.

Other experiments revealed no striking perturbation of other signaling molecules thought to regulate PI3-kinase activity. With respect to the small GTPases (26), the conversion of Rac to its GTP-bound active form by Ag was not impaired in dexamethasone-treated cells (100 nM for 18 h) as determined by the assay described in Materials and Methods (data not shown). Cdc42 activity was not detectable in RBL-2H3 cells by this assay. With respect to phosphatases that might regulate tyrosine phosphorylation of p85, Ag stimulation caused a significant decline in SHP-1 activity in untreated cells of 54 ± 3% (mean ± SEM for three
Suppression of PI3-kinase-dependent Akt phosphorylation is mediated by the glucocorticoid receptor

Akt is phosphorylated by PDK1 at Thr<sup>308</sup> and by PDK2 at Ser<sup>473</sup> in a PI3-kinase-dependent manner (42). Both amino acids were found to be phosphorylated in nonstimulated RBL-2H3 cells and the extent of these phosphorylations was reduced by treatment with dexamethasone in a dose-dependent manner (Fig. 4A). Ag stimulation induced additional phosphorylation of both sites but again phosphorylation was suppressed in a dose-dependent manner in dexamethasone-treated cells (Fig. 4B). As is also shown in Fig. 4B, the expression of Akt was not markedly affected by dexamethasone. In relative terms, the suppression of Akt phosphorylation by dexamethasone was similar for nonstimulated and stimulated cells (Fig. 4C). Approximately 50% suppression was observed with 5 nM dexamethasone in both stimulated and stimulated cells and near-maximal suppression with 10 nM dexamethasone. The extent of suppression of Akt phosphorylation was also dependent on time of exposure to dexamethasone. Suppression of phosphorylation was apparent by 6 h and was near maximal by 12 h, whereas expression of Akt protein remained unaltered (Fig. 4D).

Examination of the effects of the glucocorticoid receptor antagonist, RU486, and different types of steroids indicated that the effects of dexamethasone on Akt phosphorylation, and presumably PI3-kinase activation, were mediated through the glucocorticoid receptor. For example, RU486 blocked the inhibitory action of dexamethasone on Akt phosphorylation (Fig. 4, E and F). Also, the glucocorticoids, triamcinolone, hydrocortisone, and methyl prednisolone, like dexamethasone, inhibited Akt phosphorylation, whereas the sex steroids, progesterone, and estrogen did not. None of these steroids markedly affected expression of Akt protein (Fig. 4E).

Dexamethasone selectively inhibits activation of PI3-kinase by FceRI but not c-KIT

PI3-kinase is also activated when mast cells are stimulated by SCF through c-KIT, a tyrosine kinase growth receptor (43). To examine the effects of dexamethasone on the activation of PI3-kinase through this receptor, studies were conducted with BMMCt rather than RBL-2H3 cells. BMMCt, unlike RBL-2H3 cells, exhibit robust phosphorylation of Akt in response to SCF and this phosphorylation is blocked by LY294002 to indicate the dependency on PI3-kinase (our unpublished observation). Phosphorylation of Akt was used thus as a surrogate for PI3-kinase activation. Stimulation of BMMCt with either Ag- or SCF-induced an increase in Akt phosphorylation (Fig. 5A). Akt phosphorylation was impaired in dexamethasone-treated cells when cells were stimulated with Ag as was previously observed for RBL-2H3 cells. SCF-induced phosphorylation of Akt, in contrast, was not impaired but instead substantially enhanced in dexamethasone-treated cells. As in RBL-2H3 cells, expression of Akt was not altered by the treatment with dexamethasone. Quantitative data for all experiments are shown in Fig. 5B.

Suppression of downstream signals and degranulation

Dexamethasone inhibited Ag-induced tyrosine phosphorylation of Btk (Fig. 6A), PLCγ1, (Fig. 6B), and PLCγ2 (Fig. 6C) in a dose-dependent manner without affecting the expression of these proteins in RBL-2H3 cells. However, the phosphorylation of PLCγ2 proteins in RBL-2H3 cells. However, the phosphorylation of PLCγ2 proteins in RBL-2H3 cells.
was inhibited to a much greater extent than the phosphorylation of PLC-1. The phosphorylation of Gab2 showed intermediate sensitivity to dexamethasone. The calcium signal as determined by measurement of the increase in cytosolic Ca\(^{2+}\) in fura 2-loaded cells (Fig. 6E) and degranulation as measured by release of the granule marker, \(\beta\)-hexosaminidase (Fig. 6F) were decreased also in a dose-dependent manner. The decrease in degranulation was highly correlated with the decrease in PLC-2 phosphorylation both of which were suppressed by 50% after treatment with 1 nM dexamethasone (Fig. 6F). The calcium signal, as measured by the area under the curve, was suppressed by \(\sim 50\%\) at 5 nM dexamethasone (Fig. 6F). The extent of decrease in the calcium signal was similar whether the measurement was restricted to the first 5 min of stimulation or encompassed the entire period of stimulation as shown in Fig. 6F (data not shown).

**Inhibition of PI3-kinase impairs cytokine production as well as degranulation**

The physiologic consequences of the suppression of PI3-kinase activity were assessed by comparing the effects of dexamethasone and the PI3-kinase inhibitor, LY294002, on Akt phosphorylation, degranulation, and cytokine production. Akt phosphorylation was used as an indicator of PI3-kinase activity in vivo. Both compounds inhibited Akt phosphorylation and degranulation in a dose-dependent manner (Fig. 7). Near maximal or maximal inhibition was achieved with 20 \(\mu\)M LY294002 and 100 nM dexamethasone and such doses were used to examine the effects on expression of cytokine and chemokine mRNAs in RBL-2H3 cells (Fig. 8A) and BMMCt (Fig. 8B). RBL-2H3 cells expressed relatively low levels of mRNA for IL-3, IL-4, IL-6, TNF-\(\alpha\), and GM-CSF. Expression of mRNA for these cytokines increased >10-fold 60 min after Ag stimulation. Prior treatment of cells with dexamethasone (100 nM for 18 h) or LY294002 (20 \(\mu\)M for 30 min) largely prevented the increase in the mRNAs. Interestingly, RBL-2H3 cells expressed relatively high levels of mRNA for IFN-\(\gamma\), TGF-\(\beta\)1, TGF-\(\beta\)2, and macrophage migration inhibitory factor without stimulation. Stimulation with Ag and treatment with dexamethasone or LY294002 had little effect on levels of mRNA for these cytokines. These results indicated that the induction of cytokine/chemokine mRNA by Ag was readily suppressed by dexamethasone or LY294002, whereas the constitutive production of cytokine/chemokine mRNA was resistant to the actions of either agent. A similar pattern was apparent in BMMCt although the pattern of inducible and noninducible cytokine/chemokine mRNA was different. These cells produced mRNA for TGF-\(\beta\)1 and LIF constitutively, and Ag stimulation induced an \(\sim 2\)-fold increase in levels of these mRNAs. Dexamethasone and LY294002 totally or partially suppressed this increase. All other cytokine/chemokine mRNAs were markedly induced by Ag and this induction was partially or largely abrogated by both agents.

**Discussion**

We find that prior exposure of mast cells to low concentrations of dexamethasone markedly inhibits activation of PI3-kinase and downstream signaling events in response to Ag stimulation. This inhibition is accompanied by loss of functional responses to Ag such as degranulation and production of cytokines and is achieved with 5 to 20 nM dexamethasone or concentrations that fall within the range of therapeutic plasma levels of dexamethasone in humans, i.e., 10–20 nM (44). Dexamethasone appears to act through the glucocorticoid receptor by an undefined mechanism that is linked to failure of PI3-kinase to associate with the scaffolding adaptor protein Gab2.

The inhibitory effects of dexamethasone are receptor specific in so far as PI3-kinase-dependent phosphorylation of Akt is not inhibited but rather enhanced when cells are stimulated through the growth receptor c-KIT by SCF (data in this study). Dexamethasone treatment also enhances the responses of RBL-2H3 cells to stimulation through the G protein-coupled adenosine A\(_1\) receptor (18). Therefore, the actions of dexamethasone are not inherently inhibitory but must affect systems unique to FceRI signaling processes of which the interaction of PI3-kinase with Gab2 is a likely candidate. Analogous to the effects of dexamethasone, mast cells from Gab2\(^{−/−}\) mice exhibit substantial reduction in degranulation and cytokine gene transcription in response to activation through FceRI (21). This defect is attributable to marked impairment of PI3-kinase activation and indicates the absolute requirement for Gab2 in the activation of PI3-kinase via FceRI in mast cells. Gab2\(^{−/−}\) mast cells also exhibit defective signaling and proliferation in response to c-KIT activation but, in contrast to FceRI, Akt phosphorylation and presumably PI3-kinase activation is only partially impaired (45). Unlike FceRI, c-KIT interacts directly with PI3-kinase and may be less dependent on Gab2 for this interaction. SCF induced autophosphorylation of c-KIT at Tyr\(^{719}\) provides a docking site for the p85 subunit to promote activation of PI3-kinase and Akt phosphorylation (46–48). The suppression of PI3-kinase activity is manifested by a decrease in levels of PIP\(_3\) in intact cells and in the activity of immunoprecipitated PI3-kinase. Downstream events such as the phosphorylation of Akt, Btk, and PLC\(_{y1}\) are inhibited as well. The impairment of PLC\(_{y1}\) and PLC\(_{y2}\) phosphorylation probably accounts for the previously observed diminished Ag-induced production of inositol 1,4,5-triphosphate, calcium signal, and degranulation in dexamethasone-treated RBL-2H3 (4, 18). Interestingly, the phosphorylation of PLC\(_{y1}\) is barely inhibited by dexamethasone to suggest that PLC\(_{y2}\), but not PLC\(_{y1}\), is regulated by PI3-kinase and that PLC\(_{y2}\) rather than PLC\(_{y1}\) may be
the predominant regulator of the calcium signal and degranulation in mast cells. These conclusions are consistent with the reports that PLC/H9253 operates independently of PI3-kinase in human mast cells (49) and that BMMC from PLC/H9253 knockout mice exhibit marked impairment of inositol 1,4,5-trisphosphate production, calcium signal, and degranulation (50). However, other workers have drawn opposite conclusions on the basis of studies with the PI3-kinase inhibitor, wortmannin, in RBL-2H3 cells (51).

If PLCγ2 is the predominant regulator of the calcium signal, the fact remains that the reduction in the calcium signal did not correlate exactly with the decreases in phosphorylation of PLCγ2 (Fig. 6F). This discrepancy could mean that other potential regulators of the calcium signal in mast cells such as PLCγ1 do participate but are less susceptible to the inhibitory effects of dexamethasone as appears to be the case for PLCγ1 (Fig. 6D). We note also that the FceRI-mediated calcium signal is regulated by sphingosine kinase (52, 53), which in turn may be regulated by phospholipase D (54) neither of which was examined in this study because the signaling links to FceRI are unclear. Nevertheless, the inhibitory effects of dexamethasone on PLCγ2 phosphorylation and degranulation were highly correlated (Fig. 6F), a possible indication that PLCγ2 phosphorylation is a rate-limiting step in degranulation.

In addition to suppression of degranulation, the second possible functional consequence of suppression of PI3-kinase activation by dexamethasone is reduced cytokine production as is indicated by the similarity in the effects of dexamethasone and the PI3-kinase inhibitor, LY294002, on cytokine transcription. Previous studies

FIGURE 6. Dexamethasone inhibits phosphorylation of Btk and PLCγ as well as the calcium signal and degranulation. A–C, RBL-2H3 cells were incubated with the indicated concentrations of dexamethasone (Dex) for 18 h before stimulation with Ag (Ag) for 15 min. Immunoblots were prepared from whole cell lysates by use of Abs that recognized the activated phosphorylated (p-) forms of Btk, PLCγ1, and PLCγ2 as listed in Materials and Methods. D, Densitometric data from three experiments (mean ± SEM) are shown and compared with the effects of dexamethasone on degranulation as measured by release of β-hexosaminidase (β-Hex). Values (mean ± SEM) are a percentage of Ag-stimulated responses in the absence of dexamethasone. E, The effects of various concentrations of dexamethasone (dex) on Ag-induced increases in cytosolic free Ca²⁺ signal were determined in fura 2-loaded cells. F, The extent of suppression of the calcium signal (area under the curves as shown in E) was calculated. The values (mean ± SEM) for three experiments are compared with suppression of PLCγ2 phosphorylation and degranulation.

FIGURE 7. Dexamethasone and LY294002 inhibit degranulation and, at comparable concentrations, Akt phosphorylation. RBL-2H3 cells were incubated with IgE and the indicated concentrations of dexamethasone for 18 h or LY294002 for 30 min or left untreated before stimulation with Ag for 15 min for measurement of release of the granule marker β-hexosaminidase and phosphorylation of Akt on Ser473 (S473) as described for previous figures. Values are the mean ± SEM from three experiments.
FIGURE 8. Dexamethasone and LY294002 inhibit cytokine gene transcription. RBL-2H3 cells and BMMCt were exposed to 100 nM dexamethasone (18 h) and 20 µM LY294002 (30 min) before stimulation with Ag or not for 60 min in complete growth medium. mRNA for cytokines and chemokines were measured by a RNase-protection assay as described in Materials and Methods. mRNA levels in unstimulated cells (■) and drug-treated Ag-stimulated cells (□ and ■) are expressed as a percentage of mRNA levels in stimulated cells in the absence of dexamethasone or LY294002. Values are the mean value from at least two experiments. Mean SE is shown when more than two experiments were performed.

In addition to PI3-kinase, long-term exposure of mast cells to nanomolar concentrations of dexamethasone also results in marked suppression of the extracellular receptor-activated kinase (ERK) pathway and ERK2-dependent generation of arachidonic acid and TNF-α in RBL-2H3 cells (5, 59, 62). Other reports indicate that mitogen-activated protein kinases such as ERK2 and related functional responses are down-regulated by dexamethasone in a variety of cell types (17). A comparison of present with previous work (5, 59) indicates that dexamethasone is an equally potent suppressant of PI3-kinase activation as it is of the ERK2 pathway in RBL-2H3 cells. The disruption of these key signaling events could therefore contribute to the anti-inflammatory effects of glucocorticoids in addition to the well-documented effects of these drugs on gene transcription.

In conclusion, we have shown that treatment with dexamethasone suppresses FceRI-mediated activation of PI3-kinase, which may account for the diminished ability of mast cells to degranulate and to produce cytokines in response to Ag. We also note, contrary to present assumptions, that PLCγ2 and not PLCγ1 may be the primary regulator for the generation of a calcium signal and degranulation. Furthermore, PLCγ2 is probably activated through the Gab2-PI3-kinase multimeric signaling complex, which appears to be the primary site of action of dexamethasone, rather than the LAT complex.

References
SUPPRESSION OF PI3'-KINASE BY DEXAMETHASONE


