THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 274, No. 24, Issue of June 11, pp. 17202–17208, 1999
Printed in U.S.A.

Dexamethasone Alters Arachidonate Release from Human Epithelial Cells by Induction of p11 Protein Synthesis and Inhibition of Phospholipase A_2 Activity*

(Received for publication, December 23, 1998, and in revised form, March 26, 1999)

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The effect of the glucocorticosteroid, dexamethasone, on arachidonic acid (AA) release and on protein levels of p11 and cytosolic phospholipase A_2 ($cPLA_2$) was studied in two epithelial cell lines, HeLa cells and BEAS-2B cells. Dexamethasone treatment of HeLa cells and BEAS-2B cells increased cellular p11 protein and mRNA levels in a time- and dose-dependent manner. It had little effect on levels of $cPLA_2$ protein. In order to determine if increased p11 protein expression resulted in increased interaction between p11 and $cPLA_2$, anti-$cPLA_2$ antibodies were used to immunoprecipitate p11·$cPLA_2$ complexes and Western blots of the immunoprecipitate were used to detect p11. In cells treated with dexamethasone, more p11 was detected in the anti-$cPLA_2$ immunoprecipitate compared with control cells. Dexamethasone treatment of HeLa cells prelabeled with [^{3}H]AA decreased the release of [^{3}H]AA under basal conditions and after stimulation with the calcium ionophore A23187 (10^{-6} M). In order to determine if altering the p11 protein levels in HeLa cells independent of glucocorticosteroid treatment could also produce an effect on [^{3}H]AA release, cells were stably transfected with plasmids expressing either p11 antisense mRNA or p11 mRNA. Cloned HeLa cells expressing p11 antisense mRNA exhibited less cellular p11 protein compared with control cells and greater [^{3}H]AA release compared with cells transfected with a control vector. Cloned HeLa cells stably transfected with a p11 expression vector exhibited increased p11 cellular protein and diminished [^{3}H]AA release under basal conditions and in response to A23187. Therefore, dexamethasone alteration of epithelial cell AA release may be due in part to induction of p11 protein expression.

Phospholipase A_2s (PLA_2s)[1] are a group of enzymes that hydrolyze the ester bond of fatty acids from the *sn*-2 position of glycerophospholipids. The release of arachidonic acid (AA) from membranes by PLA_2 and its subsequent conversion into leukotrienes, prostaglandins, and other eicosanoids plays an important role in inflammation (1–4). The mammalian calcium-dependent PLA_2s can be grouped into major classes based on their molecular mass and cellular distribution, including the low molecular mass (10–14 kDa) secreted forms ($sPLA_2$) and the structurally unrelated high molecular mass (85 kDa) cytosolic PLA_2 ($cPLA_2$) (1, 3, 5).

To date, five different $sPLA_2$ isozymes have been described in mammalian cells. The 14-kDa $sPLA_2$ enzyme from synovial fluid and platelets (Group IIA) may be involved in the pathogenesis of inflammatory reactions (3, 6, 7). The 14-kDa PLA_2 lacks apparent selectivity for the *sn*-2 fatty acids of phospholipids and requires much higher Ca^{2+} concentrations (millimolar) than normal intracellular Ca^{2+} levels (nanomolar to micromolar) for activity. The 85-kDa high molecular mass $cPLA_2$ has higher selectivity to hydrolyze phospholipids containing AA esterified in the *sn*-2 position (1, 3, 5, 8–11). Its activity is regulated by phosphorylation, G-protein activation, and physiologically relevant concentrations of calcium. Because $cPLA_2$ may play a central role in producing AA and lysophospholipid for subsequent metabolism to prostaglandins, leukotrienes, hydroxyeicosatetraenoic acids, and platelet-activating factor, all potent lipid mediators of inflammation, the activation of $cPLA_2$ may play an important role in modulating the airway inflammatory response (1, 3, 5).

S-100 proteins are a family of proteins first described by Moore (12) who initially characterized a group of abundant low molecular weight (10–12 kDa) acidic proteins in neural tissue. S-100 proteins are a group of Ca^{2+}-binding proteins that are expressed in a cell type-dependent fashion. This family includes S-100α, S-100β, and p11/calpactin light chain (13). p11 was described as a member of the S-100 family of EF hand type Ca^{2+}-binding proteins but does not have the ability to bind Ca^{2+} ions due to crucial amino acid deletions and substitutions in the two EF hand loops of the protein (14, 15). p11 binds to and inhibits the phosphorylation of a 36-kDa protein known as p36, also known as annexin II as well as calpactin heavy chain (16, 17).

Glucocorticoids are effective in the treatment of immune and inflammatory disorders affecting the lung and other organs. One mechanism of glucocorticoid modulation of the inflammatory response is inhibition of the release of AA from cellular lipids (18, 19) and inhibition of prostaglandin H synthase-2 synthase or cyclooxygenase-2 expression in a number of tissues (20–24). The rate of eicosanoid synthesis may be regulated by the availability of free AA that can be metabolized into prostanoids and leukotrienes via the cyclooxygenase and lipoxygenase pathways. The decreased synthesis of bioactive eicosanoids may represent an important mechanism of the anti-inflammatory action of glucocorticoids. Glucocorticoids can induce annexins which might inhibit $sPLA_2$ activity *in vitro* (25–30). A recent study has demonstrated that p11 can directly

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[1] The abbreviations used are: PLA_2, phospholipase A_2; $cPLA_2$, cytosolic PLA_2; $sPLA_2$, secreted PLA_2; AA, arachidonic acid; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; HBSS, Hanks' balanced salt solution; RPA, ribonuclease protection assay; bp, base pair(s); GAPDH, glyceradehyde 3-phosphate dehydrogenase; BSA, bovine serum albumin; HPLC, high-pressure liquid chromatography.

interact with the COOH-terminal region of 85-kDa $cPLA_2$ and inhibit $cPLA_2$ enzyme activity (31). Therefore, it was of interest to study whether p11 plays a role in glucocorticoid induced changes in cellular arachidonate release.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in DMEM medium with 10% fetal bovine serum. BEAS-2B cells, a human bronchial epithelial cell line, were a gift from Curtis Harris and John Lechner, National Cancer Institute, Bethesda, MD. BEAS-B cells were grown in LHC-8 medium (Biofluids, Rockville, MD) without hydrocortisone or serum. All experiments were performed when cells were 80% confluent.

Immunoblot of p11 Protein—HeLa or BEAS-2B cells were grown on 175-cm^2 flasks and treated with dexamethasone (Calbiochem) (10^{-7}, 10^{-9}, and 10^{-11} M) for 24, 36 or 48 h. For time course experiments, the culture medium was changed at the same time, and all cells were harvested at the same time. Dexamethasone (10^{-7} M) was added at the indicated times prior to harvesting. At the indicated times treated and control cells were rinsed three times with cold PBS. After washing, the cells were transferred to 0.5 ml of homogenization buffer; 50 mM Hepes, pH 8.0, 1 mM EDTA, 1 mM EGTA, 100 μM leupeptin, 1 mM dithiothereitol, 10 mM phenylmethylsulfonyl fluoride, 0.5 mM soybean trypsin inhibitor, 15 mM aprotinin, and 0.5% Triton X-100. Cells in homogenization buffer were sonicated for 15 s times three using a microprobe. Total protein was assayed by BCA reagent (Pierce). Samples containing 20 μg of cell lysate protein were separated on 18% Tris-glycine gels (Novex, San Diego, CA) using Tris-glycine SDS running buffer. The separated proteins were electrophoretically transferred onto a nitrocellulose membrane (Novex), then blocked with 5% non-fat dry milk overnight. p11 protein expression was detected by using 1:2000 dilution of mouse-anti-p11 monoclonal antibody (Transduction Laboratories, Lexington, KY) and 1:5000 dilution horseradish-peroxidase-conjugated donkey-anti-mouse IgG as second antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The blot was developed using the ECL Western blotting detection system (Amersham Pharmacia Biotech).

Immunoblot of $cPLA_2$ Protein—HeLa cells grown in 175-cm^2 flasks were treated with dexamethasone (10^{-7}, 10^{-9}, and 10^{-11} M) for 24, 36, or 48 h. At the indicated times, crude cytosolic extracts of treated and control cells were prepared as described above for the immunoblot of p11 protein. Samples containing 20 μg of cell lysate protein were separated on 8% Tris-glycine gels (Novex) using Tris-glysine SDS running buffer. $cPLA_2$ protein expression was detected by using 1:1000 dilution rabbit-anti-human $cPLA_2$ polyclonal antibody (provided by the Genetics Institute, Boston, MA) and 1:5000 dilution of horseradish-peroxidase-conjugated goat-anti-rabbit IgG as a second antibody (Jackson ImmunoResearch Laboratories, Inc.). The blot was developed using the ECL Western blotting detection system.

Immunoprecipitation of Native p11 Protein from HeLa and BEAS-2B Cells—The HeLa or BEAS-2B cells grown on 175-cm^2 culture flasks were washed with cold PBS, and the cells were lysed in 0.5 ml of homogenization buffer with protease inhibitors, but without EGTA and EDTA. The crude cytosolic protein was isolated as described above for immunoblot for p11 protein. For immunoprecipitation, the isolated crude cytosolic fraction (200 μl, 400 μg of protein) was added to a microcentrifuge tube containing 1 ml HBSS (with calcium and magnesium) and 10 μl of rabbit anti-human $cPLA_2$ antibody. The samples were incubated at 4 °C for 30 min, 25 μl of Protein G Plus/Protein A-agarose (Pierce) was then added to each sample, and the mixture was incubated at 4 °C for 4 h, followed by centrifugation in a microcentrifuge at 2500 rpm for 5 min at 4 °C. The supernatant was aspirated, and the pellet was washed four times with 1.0 ml cold PBSTDS (phosphate-buffered saline, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) with repeated centrifugation. After four washings with PBSTDS, 20 μl of protein loading buffer was added to the pellet and the sample was boiled for 10 min before electrophoresis on 16% polyacrylamide gels (Novex) using Tris-glycine/SDS buffer. The separated proteins were electrophoretically transferred onto a nitrocellulose membrane blocked with 5% non-fat milk and then probed with a 1:2000 dilution of mouse anti-human p11 monoclonal antibody. The blots were then probed with a 1:5000 dilution of horseradish peroxidase-labeled donkey anti-mouse IgG and developed by using the ECL Western blotting detection system.

Ribonuclease Protection Assay (RPA) for $cPLA_2$ and p11 mRNA Levels—The HeLa cells were treated with dexamethasone (10^{-7}, 10^{-9}, and 10^{-11} M) for 24 to 48 h. Total cellular RNA was extracted from 175 cm^2 culture flasks by the single step guanidinium thiocyanate-phenol-chloroform extraction method (Tri-reagent, Molecular Research Inc., Cincinnati, OH). The RNA pellet was precipitated with isopropyl alcohol, washed with 70% ethanol, and redissolved in diethyl pyrocarbonate water. To construct the probe for $cPLA_2$ mRNA, a 306-bp product of $cPLA_2$ cDNA was amplified by polymerase chain reaction using the following sets of sense and antisense primers: 5′ primer, 5′-CTCACACCACAGAAAGTTAAAAGAT-3′(799–823); 3′ primer, 5′-AAATAAGTCGGGAGCCATAAA-3′ (1104–1084) (Biosynthesis Inc., Lewisville, TX). The product for $cPLA_2$ gene was cloned into the TA cloning vector (Invitrogen, San Diego, CA). Orientation of the insert was determined by DNA sequencing. To construct the probe for p11 mRNA, a 320-bp product of p11 cDNA was amplified by polymerase chain reaction using the following sets of sense and antisense primers: 5′ primer, 5′-ACCACACCAAAATGCCATCTC-3′(101–121); 3′ primer, 5′-CTGCTCATTTCTGCCTACTT-3′ (400–419) (Genosys Biotechnologies, Inc., The Woodlands, TX). The product for p11 was cloned into the pGEM-T Easy Vector (Promega, Madison, WI). Orientation of the insert was determined by DNA sequencing. The $cPLA_2$ and glyceradehyde 3-phosphate dehydrogenase (GAPDH) RNA probes were prepared by *in vitro* transcription using T7 polymerase with [α-^{32}P]CTP. The p11 RNA probes was prepared by *in vitro* transcription using SP6 polymerase with [α-^{32}P]CTP. An RPA assay kit (RPAII, Ambion, Austin Texas) was used. Hybridization was performed at 45 °C for 16 h and with 10 μg (for GAPDH) or 20 μg (for $cPLA_2$) and 40 μg (for p11) of total RNA. 10^4 dpm (for GAPDH) and 2×10^4 dpm (for $cPLA_2$ and p11) of ^{32}P-labeled RNA probe were used. After hybridization, the unhybridized RNA was digested by addition of 1:100 diluted RNaseA/T1 mix at 37 °C for 60 min. Digestion was terminated by the addition of RNase inactviation and precipitation mixture. The protected RNA fragment was analyzed by autoradiography after separation on 6% polyacrylamide, 8 M urea gels (Novex).

Effect of RU486 on Dexamethasone-induced p11 Expression—The HeLa cells grown on 175-cm^2 flasks were treated with dexamethasone (10^{-7} M) with or without the glucocorticoid receptor antagonist, RU486 (10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, and 10^{-12} M) for 24 h. At the end of incubation time, crude cytosolic extracts of treated and control cells were prepared and Western blots were done as described in the experimental procedures section for immunoblot of p11 protein.

Arachidonic Acid Release from Dexamethasone-treated Cells—The HeLa cells grown on T-75-cm^2 cultured flasks were labeled for 18 h with 1 μCi/ml [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid ([^{3}H]AA)(214 Ci/mmol; Amersham Pharmacia Biotech) in DMEM media with 10% fetal calf serum. Subsequently, some cultures were treated with dexamethasone (10^{-7} M) for 24 h, while others were maintained as controls. Following 20-h incubation with dexamethasone, all cells were relabeled with 1 μCi/ml [^{3}H]AA for 4 h before harvesting the cells. For studies of AA release after calcium ionophore stimulation, following three washes, 12 ml of calcium ionophore A23187 (10^{-6} M) (Calbiochem) in HBSS(+) with 0.5% BSA or HBSS(+) with 0.5% BSA alone were added to each flask, and the cells were incubated at 37 °C for 30 min. The supernatant was harvested for HPLC analysis. The samples for HPLC analysis were extracted by octadecylsilane C_{18} cartridges (Sep-Pak C_{18}; Waters Associates, Milford, MA) and chromatographed by reverse phase HPLC. Individual Sep-Pak C_{18} cartridges were prepared with 15 ml of methanol followed by 5 ml of 5 mM EDTA and 10 ml of water. Samples were loaded onto the cartridges washed with 10 ml of water and eluted with 4 ml of methanol. The methanol fraction was collected and evaporated to dryness under steady flow nitrogen gas and resuspended in 200 μl of methanol for analysis by HPLC. An ultrasphere C_{18} column (4.7 × 250 mm) (Beckman Instruments) with 5-μm particle size was used. A gradient program was used with mobile phase A, water/acetonitrile/phosphoric acid (75:25:0.025), and mobile phase B, methanol/acetonitrile/trifluoroacetic acid (60:40:0.0016), at a flow rate of 1.5 ml/min. The AA fraction of HPLC elution was collected and measured for radioactivity.

Stable Transfection of a p11 Antisense Plasmid in HeLa Cells—A 321-bp cDNA corresponding to bases 115–436 of the p11 cDNA sequence was used to construct the antisense p11 expression plasmid, the insert was cloned into the mammalian expression vector pcDNA3.1(+) (Invitrogen) in the antisense orientation, giving rise to ASp11-pcDNA3.1(+). The identity and orientation of construct was confirmed by DNA sequencing. HeLa cells grown in 175-cm^2 flasks were exposed to 120 μl of LipofectAMINE Reagent (Life Technologies, Inc.) with 20 μg of ASp11-pcDNA3.1(+) plasmid after repeated washing with serum-free DMEM medium. Control cells were transfected with pcDNA3.1(+) expression plasmid alone. Cells were exposed to the mixture of LipofectAMINE and plasmid for 4 h. Following removal of the transfection reagent, fresh DMEM with 10% serum and 1000 μg/ml Geneticin (G418 sulfate) (Calbiochem) was added to each

flask. Subsequent cultures of selected HeLa cells were routinely grown in the presence of selective pressure. Transfected HeLa cells were cloned by limiting dilution and clones used for Western blot and AA release.

For [^{3}H]AA release studies, equal numbers of cells transfected with pcDNA3.1(+) vector alone as control, and the cells transfected with the p11 antisense plasmid ASp11-pcDNA3.1(+) were grown in T-75-cm^2 culture flasks. Cells were labeled for 18 h with 1 μCi/ml [^{3}H]AA in DMEM medium with 10% fetal calf serum and 1000 μg/ml Geneticin. Following repeated washing with media, 12 ml of fresh medium with 10% serum and 1000 μg/ml Geneticin were added to each flask. For studies of AA release after calcium ionophore stimulation, following repeated washing with HBSS(+) with 0.5% BSA for three times, 12 ml of calcium ionophore A23187 (10^{-6} M) in HBSS(+) with 0.5% BSA or HBSS with 0.5% BSA without A23187 were added to each flask, and the cells were incubated at 37 °C for 30 min. The supernatants were extracted by Sep-Pak C_{18} cartridges and chromatographed by reverse phase HPLC as described above. The AA fraction of HPLC elution was collected and measured for radioactivity.

Stable Transfection of a p11 Expression Plasmid in HeLa Cells—A cDNA containing the coding region of the p11 gene was cloned into the mammalian expression vector pcDNA3.1(+) (Invitrogen) to create p11-pcDNA3.1(+). The identity and orientation of construct was confirmed by DNA sequencing. The pcNDA3.1(+) vector carries the human cytomegalovirus immediate early enhancer-promoter sequences to promote constitutive expression of the cloned p11 insert in mammalian cells. The HeLa cells grown in 175-cm^2 flasks were exposed to 120 μl of LipofectAMINE Reagent (Life Technologies, Inc.) with 20 μg of p11-pcDNA3.1(+) plasmid after repeated washing with serum-free DMEM medium. Control cells were transfected with pcDNA3.1(+) expression plasmid alone. Cells were exposed to the mixture of LipofectAMINE and plasmid for 4 h. Following removal of the transfection reagent, fresh DMEM with 10% serum and 1000 μg/ml Geneticin (G418 sulfate) (Calbiochem) was added to HeLa cells. Subsequent cultures of selected HeLa cells were routinely grown in the presence of selective pressure. Transfected HeLa cells were cloned by limiting dilution and clones were used for Western blot and AA release after four passages.

For [^{3}H]AA release, equal numbers of cells transfected with pcDNA3.1(+) vector alone as control and the cells transfected with the p11 expression plasmid p11-pcDNA3.1(+) were grown in T-75-cm^2 culture flasks. Cells were labeled for 18 h with 1 μCi/ml [^{3}H]AA in DMEM medium with 10% fetal calf serum with 1000 μg/ml Geneticin. For studies of AA release after calcium ionophore stimulation, following three washes with HBSS(+) with 0.5% BSA, 12 ml of calcium ionophore A23187 (10^{-6} M) in HBSS(+) with 0.5% BSA or HBSS(+) with 0.5% BSA alone were added to each flask, and the cells were incubated at 37 °C for 30 min. The supernatants were collected, extracted on Sep-Pak C_{18} cartridges and chromatographed on reverse phase HPLC as described above. The AA fraction of HPLC elution was collected and measured for radioactivity.

RESULTS

Dexamethasone Increases p11 Protein Levels in Human HeLa Cells and BEAS-2B Cells—The effect of dexamethasone treatment on human epithelial cell expression of p11 was studied by Western blot of two different epithelial cell lines, HeLa cells and BEAS-2B cells. Fig. 1*A* demonstrates the effect of dexamethasone treatment of HeLa cells on cellular p11 accumulation. Treatment of cells with dexamethasone (10^{-7} M) for 24–48 h resulted in a significant increase in p11 protein expression in cell lysates. In addition, treatment of cells with 10^{-7}, 10^{-9}, and 10^{-11} M dexamethasone for 24 h resulted in a dose-related increase cellular p11 protein levels (Fig. 1*B*). Treatment of BEAS-2B cells with dexamethasone (10^{-7} M) for 24–48 h also resulted in a significant increase in p11 protein expression in cell lysates (Fig. 1*C*).

Effect of Dexamethasone on Steady State Levels of p11 mRNA—Steady state levels of mRNA for p11 were measured by RPA of total cellular RNA extracted from HeLa cells that were incubated without or with dexamethasone (10^{-7} M) for 24–48 h. As shown in Fig. 2*A*, these cells produce p11 mRNA and the steady state level of p11 mRNA was increased by dexamethasone treatment over 24–48 h. In addition, dexamethasone in concentrations of 10^{-7} to 10^{-11} M induced a dose-related change in p11 mRNA levels (Fig. 2*B*).

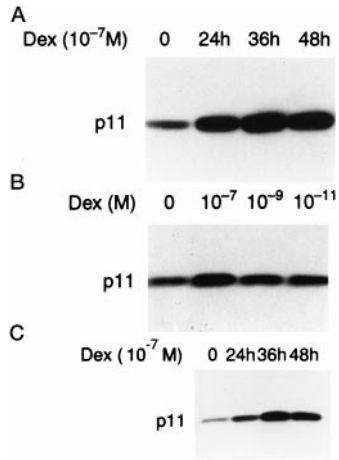

FIG. 1. **The effect of dexamethasone on p11 protein levels.** *A*, the effect of dexamethasone on p11 protein levels in HeLa cells. Cells were grown to near confluence and then treated with dexamethasone (10^{-7} M) for 24–48 h. Cell lysates from treated and untreated cells were processed as described under "Experimental Procedures," and 20 μg of total protein was subjected to gel electrophoresis and immunoblotting. *B*, the dose effect of dexamethasone on p11 protein levels in HeLa cells. Cells were grown to near confluence and then treated with dexamethasone (10^{-7} to 10^{-11} M) for 24 h. Cell lysates from treated and untreated cells were processed as described under "Experimental Procedures" and 20 μg of total protein was subjected to gel electrophoresis and immunoblotting. *C*, the effect of dexamethasone on p11 protein levels in BEAS-2B cells. Cells were grown to near confluence and then treated with dexamethasone (10^{-7} M) for 24–48 h. Cell lysates from treated and untreated cells were processed as described under "Experimental Procedures," and 20 μg of total protein was subjected to gel electrophoresis and immunoblotting.

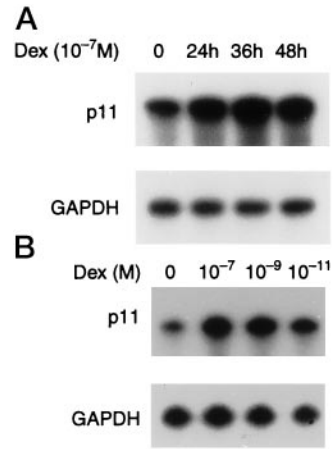

FIG. 2. **The effect of dexamethasone on p11 mRNA levels in HeLa cells.** *A*, the effect of dexamethasone on p11 mRNA levels. HeLa cells were treated with dexamethasone (10^{-7} M) for 24, 36, and 48 h before total RNA was extracted. Ten μg and 40 μg of the total RNA were hybridized to GAPDH and p11-specific radiolabeled cRNA probes, respectively, and assayed by RPA. The protected fragments of p11 (320 bp) and GAPDH were visualized by autoradiography. The result shown is representative of three separate experiments demonstrating the same result. *B*, the dose effect of dexamethasone on p11 mRNA levels. The HeLa cells were treated with dexamethasone (10^{-7}, 10^{-9}, and 10^{-11} M) for 24 h before total RNA was extracted. Ten μg or 40 μg of the total RNA were hybridized to GAPDH and p11-specific radiolabeled RNA probes and assayed by RPA. The protected fragments of p11 (320 bp) were visualized by autoradiography. The result shown is representative of three separate experiments.

Effect of Dexamethasone on cPLA$_2$ Protein and mRNA Levels in HeLa Cells—The effect of dexamethasone treatment on human epithelial cell expression of cPLA$_2$ was studied by Western blot of cell lysates. Treatment of cells with dexamethasone

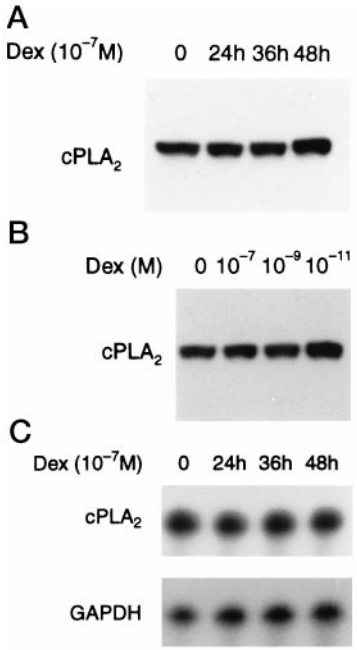

FIG. 3. **The effect of dexamethasone on cPLA$_2$ protein and steady state mRNA levels.** *A*, the effect of dexamethasone on cPLA$_2$ protein levels in HeLa cells. Cells were grown to near confluence and then treated with dexamethasone (10^{-7} M) for 24–48 h. Cell lysates from treated and untreated cells were processed as described under "Experimental Procedures," and 20 μg of total protein was subjected to gel electrophoresis and immunoblotting. *B*, the dose effect of dexamethasone on cPLA$_2$ protein levels in HeLa cells. Cells were grown to near confluence and then treated with dexamethasone (10^{-7} to 10^{-11} M) for 24 h. Cell lysates from treated and untreated cells were processed as described under "Experimental Procedures," and 20 μg of total protein was subjected to gel electrophoresis and immunoblotting. *C*, the effect of dexamethasone on cPLA$_2$ mRNA levels. HeLa cells were treated with dexamethasone (10^{-7} M) for 24, 36, and 48 h before total RNA was extracted. Ten μg and 20 μg of the total RNA were hybridized to GAPDH and cPLA$_2$-specific radiolabeled cRNA probes, respectively, and assayed by RPA. The protected fragments of cPLA$_2$ (306 bp) and GAPDH were visualized by autoradiography. The result shown is representative of three separate experiments.

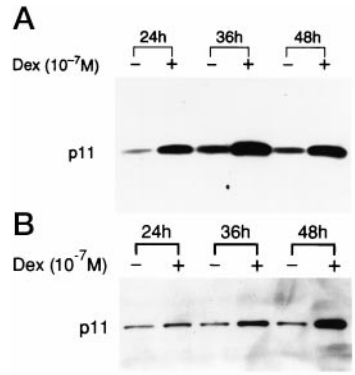

FIG. 4. **Immunoprecipitation of p11·cPLA$_2$ complex.** *A*, immunoprecipitation of the p11·cPLA$_2$ complex from HeLa cells. Cell lysates from untreated cells and cells treated with dexamethasone (10^{-7} M) for 24, 36, and 48 h were incubated with rabbit anti-human cPLA$_2$, 25 μl of Protein G Plus/Protein A-agarose beads was then added to each sample for further incubation. The beads were collected, washed, and subjected to SDS-polyacrylamide gel electrophoresis. The precipitated p11 protein was then detected by Western blotting analysis as described under "Experimental Procedures." The position of p11 protein is indicated. *B*, immunoprecipitation of the p11·cPLA$_2$ complex from BEAS-2B cells. Lysates from untreated cells and cells treated with dexamethasone (10^{-7} M) for 24, 36, and 48 h were incubated with rabbit anti-human cPLA$_2$, and 25 μl of Protein G Plus/Protein A-agarose beads was then added to each sample for further incubation. The beads were collected, washed, and subjected to SDS-polyacrylamide gel electrophoresis. The precipitated p11 protein was then detected by Western blotting analysis as described under "Experimental Procedures." The position of p11 protein is indicated.

(10^{-7} M) for 24–48 h had no effect on cPLA$_2$ protein expression (Fig. 3*A*). In addition, treatment of cells with 10^{-7}, 10^{-9}, and 10^{-11} M dexamethasone for 24 h did not result in a change in cPLA$_2$ protein levels (Fig. 3*B*). The effect of dexamethasone treatment on human epithelial cell expression of cPLA$_2$ was also studied by RPA of HeLa cells treated with dexamethasone. Fig. 3*C* demonstrates the effect of dexamethasone treatment of HeLa cells on steady state levels of cPLA$_2$ mRNA. Treatment of cells with dexamethasone (10^{-7} M) for 24–48 h had no clear effect on levels of cPLA$_2$ mRNA.

Dexamethasone Increases p11 Bound to cPLA$_2$—The above results demonstrated that dexamethasone treatment increased p11 expression, but had little or no effect on cPLA$_2$ expression. To further investigate the interaction between p11 and cPLA$_2$ in human epithelial cells, immunoprecipitation of the p11·cPLA$_2$ complex from HeLa cells and BEAS-2B cells was performed. As shown in Fig. 4, *A* and *B*, p11 was precipitated from the HeLa cell and BEAS-2B cell lysates by rabbit anti-human cPLA$_2$ polyclonal antibody followed by the addition of Protein G Plus/Protein A-agarose. Immunoblots of the purified complex were developed for p11 protein. There was more p11 coprecipitated with cPLA$_2$ after dexamethasone treatment. This result demonstrated that dexamethasone treatment resulted not only in an increase in cellular p11 protein but also in an increase in p11 bound to cPLA$_2$.

RU486 Inhibits Dexamethasone-induced p11 Protein Increases—In an attempt to determine if the effect of dexamethasone on p11 protein levels is mediated via a glucocorticoid receptor interaction, RU486 (10^{-7} to 10^{-12} M) was incubated with cells prior to and concomitant with the dexamethasone treatment. Treatment with RU 486 resulted in a dose-dependent inhibition of the dexamethasone-induced increases in p11 protein levels. Fig. 5 shows the effect of RU486 (10^{-10}-10^{-12} M) on dexamethasone-induced p11 protein levels.

Dexamethasone Inhibits AA Release from the HeLa Cells—The results from RPA and Western blot studies indicated that dexamethasone treatment had an effect on p11 mRNA levels and protein production but little or no effect on the mRNA expression or protein level of cPLA$_2$. In these cells, dexamethasone treatment does alter the release of ^{3}H-labeled AA both at base line and after exposure to the calcium ionophore A23187. Fig. 6 demonstrates that labeled AA release from dexamethasone-treated HeLa cells (*HD*) is lower than that from untreated HeLa cells (*HC*). After treatment with A23187, the release of labeled AA from dexamethasone-treated HeLa cells (*HD*+*A*) is significantly decreased compared with untreated control cells (*HC*+*A*).

Antisense Inhibition of p11 Increases AA Release—We have shown that dexamethasone increases p11 expression and inhibits PLA$_2$ activity *in vitro*. It has been reported that p11 can bind to cPLA$_2$ and inhibit cPLA$_2$ activity *in vitro*. In order to study whether dexamethasone might alter cPLA$_2$ activity in part by increasing p11 expression in human cells, we performed two studies. First, we constructed a p11 antisense plasmid and then stably transfected HeLa cells to examine the AA release in these cells. Western blot studies of cloned transformed cells showed that p11 protein production was decreased in HeLa cells which were transfected with ASp11-pcDNA3.1(+) plasmid compared with HeLa cells, which were transfected with pcDNA3.1(+) plasmid alone (Fig. 7*A*). There was no change in cPLA$_2$ expression in these cells (Fig. 7*B*). [^{3}H]AA release from the HeLa cells that were permanently transfected with p11 antisense plasmid was increased both at base line and

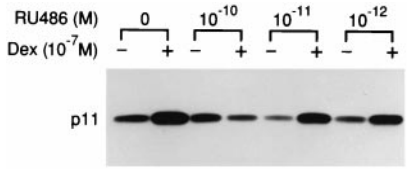

FIG. 5. **The effect of RU486 treatment on dexamethasone-induced p11 protein levels.** Cells were grown to near confluence. RU486 was incubated with cells prior to and concomitant with or without dexamethasone (10^{-10} to 10^{-12} M) for 24 h. Cell lysates from treated and untreated cells were processed as described under "Experimental Procedures," and 20 μg of total protein was subjected to gel electrophoresis and immunoblotting.

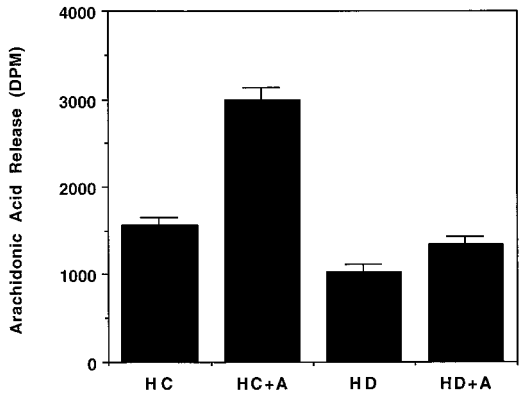

FIG. 6. **[^{3}H]AA release from dexamethasone treated HeLa cells.** The cells grown in T-75-cm^2 flasks were labeled for 18 h with 1 μCi/ml [^{3}H]AA in 12 ml of DMEM medium and then treated with 10^{-7} M dexamethasone for 24 h. After repeated washing, the cells were then incubated with 10^{-6} M ionophore A23187 in 12 ml of HBSS (containing 1.3 mM Ca^{2+}) for 30 min, and the supernatants were extracted by Sep-Pak C_{18} cartridges and chromatographed by HPLC as described under "Experimental Procedures." Data were expressed as AA release measured separately from 11–12 individual flasks from two separate sets of experiments. *HC* = HeLa control cells; *HD* = HeLa cells treated with dexamethasone; *A* = treatment with A23187. $p < 0.001$ for HC *versus* HD; $p < 0.001$ for HC+A *versus* HD+A.

after exposure to the calcium ionophore A23187 compared with control cells (Fig. 8).

Increased p11 Expression Inhibits AA Release—In order to determine whether dexamethasone inhibition of cellular PLA_2 activity might be related in part to increasing p11 expression in human epithelial cells, we constructed a p11 expression plasmid and stably transfected HeLa cells to examine the effect of increased p11 expression on AA release in these cells. The effect of the p11 expression plasmid on cellular p11 protein is demonstrated in Fig. 9A. Western blot results showed that p11 protein production was increased in HeLa cells that were transfected with p11-pcDNA3.1(+) plasmid compared with HeLa cells that were transfected with pcDNA3.1(+) plasmid alone. There was no change in $cPLA_2$ expression (Fig. 9*B*). AA release from the HeLa cells which were permanently transfected with p11 expression plasmid was decreased both at base line and after exposure to the calcium ionophore A23187 (Fig. 10). Therefore, dexamethasone treatment increases p11 protein expression and reduces cellular arachidonate release. Furthermore, increasing cellular p11 protein production independent of dexamethasone treatment reduces cellular AA release as well.

DISCUSSION

p11, or calpactin light chain, is a member of the S-100 family small calcium binding proteins; however, it has several unique features. S-100 proteins contain two EF hands that function as calcium binding domains (13). p11 does not have the ability to bind Ca^{2+} ions due to amino acid deletions and substitutions in

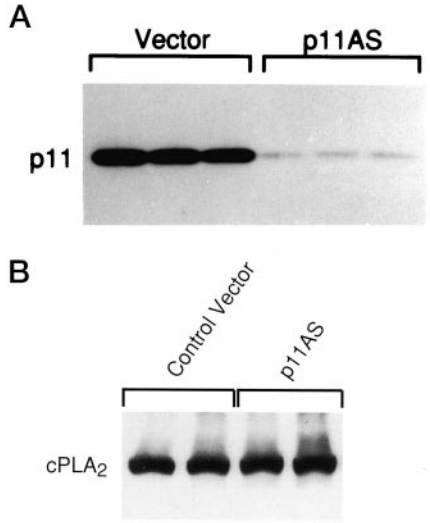

FIG. 7. **The p11 and $cPLA_2$ protein levels in HeLa cells transfected with a p11 antisense plasmid.** *A*, cell lysates from cells transfected with the p11 antisense plasmid or cells transfected with a control vector were processed as described under "Experimental Procedures," and 20 μg of cell lysate protein was subjected to gel electrophoresis and immunoblotting for p11 protein. Three different samples of cell lysates of p11 antisense cells and control cells were processed. B, protein levels of $cPLA_2$ in HeLa cells transfected with a p11 antisense plasmid. Cell lysates from cells transfected with the p11 antisense plasmid or cells transfected with a control vector were processed as described under "Experimental Procedures," and 20 μg of cell lysate protein was subjected to gel electrophoresis and immunoblotting for $cPLA_2$.

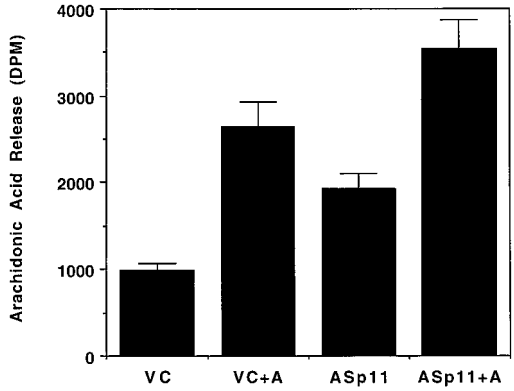

FIG. 8. **[^{3}H]AA release from HeLa cells transfected with a p11 antisense plasmid.** The cells grown in T-75-cm^2 flasks were labeled for 18 h with 1 μCi/ml [^{3}H]AA in 12 ml of DMEM with Geneticin. After repeated washing, some cells were then incubated with 10^{-6} M ionophore A23187 in 12 ml of HBSS (containing 1.3 mM Ca^{2+}) or with HBSS without A23187 for 30 min, and the supernatants were extracted by Sep-Pak C_{18} cartridges and chromatographed by HPLC as described under "Experimental Procedures." Data were expressed as AA release measured separately from 10 to 12 individual flasks in each group. *VC* = vector control cells; *ASp11* = HeLa cells transfected with a plasmid expressing p11 antisense mRNA; *A* = HeLa cells treated with A23187. $p < 0.001$ for VC *versus* ASp11; $p < 0.05$ for VC+A *versus* ASp11+A.

the two EF hand motifs (14, 15). Instead, p11 is present in a variety of cells separately or as a heterotetramer binding to annexin II. The heterotetramer is composed of two copies of the 36-kDa heavy chain, annexin II subunits and two copies of 11-kDa light chain, p11 subunits as $(p36)_2(p11)_2$ (32, 33).

Glucocorticosteroids are potent anti-inflammatory agents. This anti-inflammatory effect may be produced via a variety of mechanisms. A group of structurally related, calcium-dependent phospholipid-binding proteins, annexins, which were formerly known as lipocortins or calpactins, had been shown to be

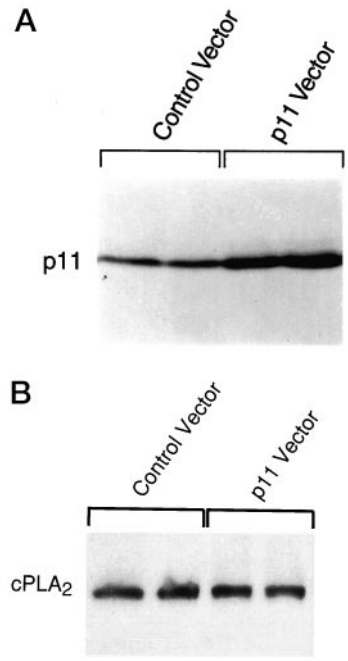

FIG. 9. **The p11 and cPLA$_2$ protein levels in HeLa cells transfected with a p11 expression plasmid.** *A*, cell lysates from cells transfected with the p11 expression vector or cells transfected with control vector were processed as described under "Experimental Procedures," and 20 μg of total protein was subjected to gel electrophoresis and immunoblotting for p11 protein. Two cell lysates from cells transfected with the p11 expression vector and cells transfected with the control vector were processed. *B*, protein levels of cPLA$_2$ in HeLa cells transfected with a p11 expression vector or cells transfected with a control vector. Cell lysates from cells transfected with the p11 expression or cells transfected with a control vector were processed as described under "Experimental Procedures," and 20 μg of cell lysate protein was subjected to gel electrophoresis and immunoblotting for cPLA$_2$.

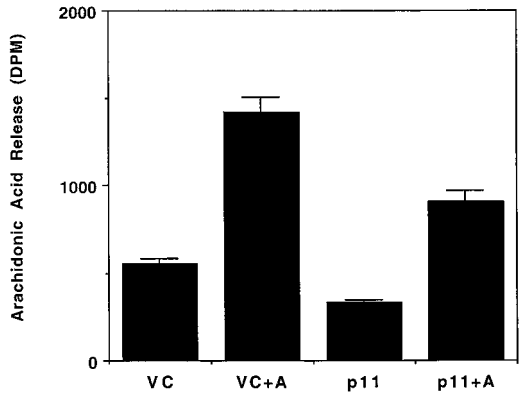

FIG. 10. **[^{3}H]AA release from HeLa cells transfected with a p11 expression plasmid.** The cells grown in T-75-cm^2 flasks were labeled for 18 h with 1 μCi/ml [^{3}H]AA in 12 ml of DMEM with Geneticin. After repeated washing, the cells were then incubated with or without 10^{-6} M ionophore A23187 in 12 ml of HBSS (containing 1.3 mM Ca^{2+}) for 30 min, and the supernatants were extracted by Sep-Pak C_{18} cartridges and chromatographed by HPLC as described under "Experimental Procedures." Data were expressed as AA release measured separately from nine individual flasks in each group. *VC* = HeLa cells transfected with control vector; *p11* = HeLa cells transfected with a p11 expression vector; *A* = HeLa cells treated with A23187. $p < 0.001$ for VC *versus* p11; $p < 0.001$ for VC+A *versus* p11+A.

inducible by glucocorticoids. Annexin I has been reported to inhibit sPLA$_2$ activity *in vitro* (25–30). These observations led to the hypothesis that the inhibition of sPLA$_2$ by annexins is the mechanism of the anti-inflammatory action of glucocorticoids. Subsequent studies failed to show a direct interaction between the 14-kDa PLA$_2$ and annexins. Instead, this inhibition may be dependent on the concentration of substrate (34, 35), the extent of inhibition being more closely related to the inhibitor:substrate rather than the inhibitor:enzyme ratio. In addition, glucocorticoid treatment suppresses the induction of Group II sPLA$_2$ expression in a variety of cells (36–40).

cPLA$_2$ selectively hydrolyzes AA from the *sn*-2-ester bond of membrane phospholipids. cPLA$_2$ may play an important role in the production of free fatty acids and lysophospholipids, precursors of eicosanoids and PAF, all of which may function as intracellular second messengers or potent inflammatory mediators (1, 3, 5). It has been reported that dexamethasone treatment reduces changes in cPLA$_2$ protein and mRNA levels induced by TNF treatment of HeLa cells (41). Dexamethasone may have other effects on AA metabolism and at earlier time points, including effects perhaps not requiring transcription such as inhibition of phosphorylation of cPLA$_2$ (42). We did not document an effect of dexamethasone on unstimulated expression of cPLA$_2$; however, we did note an effect of dexamethasone on cellular p11 protein and mRNA levels. Because it has been demonstrated that p11 can directly interact with the carboxyl region of cPLA$_2$ and inhibit its activity *in vitro* (31), we hypothesized that a part of the effect of dexamethasone on cellular AA release might be mediated by a dexamethasone-induced change in p11 protein levels.

Four lines of evidence suggest that dexamethasone may alter cellular arachidonate release in part by induction of p11 protein expression. First, studies in two different cell lines demonstrate that dexamethasone induces human epithelial cell p11 gene expression and protein production. This effect was not associated with a reduction of cPLA$_2$ expression in HeLa cells. RU486, an antagonist that competes with glucocorticoids for binding to the glucocorticoid receptor (43, 44), blocked the stimulatory effect of dexamethasone on p11 protein production, suggesting that dexamethasone-induced p11 gene expression and subsequent protein synthesis occurs via a glucocorticoid receptor-mediated pathway. Second, in dexamethasone-treated cells, there was increased p11 binding to cPLA$_2$ as evidenced by p11 which was precipitated by anti-cPLA$_2$ antibody as a p11·cPLA$_2$ complex. Third, cells stably transfected with a plasmid that expresses p11 antisense mRNA and that subsequently express less p11 protein have enhanced release of prelabeled AA both at base line and after stimulation with the ionophore A23187. Fourth, we studied the effect of p11 on AA release in the setting of overexpression of p11 protein in a human epithelial cell line, Hela cells. The release of prelabeled AA from cells that overexpressed p11 was significantly lower than that from control cells. Therefore, overexpression of p11 inhibits PLA$_2$ activity and reduces the release of AA from [^{3}H]AA-prelabeled cells. Thus, manipulation of p11 levels independent of corticosteroid therapy also alters AA release from permanently transfected cells.

AA release from cell membranes may be a complex process affected by a variety of stimuli and involving multiple enzymes and regulatory proteins. We suggest that one of these effects may be related to modulation of p11 protein production and binding to cPLA$_2$.

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