Lipocortin-independent effect of dexamethasone on phospholipase activity in a thymic epithelial cell line

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In the cloned rat thymic endocrine epithelial cell line TEA3A1, treatment with dexamethasone leads to decreased levels of prostaglandin E_2 , prostaglandin $F_{2\alpha}$, and thromboxane B_2 . Dexamethasone treatment also leads to a decrease of both calcium-dependent and calcium-independent phospholipase A_2 activity measured in a cell-free assay. Dexamethasone-treated cells also have increased levels of lipocortin-I, a putative modulator of phospholipase A_2 activity. The property of calcium-dependent binding of lipocortin to the particulate fraction was used to prepare cytosolic and particulate subcellular fractions which contained phospholipase A_2 activity but no lipocortin-I. Dexamethasone decreased phospholipase A_2 activity in both cytosolic and particulate fractions even in the absence of lipocortin, suggesting the presence of a lipocortin-independent mechanism.

INTRODUCTION

Arachidonic acid metabolites are known to be potent modulators of T cell growth and function [1–3]. Prostaglandins are also believed to play an important role in T cell development in the thymus [4–6]. The fact that prostaglandin levels in the thymus are 4–10-fold higher than in plasma [4,7] also suggests the importance of prostaglandins in thymic function.

Thymic epithelial cells have been postulated to play an important role in regulating thymic prostaglandin levels [8]. Recently, we have cloned a rat thymic epithelial cell line, TEA3A1. TEA3A1 cells synthesize the thymic hormone thymosine α_1 , as well as expressing a marker for endocrine thymic epithelium [9]. We are using the cell line as an experimental model to study regulation of arachidonate metabolism.

In this paper we examine some aspects of regulation by glucocorticoids. Glucocorticoid is a known inhibitor of arachidonate release in other cell types and tissues [10,11], and has been reported to inhibit release of prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) by primary cultures containing predominantly thymic epithelial cells [12,13]. This effect of glucocorticoids has been shown (in other tissues) to require protein synthesis, and has been presumed to be mediated by the induction and secretion of phospholipase A_2 (PLA_2) inhibitory proteins known as lipocortins [10,11]. Included in this family are well-characterized intracellular proteins which are tyrosine kinase substrates, lipocortin-I and lipocortin-II [14]. Lipocortin-I has been found in high concentrations in thymic epithelium and in TEA3A1 cells [15]. However, the involvement of intracellular lipocortin-I and related proteins as mediators of glucocorticoid inhibition of arachidonate release is still open to question. It has been shown recently that in a cell-free assay system used to measure PLA_2 inhibition, lipocortins act by binding to

the substrate rather than by directly inhibiting the enzyme [16,17].

The current study finds that glucocorticoid inhibits prostaglandin production by TEA3A1 cells and also raises the level of lipocortin-I. We also show the novel finding that glucocorticoid decreases the level of PLA_2 activity in a cell-free assay, and that this effect is independent of lipocortin-I.

MATERIALS AND METHODS

Materials

All radiolabelled substrates were purchased from New England Nuclear Research Products (Boston, MA, U.S.A.). Unlabelled L - α -palmitoyl-2-oleoyl lecithin was obtained from Avanti Polar Lipids (Birmingham, AL, U.S.A.). Calcium-free WJJC 404 medium was generously supplied by Kyokuto Pharmaceutical Co., Ltd. (Tokyo, Japan). The composition of this medium has been described by McKeehan *et al.* [18]. Preparation and characterization of specific antiserum to lipocortin-I (35 kDa epidermal growth factor receptor substrate) has been reported [19,20].

Cell culture

Culture and maintenance of the rat endocrine thymic epithelial cell line (TEA3A1) has been described in detail [9]. Stock cultures are grown and passaged in WJJC 404A medium (7.5% DMEM in calcium-free WJJC 404) supplemented with 10 μ g of insulin/ml, 10 μ g of transferrin/ml, 10 μ M-dexamethasone, 10 ng of epidermal growth factor/ml, and 2% iron-supplemented calf serum (sCS; Hyclone Labs, Logan, UT, U.S.A.). For experimental purposes, TEA3A1 cells are grown to confluence in a 1:1 mixture of DMEM and WJJC 404A supplemented with insulin and transferrin (DW-IT) plus 2%

Abbreviations used: PGE_2 , prostaglandin E_2 ; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; TXB_2 , thromboxane B_2 ; PLA_2 , phospholipase A_2 ; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; DW-IT, DMEM: WJJC404A medium (1:1, v/v) supplemented with insulin and transferrin; sCS, iron-supplemented calf serum.

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sCS. Dexamethasone was added to cultures either at the beginning or at various times before performing assays.

Enzyme assays

Confluent TEA3A1 cultures (dexamethasone-treated and controls) were washed with phosphate-buffered saline (PBS; 137 mM-NaCl/3 mM-KCl/8 mM-Na₂HPO₄/1.5 mM-KH₂PO₄), then scraped into assay buffer containing 10 mM-Tris (pH 7.5), 2 mM-EDTA, aprotinin (1 µg/ml), pepstatin (1 µg/ml) and leupeptin (1 µg/ml). The cell suspension was sonicated briefly. In some experiments the cell lysate was separated into supernatant and pellet fractions by centrifugation at 100 000 *g* for 45 min with or without added 2 mM-free calcium. Lipocortins are known to associate with the particulate fraction in the presence of calcium, or to be extracted into the supernatant fraction with calcium chelators. Samples which did not receive calcium prior to centrifugation had calcium added (2 mM excess) before incubation with substrate, unless stated otherwise. Aliquots of 150 µl of whole or fractionated cell preparation were mixed with 50 µl of radiolabelled substrate solution containing 40 or 80 nmol (0.025 µCi) of substrate sonicated in 0.5% Triton X-100. The final concentration of substrate present in the incubation mixture was 200 or 400 µM in addition to endogenous phospholipids present in cell lysates or particulate fractions. The radiolabelled substrates used included: 1-palmitoyl-2-[1-¹⁴C]oleoyl-phosphatidylcholine; 1-palmitoyl-2-[1-¹⁴C]arachidonyl-phosphatidylcholine; and 1-hexadecyl-2-[1-³H]arachidonyl-phosphatidylcholine. After incubating at 37 °C for 1 h, the reaction was stopped and reaction products were separated from unreacted substrate by a modified Dole's [21] procedure and measured by liquid scintillation counting. Blank values using substrate without cellular enzyme preparations were determined and subtracted. The amount of protein in each sample was determined by a modified Lowry assay [22]. One unit of PLA₂ activity is defined as the release of 1 nmol of free fatty acid/h from the exogenous substrate.

Preliminary experiments established that the rate of hydrolysis of the substrate was proportional to the amount of substrate used (up to 1 mg of protein for a 60 min assay) and linear with respect to time for this period. With the maximal cellular PLA₂ activity measured, approx. 30% of the substrate was hydrolysed by 60 min.

Radioimmunoassay of arachidonate metabolites

Media from cells cultured in the presence or absence of dexamethasone were assayed separately for PGE₂, PGF_{2α} and thromboxane B₂ (TXB₂) by radioimmunoassay, a modification of the procedure described by Campbell & Oieda [23]. Antibody (100 µl), ³H-labelled tracer (15000 c.p.m., 25 pg, 100 µl) and the sample to be tested or unlabelled standard (100 µl), all in assay buffer (PBS+0.1% gelatin) were mixed and incubated overnight at 4 °C. Dextran-charcoal suspension (300 µl) was added to remove the unbound antigen, and the mixture was centrifuged. Supernatant (0.5 ml) was added to 5.0 ml of scintillation cocktail and the radioactivity was measured on a scintillation counter. A standard curve was produced for each experiment. The cross-reactivities of the antisera used in these assays has been determined to be less than 0.5% (radioimmunoassay for PGE₂ also measures PGA₂, a spontaneous derivative of PGE₂).

Western blot for lipocortin-I

TEA3A1 cells were cultured to confluence in DW-IT plus 2% sCS with or without 100 nM-dexamethasone. Cultures were washed twice in PBS, then scraped and sonicated in cold 0.01 M-Tris buffer (pH 7.4) containing 0.01 M-EDTA. The cell sonicate was incubated on ice for 15 min and then centrifuged at 100 000 *g*, 4 °C for 45 min. An aliquot of supernatant was used for protein determination and the remainder was mixed with SDS sample buffer and boiled for 10 min. Samples containing 10 µg of total protein were run on SDS/polyacrylamide gel electrophoresis (SDS/PAGE) (10% acrylamide) by the method of Laemmli [24] and blotted on to nitrocellulose [25]. Nitrocellulose filters were blocked with 1% gelatin in PBS, incubated for 1 h in anti-(human lipocortin-I) antiserum (diluted 1:1000 in 1% gelatin in PBS), washed with 0.02% Tween-20 in PBS, incubated for 30 min in horseradish-peroxidase-conjugated goat anti-rabbit Ig antiserum (diluted 1:1000 in 1% gelatin in PBS; Cooper Biomedical, Malvern, PA, U.S.A.), then washed again in 0.02% Tween-20 in PBS. The presence of immunoreactive lipocortin-I on the nitrocellulose filter was revealed by incubation in solution containing the peroxidase substrate, 4-chloro-1-naphthol.

RESULTS

Effects of dexamethasone on arachidonate metabolism in intact TEA3A1 cells

The levels of several individual arachidonate metabolites released into the medium in response to dexamethasone were determined by radioimmunoassay (Table 1). It was found the dexamethasone treatment decreased levels of PGE₂ (78%), PGF_{2α} (77%) and TXB₂ (77%). The time-course for dexamethasone inhibition of PGE₂ production (Fig. 1a) shows an effect by 6–12 h which reaches a maximum by 36 h. The dose-response (Fig. 1b) shows that half-maximal inhibition occurs with 10 nM-dexamethasone.

Effect of dexamethasone treatment on PLA₂ activity

In determining whether or not the effects of dexamethasone on arachidonate metabolism in intact TEA3A1 cells could be correlated with an inhibitory effect on cellular PLA₂, we wished to avoid the complication of apparent inhibition which is due to substrate sequestering rather than regulation of enzyme activity (see Introduction). Therefore, we used high concen-

Table 1. Dexamethasone decreases levels of PGE₂, PGF_{2α} and TXB₂ secreted into the medium

Treatment	Concentration in medium (ng/ml)		
	PGE ₂	PGF _{2α}	TXB ₂
Control	2.6 ± 0.3	1.4 ± 0.3	0.77 ± 0.12
Dexamethasone	0.58 ± 0.05	0.3 ± 0.03	0.18 ± 0.02

Confluent cultures of TEA3A1 grown for 4 days with or without 10⁻⁶ M-dexamethasone were analysed for PGE₂, PGF_{2α}, and TXB₂ both in the cells and in conditioned media using radioimmunoassays. Each value was determined by assaying triplicate cultures, and values are means ± S.E.M.

Fig. 1. Dexamethasone decreases PGE₂ production by TEA3A1 cells

Confluent TEA3A1 cells cultured in DW-IT plus 2% sCS were incubated in fresh medium plus: (a) dexamethasone (1 μ M) for varying lengths of time, or (b) various concentrations of dexamethasone for 24 h. PGE₂ levels in the cells were measured by radioimmunoassay for triplicate cultures.

trations of added substrate (200 μ M) in our assay system which, in addition to the effects of endogenous phospholipids present in the cell lysate, make this possibility very unlikely. Other conditions of the assay such as detergent and buffer concentrations were chosen to optimize activity for these particular cells with the substrates that were used. Using the substrate 1-palmitoyl-2-[1-¹⁴C]arachidonyl-phosphatidylcholine, it was found that PLA₂ activity in whole lysates from dexamethasone (1 μ M)-treated cells was decreased by 55% (Table 2).

Since it is possible that enzymes may be present in the crude cell lysate which could release labelled fatty acid from the substrate by alternative indirect pathways, a whole lipid extract of the reaction mixture was further analysed by t.l.c. (results not shown). No evidence could be found for phosphatidylcholine-reactive phospholipase C activity under these conditions. However, the presence of significant amounts of labelled lysophosphatidylcholine in the reaction mixture indicated the presence of PLA₁ activity. Since PLA₁, in conjunction with lysophospholipid lipase, could contribute to the production of free arachidonate, a second substrate, 1-hexadecyl-2-[1-³H]arachidonyl-phosphatidylcholine, which is resist-

Table 2. Dexamethasone treatment decreases PLA₂ activity in TEA3A1 cells at high substrate concentration

Confluent TEA3A1 cells cultured in DW-IT plus 2% sCS were incubated for 24 h with serum-free DW-IT plus or minus dexamethasone (10⁻⁶ M). Quadruplicate cultures (whole cell lysates) were assayed with both substrates according to the enzyme assay procedure described in the Materials and methods section. Values represent means $\pm$ S.E.M. PC, phosphatidylcholine.

Substrate	PLA ₂ activity (units/mg)	
	Control	Dexa-methasone (10 ⁻⁶ M)
1-palmitoyl-2-[1- ¹⁴ C]arachidonyl-PC (200 μ M)	13.3 $\pm$ 1.3	6.0 $\pm$ 0.3
1-hexadecyl-2-[1- ³ H]arachidonyl-PC (200 μ M)	7.7 $\pm$ 0.6	2.6 $\pm$ 0.1

ant to PLA₁ hydrolysis, was used. The use of this substrate confirmed that dexamethasone treatment does decrease PLA₂ activity (66%, Table 2).

Dexamethasone increases levels of lipocortin-I in TEA3A1 cells

Since we have been able to show that the dexamethasone effect on arachidonate metabolism in TEA3A1 cells correlates with a decrease in cellular PLA₂ activity (which is not due to substrate sequestering), we were naturally interested in the possible role of lipocortin-I, which is abundant in these cells. Although glucocorticoids are known to induce secreted lipocortins in other types of cells [10,11], glucocorticoid induction of the intracellular lipocortin-I has not been shown, to our knowledge. Using specific antiserum for lipocortin-I, we were able to demonstrate that TEA3A1 cells cultured with dexamethasone do contain higher levels of lipocortin-I as analysed by Western blot (Fig. 2).

The dexamethasone effect on PLA₂ activity in the cell-free enzyme assay is independent of lipocortin

Lipocortins I and II and related proteins share the property of calcium-dependent binding to phospholipid [14,16,20,26]. When subcellular fractions are prepared in the presence of calcium, lipocortin is found in the particulate fraction; in the presence of EDTA, lipocortin appears in the soluble fractions [26]. This property has been used in the purification of lipocortin-I [20] and was confirmed here by Western blotting (Fig. 3). In Table 3, subcellular fractions prepared in the presence of either free calcium or EDTA were assayed for PLA₂ using substrate at 400 μ M concentration. Although specific activities vary between experiments, it can be seen that within each experiment, the dexamethasone effect on PLA₂ activity is just as pronounced in cell fractions from which lipocortin was absent (both supernatants and pellets). The lower apparent specific activity in the pellet fractions as compared with supernatants reflects the presence of endogenous phospholipid in the pellet, which would compete with the radiolabelled substrate.

Fig. 2. Dexamethasone increases levels of lipocortin-I in TEA3A1 cells

(a) Tris/EDTA extracts of confluent A431 and TEA3A1 cultures run on 10% acrylamide SDS/polyacrylamide-gel electrophoresis. Nitrocellulose blots were reacted with specific antiserum to lipocortin-I; 20 μ g of total protein per lane. (b) Tris/EDTA extracts of confluent TEA3A1 cultures grown in DW-IT medium + 2% sCS (lanes 1–3) or same medium plus 100 nM-dexamethasone (lanes 4–6); 10 μ g of total protein per lane.

Fig. 3. Removal of lipocortin-I from TEA3A1 subcellular fractions

TEA3A1 cells were grown to confluence in DW-IT media containing 2% sCS and 1 μ M-dexamethasone. The supernatant and pellet fractions were prepared either in the presence of EDTA or 2 mM-free calcium. Lanes: 1, EDTA supernatant; 2, EDTA pellet; 3, calcium supernatant; 4, calcium pellet.

Dexamethasone may affect several distinct phospholipases in TEA3A1 cells

Further experiments were carried out to characterize the dexamethasone-sensitive phospholipase activities in both supernatant and pellet fractions as to calcium dependence. Fractions were prepared in the presence of EDTA and were then assayed for PLA₂ in the presence or absence of 2 mM excess free calcium (Table 4). Dexamethasone treatment consistently decreased phospholipase activities in the supernatant assayed in either the presence or absence of calcium. Pellet activity in the presence of calcium was also decreased by dexamethasone. The same effect was seen whether oleic or

Table 3. The effect of dexamethasone on PLA₂ activity in the cell-free assay system is independent of the presence of lipocortins

TEA3A1 cells were grown to confluence in DW-IT plus 2% sCS, plus or minus dexamethasone (100 nM). Lipocortins were removed from the supernatant fraction of cell lysates by addition of calcium (2 mM excess) prior to centrifugation. Conversely, lipocortins were removed from the pellet fractions by preparation in the presence of 2 mM-EDTA without calcium. In each experiment duplicate PLA₂ assays were performed on subcellular fractions from duplicate cultures (each value represents the mean $\pm$ S.E.M. of 4 assays). The substrate was 1-palmitoyl-2-[1-¹⁴C]oleoyl-phosphatidylcholine at a concentration of 400 μ M.

Cell fraction	PLA ₂ activity (units/mg)					
	Experiment 1		Experiment 2		Experiment 3	
	Control	Dexamethasone	Control	Dexamethasone	Control	Dexamethasone
EDTA supernatant	28.7 $\pm$ 1.0	12.7 $\pm$ 0.7	12.8 $\pm$ 0.4	7.4 $\pm$ 0.4	20.2 $\pm$ 0.2	12.2 $\pm$ 0.3
Calcium supernatant (no lipocortin)	22.3 $\pm$ 1.1	12.3 $\pm$ 0.2	9.1 $\pm$ 0.2	4.1 $\pm$ 0.1	23.5 $\pm$ 0.5	13.7 $\pm$ 0.4
EDTA pellet (no lipocortin)	4.2 $\pm$ 0.2	1.6 $\pm$ 0.0	2.5 $\pm$ 0.0	1.1 $\pm$ 0.1	4.9 $\pm$ 0.4	2.1 $\pm$ 0.0
Calcium pellet	4.6 $\pm$ 0.1	3.0 $\pm$ 0.1	3.6 $\pm$ 0.0	1.8 $\pm$ 0.1	4.4 $\pm$ 0.4	3.4 $\pm$ 0.3

Table 4. Dexamethasone may affect several distinct cellular phospholipases

TEA3A1 cells were grown to confluence in DW-IT plus 2% sCS, then incubated in serum-free DW-IT plus or minus dexamethasone as indicated. Cell lysates were fractionated in the absence of calcium. Enzyme assays were performed plus or minus 2 mM-free calcium. Each value represents the mean $\pm$ S.E.M. from duplicate assays performed on duplicate cultures.

Cell fraction	Assay conditions	PLA ₂ activity (units/mg)					
		Experiment 1 Substrate: 1-palmitoyl- 2-oleoyl-[1- ¹⁴ C]PC (200 μ M)		Experiment 2 Substrate: 1-palmitoyl- 2-arachidonyl-[1- ¹⁴ C]PC (200 μ M)		Experiment 3 Substrate: 1-palmitoyl- 2-arachidonyl-[1- ¹⁴ C]PC (200 μ M)	
		Control	Dexamethasone (10 ⁻⁷ M, 48 h)	Control	Dexamethasone (10 ⁻⁷ M, 48 h)	Control	Dexamethasone (10 ⁻⁶ M, 24 h)
Supernatant	2 mM-Ca	27.8 $\pm$ 0.3	16.8 $\pm$ 0.9	13.6 $\pm$ 0.2	7.8 $\pm$ 0.1	25.5 $\pm$ 1.3	18.0 $\pm$ 0.5
Supernatant	No Ca	15.1 $\pm$ 0.5	10.3 $\pm$ 0.3	6.2 $\pm$ 0.1	3.5 $\pm$ 0.1	9.9 $\pm$ 0.4	8.3 $\pm$ 0.3
Pellet	2 mM-Ca	3.1 $\pm$ 0.0	1.7 $\pm$ 0.1	3.3 $\pm$ 0.1	1.7 $\pm$ 0.1	8.5 $\pm$ 0.3	4.3 $\pm$ 0.3
Pellet	No Ca	3.2 $\pm$ 0.1	1.8 $\pm$ 0.1	1.4 $\pm$ 0.1	1.3 $\pm$ 0.1	3.2 $\pm$ 0.1	2.5 $\pm$ 0.1

arachidonic acid was in the *sn*-2 position of the substrate. The results on pellet PLA₂ activity in the absence of calcium were inconclusive.

DISCUSSION

The results shown here may shed light on a novel aspect of glucocorticoid regulation of eicosanoid production. As expected, dexamethasone treatment of TEA3A1 caused reduced levels of PGE₂, PGF_{2 α} and TXB₂ to be released into the medium. The effect was dosage- and time-dependent. At the same time, dexamethasone treatment reduced PLA₂ activity measured in a cell-free assay. This decrease in activity was seen equally with exogenous substrate concentrations of 200 μ M and 400 μ M (actual substrate concentrations including endogenous phospholipids in the cell lysate would be several-fold higher). It has been reported that lipocortin inhibition of pancreatic PLA₂ was abolished at substrate concentrations above 8 μ M [16], while inhibition of some intracellular PLA₂ by lipocortin was not observed when substrate concentrations were increased to 50 μ M [17]. It is therefore unlikely that the effect on dexamethasone reported here could be due to sequestering of substrate by a lipocortin-like inhibitor.

Thin-layer chromatographic analysis of the reaction products of *sn*-2-labelled phosphatidylcholine substrates with TEA3A1 cell lysates (results not shown) indicated the presence of PLA₁ activity. Since an alternative pathway of arachidonate production could involve PLA₁ plus lysophospholipid lipase, it is possible that dexamethasone could be acting on this pathway instead of on PLA₂ as is usually supposed. The use of an *sn*-1 ether-linked phosphatidylcholine substrate, which is resistant to PLA₁ hydrolysis, confirmed that dexamethasone treatment decreases PLA₂ activity.

Western blotting experiments using antiserum to lipocortin-I indicate that dexamethasone treatment leads to higher levels of lipocortin-I in TEA3A1 cells. The property of lipocortins of binding to membranes in a calcium-dependent manner [17,23,29] was used to prepare subcellular fractions containing PLA₂ activity with or without endogenous lipocortin. Dexamethasone treatment

was found to decrease PLA₂ activity in both supernatant and pellet fractions in the absence of lipocortins. Furthermore, both calcium-dependent and calcium-independent phospholipase activities in the supernatant were decreased by dexamethasone treatment.

These results, obtained with a cell-free assay system, do not rule out a role for lipocortins as phospholipase regulators in intact TEA3A1 cells. They do demonstrate, at least, that another mechanism for dexamethasone inhibition of PLA₂ activity does exist. The effect of dexamethasone could be due to decreases in the level of the PLA₂ enzymes themselves, or could be mediated through other types of regulators which do not have the property of calcium-dependent binding to membranes which is found with lipocortins.

The authors would like to thank Lisa Johnson for technical help, Marina LaDuke for artwork and Julie Lamb for preparation of the manuscript. This work was supported by grants DK38639-01A1 and PO1 CA37589 from NIH.

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Received 17 November 1988/2 February 1989; accepted 16 February 1989