

Attenuation of glucocorticoid functions in an Anx-A1^{-/-} cell line

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The Ca²⁺- and phospholipid-binding protein Anx-A1 (annexin 1; lipocortin 1) has been described both as an inhibitor of phospholipase A₂ (PLA₂) activity and as a mediator of glucocorticoid-regulated cell growth and eicosanoid generation. Here we show that, when compared with Anx-A1^{+/+} cells, lung fibroblast cell lines derived from the Anx-A1^{-/-} mouse exhibit an altered morphology characterized by a spindle-shaped appearance and an accumulation of intracellular organelles. Unlike their wild-type counterparts, Anx-A1^{-/-} cells also overexpress cyclooxygenase 2 (COX 2), cytosolic PLA₂ and secretory PLA₂ and in response to fetal calf serum, exhibit an exaggerated release of eicosanoids, which is insensitive to dexamethasone (10⁻⁸–10⁻⁶ M) inhibition. Proliferation and serum-induced progression of Anx-A1^{+/+} cells from G₀/G₁ into S phase, and the associated

expression of extracellular signal-regulated kinase 2 (ERK2), cyclin-dependent kinase 4 (cdk4) and COX 2, is strongly inhibited by dexamethasone, whereas Anx-A1^{-/-} cells are refractory to the drug. Loss of the response to dexamethasone in Anx-A1^{-/-} cells occurs against a background of no apparent change in glucocorticoid receptor expression or sensitivity to non-steroidal anti-inflammatory drugs. Taken together, these observations suggest strongly that Anx-A1 functions as an inhibitor of signal-transduction pathways that lead to cell proliferation and may help to explain how glucocorticoids regulate these processes.

Key words: annexin, arachidonic acid, cell proliferation, JACRO cells, lipocortin, lung fibroblast.

INTRODUCTION

The annexins are a superfamily of proteins that are each characterized by a canonical repeating 70-amino acid calcium domain and a unique N-terminal tail [1]. The almost ubiquitous expression of annexins in plant and animal tissues suggests that these proteins play a fundamental role in the biology of many different cell types [2].

Anx-A1 (annexin 1; lipocortin 1), a 37 kDa member of the annexin family, is implicated in the control of cell growth [3,3a] and differentiation [4], signal transduction and arachidonic acid release [5,6], as well as intracellular vesicle trafficking [7,8]. In mammals, glucocorticoids regulate the synthesis and cellular disposition of Anx-A1 and work from several groups has provided evidence for the involvement of Anx-A1 in the regulation by these drugs of leucocyte migration [9], acute [10] and chronic [11] inflammation, ischaemic damage [12], pain [13] and fever [14]. In the rodent anterior pituitary gland, the inhibition of corticotropin ('ACTH') [15] and other hormone [16] release by glucocorticoids is mediated through an Anx-A1-dependent mechanism.

Previous studies investigating Anx-A1 function have relied upon techniques such as immuno-neutralization, antisense oligonucleotides, recombinant proteins and peptidomimetics to elucidate the range of actions of this protein. Following the creation of the Anx-A1-null mouse [17] we have generated three fibroblast cell lines (JACRO cell lines) derived from the lung tissue of wild-type Anx-A1^{+/+}, heterozygote Anx-A1^{+/-} and knockout Anx-A1^{-/-} mice. We have compared the morphology, protein expression, proliferation, pro-inflammatory mediator release, cell-cycle progression and cell signalling of these

cell lines and in particular contrasted their responsiveness to dexamethasone.

MATERIALS AND METHODS

Isolation of JACRO cell lines

Whole lung tissue was removed from male Anx-A1^{+/+}, Anx-A1^{+/-} and Anx-A1^{-/-} mice, minced into approx. 1 mm³ pieces with crossed scalpel blades and resuspended in PBS containing 10 mM EDTA, 134 units · ml⁻¹ collagenase and 0.05 % trypsin and agitated for 1 h. Dispersed cells were washed in PBS and resuspended into Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 containing 10 % fetal calf serum (FCS) with 0.1 % penicillin and streptomycin and incubated at 37 °C, in an atmosphere of 5 % CO₂ in O₂ in T25 flasks (Greiner Bio-one, Stonehouse, Gloucs., U.K.) for 14 h. The cell-culture supernatant containing cell debris and unattached cells was discarded, whereas the monolayers of attached cells were washed with PBS and incubated in fresh DMEM/F-12/10 % FCS. Each flask of cells was cultured until approx. 70 % confluence, split (1:3) and this was repeated for approx. 20 passages. Immunostaining of the cell monolayers and Western blotting of cell lysates showed near-identical expression of vimentin intermediate filaments but with no evidence of cytokeratin expression in each cell line (results not shown).

Electron microscopy

Cells were fixed for 1 h in 3 % glutaraldehyde in 0.1 M sodium cacodylate, washed in the same buffer and post-fixed for 30 min

Abbreviations used: GR, glucocorticoid receptor; cPLA₂, cytosolic phospholipase A₂; sPLA₂, secretory PLA₂; COX 2, cyclooxygenase 2; FCS, fetal calf serum; ERK2, extracellular signal-regulated kinase 2; cdk4, cyclin-dependent kinase 4; DMEM, Dulbecco's modified Eagle's medium; JNK, c-Jun N-terminal kinase.

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in 1% osmium tetroxide. Following dehydration in a graded series of alcohols and propylene oxide, cells were embedded in Araldite. Ultra-thin sections were stained with uranyl acetate and lead citrate and viewed in a Jeol 1200 EX II electron microscope at an accelerating voltage of 60 kV.

Western blotting

Cell monolayers were dispersed with 0.05% trypsin in PBS/10 mM EDTA and cell pellets were snap-frozen in 3 ml of PBS containing 10 mM EDTA, 1 mg·ml⁻¹ soya bean trypsin inhibitor, 0.01% leupeptin, 1 mM PMSF and 1 mM sodium orthovanadate. Once thawed, cell lysates were clarified by centrifugation at 13 000 *g* for 5 min. Protein concentrations were measured by Bradford assay and identical concentrations incubated with 250 µl of sample buffer for 5 min at 90 °C prior to SDS/PAGE analysis by Western blotting and detection by diaminobenzidine. A Snapscan scanner (Agfa) scanned the completed Western blots and the image composite was transferred into Microsoft Power Point running on an Apple Macintosh computer. The NIH Image 1.54 image-analysis program was used to measure the density of bands and reported them as percentage changes within each blot. The values given are semi-quantitative and are only meant to give some numerical guide to the ratio of band intensities. The blots presented are typical examples of at least three such experiments. Although overall band intensities varied

between experiments, the ratios of band intensities remained the same.

Cell proliferation

Subconfluent cell cultures were seeded into 12-place multi-well plates (Falcon) at a density of 5×10^4 cells·ml⁻¹·well⁻¹ in DMEM/F-12 containing 10% FCS. Following incubation overnight, the cells were washed in 2 ml of sterile PBS and 1 ml of fresh DMEM/F-12 containing FCS, dexamethasone, indomethacin or vehicle control. On day 2, cells were replenished with fresh experimental medium containing test agents or vehicle control. On day 3, medium was removed from each well and 1 ml of PBS containing 0.05% trypsin and 0.02% EDTA was added to each well. A Coulter Multisizer II was used to count the dispersed cells. The percentage inhibition of cell proliferation for each culture was calculated relative to control wells. We used Trypan Blue to determine cell viability and the data reported are due to inhibition of cell proliferation and not cell death.

Transfection procedure

Murine Anx-A1 cDNA was subcloned directionally between *NotI* and *SmaI* sites in pCMVpA (Clontech, Palo Alto, CA, U.S.A.). Supercoiled plasmid DNA (Anx-A1 or plasmid control) was introduced into Anx-A1^{-/-} cells (1 µg/10⁵ cells) without added carrier DNA using the FuGENE 6 transfection reagent (Roche Molecular Biochemicals) in serum-free DMEM/F-12

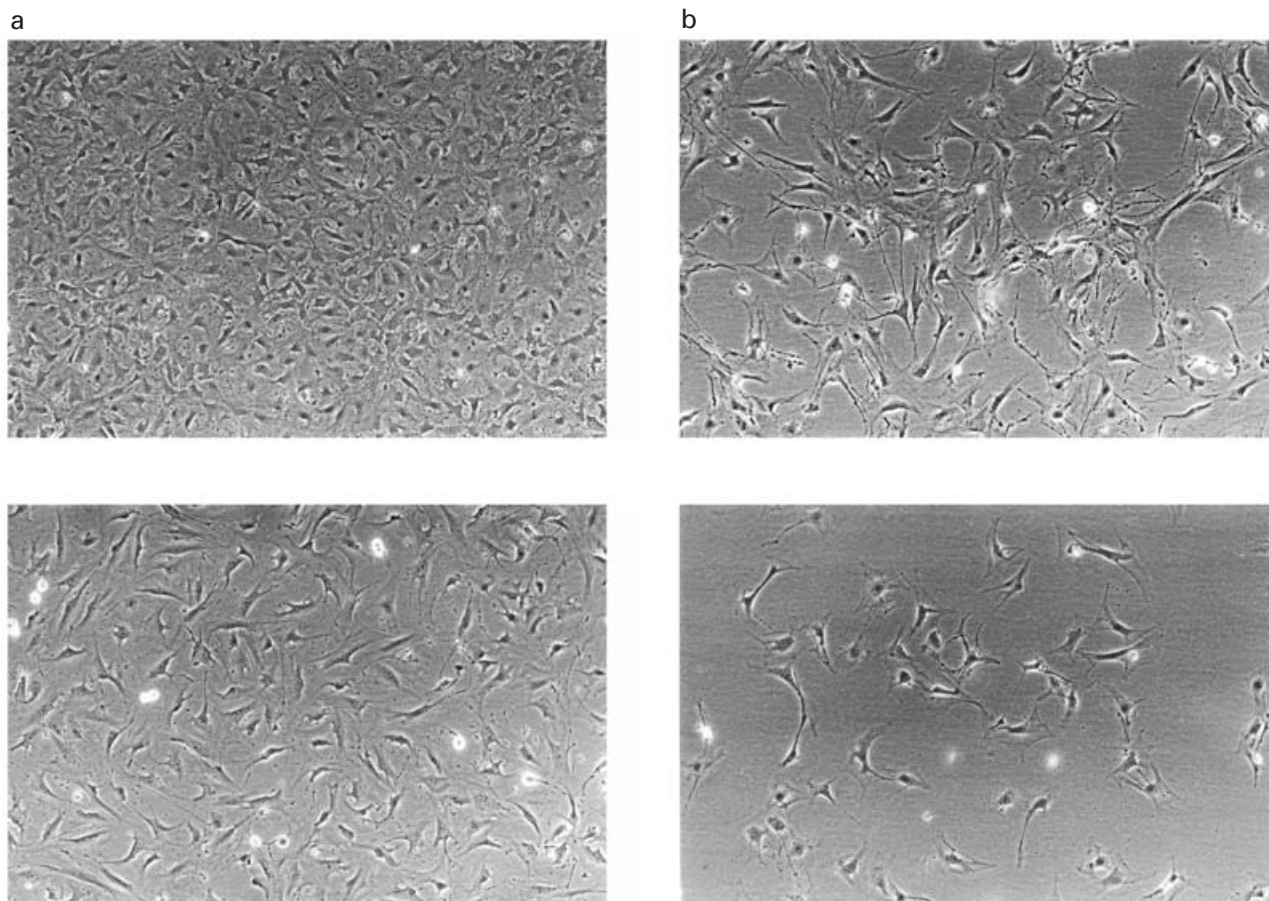


Figure 1 For legend, see facing page

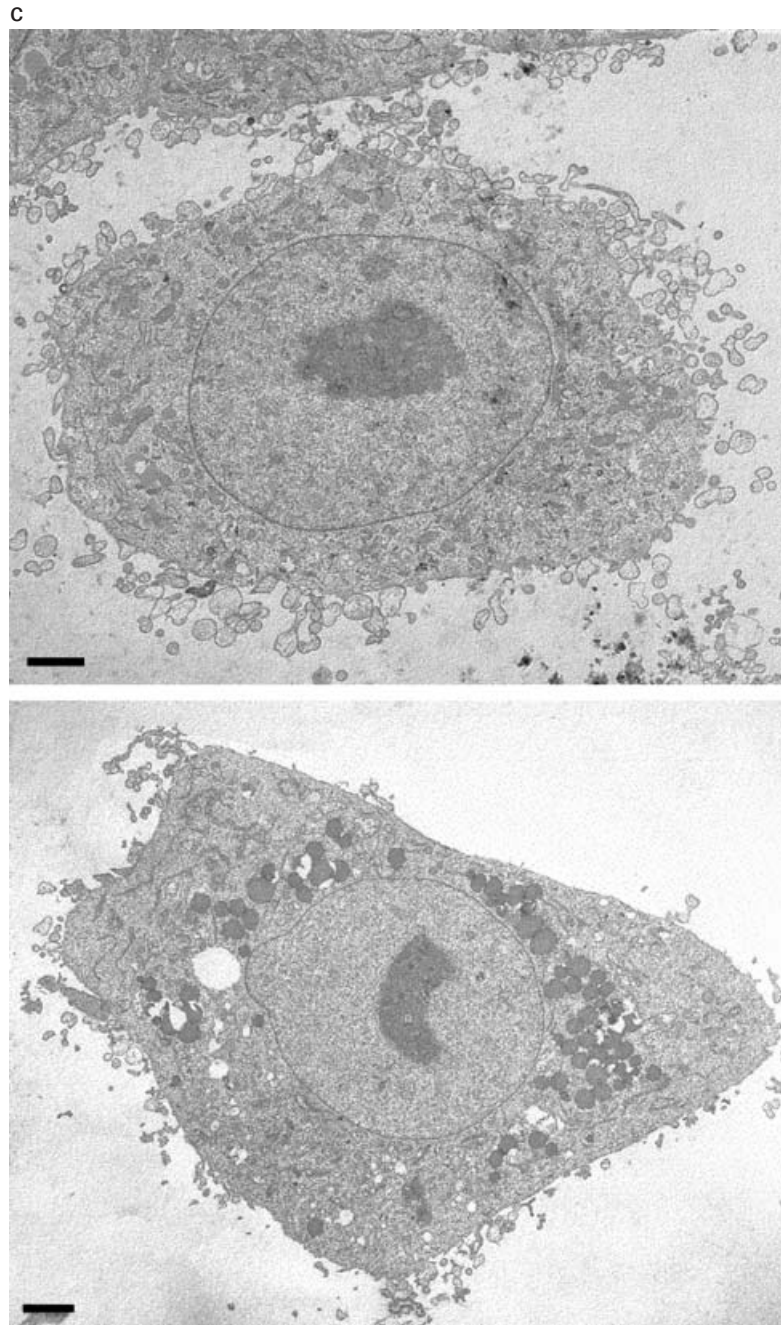


Figure 1 Differences in morphology between *Anx-A1*^{+/+} and *Anx-A1*^{-/-} cells

(a) Phase-contrast photomicrographs of (upper panel) confluent and (lower panel) subconfluent monolayers of wild-type JACRO *Anx-A1*^{+/+} cells ($\times 70$ magnification). (b) Phase-contrast photomicrographs of (upper panel) confluent and (lower panel) subconfluent monolayers of JACRO *Anx-A1*^{-/-} cells ($\times 70$ magnification) showing a more spindle-shaped appearance compared with wild-type cells. *Anx-A1*^{+/-} cells showed an intermediate response between *Anx-A1*^{+/+} and *Anx-A1*^{-/-} cells (results not shown). (c) Electron microscopy of (upper panel) *Anx-A1*^{+/+} and (lower panel) *Anx-A1*^{-/-} cells showing the presence of many intracellular lipid bodies in the latter (scale bars, 1 μm).

medium. After 24 h the medium was replaced with fresh serum-free DMEM/F-12 for a further 48 h, after which the cells were counted as above. Transfection efficiency was determined by β -galactosidase assay (Promega).

Measurement of arachidonic acid release

Subconfluent cell cultures were seeded into 12-place multi-well plates at a density of 1×10^5 cells \cdot ml⁻¹ \cdot well⁻¹ in DMEM/F-12

containing 10 % FCS, and incubated overnight. [³H]Arachidonic acid in ethanol was evaporated to dryness under N₂ and re-suspended in an appropriate volume of DMEM/F-12 (without Phenol Red) and after vortex mixing stood at 37 °C for 1 h. After the cells had been washed with PBS, 9.25 KBq of [³H]arachidonic acid in 0.5 ml of DMEM/F-12 (without Phenol Red or FCS) was added to each well and incubated overnight. The medium containing free [³H]arachidonic acid was then removed and the cells washed three times with 1 ml of DMEM/F-12 containing

1 mg · ml⁻¹ BSA. The cells thus labelled with [³H]arachidonic acid were then treated for 3 h with dexamethasone or vehicle control (ethanol). Then 10 % FCS was added for 30 min. After incubation, 0.4 ml of medium was removed from each well for scintillation counting.

Cell-cycle analysis

Lung fibroblasts from Anx-A1^{+/+} and Anx-A1^{-/-} mice were cultured in FCS-free medium for 24 h to arrest cells in G₀ (0 h). We initiated the cell cycle by replacing this with fresh medium containing 10 % FCS together with either 0.1 μM dexamethasone or vehicle control. At indicated time points, cells were trypsinized, washed in ice-cold PBS and fixed by using ice-cold acetone-free methanol (the methanol/PBS ratio was 2:1). We stained cellular DNA with 0.001 % propidium iodide (Calbiochem) in PBS containing 0.1 % Triton X-100 and 0.037 % (w/v) EDTA followed by the addition of 100 units/ml ribonuclease (Sigma) to remove RNA. Samples were filtered through a 35 μm nylon mesh and we assessed the percentage of cells in the G₀/G₁, S and G₂/M phases using flow-assisted cell sorting analysis (FACS; Becton Dickinson). Data were analysed using CellQuest (Becton Dickinson).

RESULTS

Anx-A1^{-/-} mice were created as described in [17]. We established cell lines (JACRO cells) from whole lung homogenates of tissue obtained post-mortem from litter-mate male Anx-A1^{+/+}, Anx-A1^{+/-} and Anx-A1^{-/-} mice, grew each cell line as an adherent monolayer and observed that they all exhibited the intermediate filament staining characteristic of cells of a fibroblastic origin. We found that Anx-A1^{-/-} cells had an altered morphology compared with Anx-A1^{+/-} or Anx-A1^{+/+} cells having a more spindle-like appearance (Figure 1). Electron microscopy indicated that Anx-A1^{-/-} cells maintained ultrastructural integrity but appeared to contain abundant numbers of lipid vesicles (67 ± 12, *n* = 15) not seen in the Anx-A1^{+/+} cells (Figure 1c). Trypan Blue staining for each cell line was similar (typically < 5 % at 70 % confluence) and there was no apparent difference in viability between genotypes.

We screened the cell lines for the presence of several other annexins, phospholipases and cyclo-oxygenase 2 (COX 2) and used actin as an internal control on all our blots (Figure 2, left-hand panel). We confirmed that Anx-A1 is absent from Anx-A1^{-/-} cells and reduced in Anx-A1^{+/-} cells. Other annexin family members, such as Anx-2, Anx-4 and Anx-6, as well as other unrelated proteins, including cytosolic phospholipase A₂ (cPLA₂), secretory PLA₂ (sPLA₂) and COX 2, were overexpressed in Anx-A1^{-/-} cells (Figure 2, right-hand panel). Expression of calcium-independent PLA₂, glucocorticoid receptor (GR) and actin were unchanged.

We next examined the growth-inhibitory effects of dexamethasone and indomethacin on each cell line. Anx-A1^{+/+} cells were growth-inhibited by dexamethasone in a concentration-dependent (0.01–1 μM) manner (Figure 3a) and similarly inhibited by indomethacin (0.1–10 μM; Figure 3b). However, we found that Anx-A1^{-/-} cells were not significantly affected at these concentrations of dexamethasone but retained a near-identical response to indomethacin (Figures 3a and 3b). Anx-A1^{-/-} cells transfected with an Anx-A1 construct were growth-inhibited whereas a control plasmid was without effect (Figure 3c). In cells that were serum-starved for 24 h we also saw that the addition of 10 % FCS stimulated the proliferation of Anx-A1^{+/+} cells by an additional 90 %. In Anx-A1^{-/-} cells the FCS effect

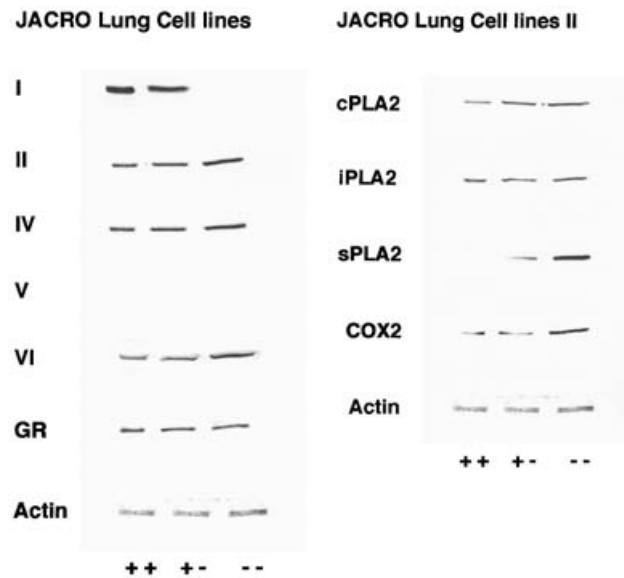


Figure 2 Overexpression of annexins, cPLA₂, sPLA₂ and COX 2 in Anx-A1^{-/-} cells

Western blot of protein equivalents from Anx-A1^{+/+}, Anx-A1^{+/-} and Anx-A1^{-/-} cell lines. The blots shown are typical of three separate experiments. Left-hand panel: Anx-A1 protein was expressed in Anx-A1^{+/-} cells at 70 % of the level found in wild-type cells and was undetectable in Anx-A1^{-/-} cells. Densitometry revealed that Anx-2A (II) expression was up-regulated by an additional 20 % in Anx-A1^{+/-} cells and by 144 % in Anx-A1^{-/-} cells. Similarly we found the expression of Anx-4A (IV) and Anx-6A (VI) also to be up-regulated by 86 % and 131 % respectively in Anx-A1^{-/-} cells. Anx-5A (V) was only weakly detectable in all three cell lines. There was no apparent change in actin or GR expression between the cell lines. Right-hand panel: the expression of cPLA₂ and sPLA₂ (but not calcium-independent PLA₂) was up-regulated by 146 and 300 % respectively in Anx-A1^{-/-} cells. COX 2 was also up-regulated by 220 % in Anx-A1^{-/-} cells.

was greatly enhanced (160 %). The addition of 10 μM indomethacin significantly reduced the FCS-induced growth response in both cell types to an equal extent; in contrast, 1 μM dexamethasone reduced FCS-stimulated proliferation of Anx-A1^{+/+} but not of Anx-A1^{-/-} cells (Figure 3d). In general, heterozygote Anx-A1^{+/-} cells exhibited an intermediate response.

To probe further the effect of Anx-A1 gene deletion on cellular behaviour we examined the cell-cycle progression of each JACRO cell line using FACS analysis. Serum-starved Anx-A1^{+/+} cells stimulated with FCS for 16 h moved from G₀/G₁ into S phase (Figure 4A) and the concomitant administration of 0.1 μM dexamethasone significantly suppressed this response (Figure 4B). In contrast, we found that serum-starved Anx-A1^{-/-} cells required at least 24 h stimulation with FCS to move from G₀/G₁ into S phase (Figure 4C). Unaccountably this progression was greatly enhanced in the presence of 0.1 μM dexamethasone (Figure 4D). Serum-starved Anx-A1^{+/-} cells stimulated with FCS moved into the S phase at 16 h like their wild-type counterparts yet dexamethasone did not suppress this response; rather, there was a less significant enhancement of progression compared with the knockout cells.

The expression of cell-cycle intermediates mirrored the cell-proliferation data. COX 2, extracellular signal-regulated kinase 2 (ERK2), cyclin D1, cyclin-dependent kinase 4 (cdk4) and phosphorylated c-Jun N-terminal kinase (JNK) were increased in Anx-A1^{+/+} cells (Figure 5, top panel) within 4 h of FCS treatment and apparent in both genotypes by 24 h (Figure 5, middle panel). We found that basal levels of COX 2 were higher (by 32 %) in serum-starved Anx-A1^{-/-} cells whereas cdk4 levels were lower

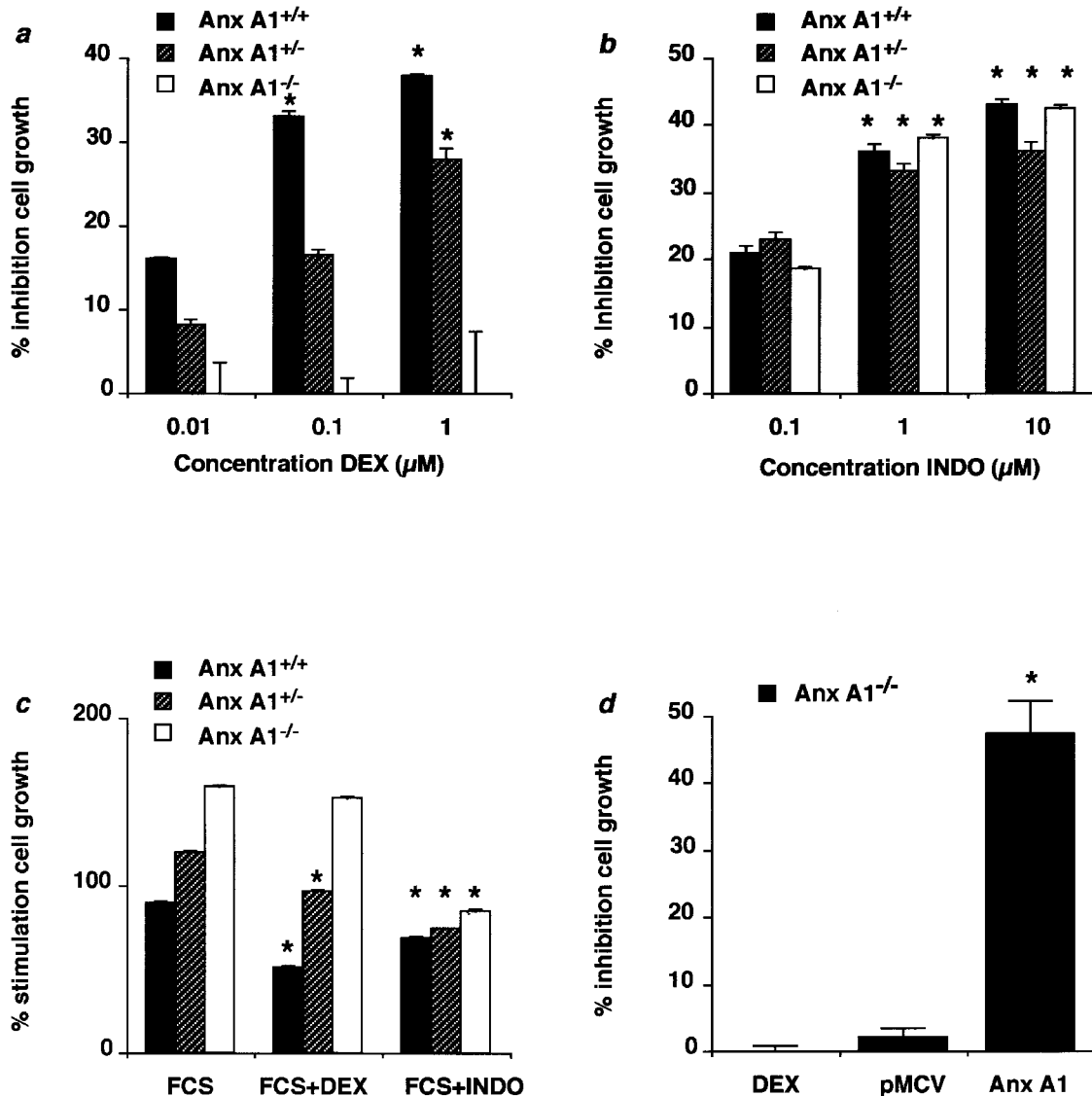


Figure 3 Growth of Anx-A1^{-/-} cells is not inhibited by dexamethasone

(a) Anx-A1^{+/+} cells are growth-inhibited by dexamethasone (DEX; 0.01–1 μM) in a concentration-dependent manner whereas Anx-A1^{-/-} cells are not significantly affected. Anx-A1^{+/-} cells show an intermediate response. (b) Anx-A1^{+/+}, Anx-A1^{+/-} and Anx-A1^{-/-} cells are all growth-inhibited by indomethacin (INDO; 0.1–10 μM) to an equivalent extent. (c) In cells that were serum-starved for 24 h we also saw that the addition of 10 % FCS stimulated the proliferation of Anx-A1^{+/+} cells by an additional 90 % and of Anx-A1^{+/-} cells by 120 %. In Anx-A1^{-/-} cells the FCS effect was greatly enhanced (160 %). The addition of 10 μM indomethacin significantly reduced the FCS-induced growth response in all cell types to an equal extent; in contrast 1 μM dexamethasone reduced FCS-stimulated proliferation of Anx-A1^{+/+} and Anx-A1^{+/-} cells but not Anx-A1^{-/-} cells. (d) Anx-A1^{-/-} cells transfected with an Anx-A1 construct were significantly growth-inhibited whereas both a control plasmid (pMCV) and 1 μM dexamethasone had no significant effect. In all panels, results are typical of three separate experiments. **P* < 0.001, relative to vehicle control (Student's *t* test).

(reduced by 90 %). The induction, by FCS, of COX 2, ERK2, cdk4 and JNK phosphorylation (but not cyclin D1) was reversed by 0.1 μM dexamethasone in Anx-A1^{+/+} cells to basal levels by 4 h (Figure 5, top panel). The effects of dexamethasone were blocked in the presence of 1 μM RU486, suggesting that occupation of GRs is required. In contrast, we found the induction of COX 2, ERK2, cdk4, cyclin D1 and the phosphorylation of JNK to be unaffected by the presence of 0.1 μM dexamethasone in Anx-A1^{-/-} cells at either 4 or 24 h of treatment (Figure 5, middle panel).

To complement the changes we observed in COX 2 and cPLA₂ we measured the release of [³H]arachidonic acid from pre-labelled cells that had been serum starved for 24 h. We found that the addition of 10 % FCS increased the release of this fatty acid

from Anx-A1^{+/+} cells by an additional 56 %, and that this release was significantly (*P* < 0.001) inhibited in the presence of dexamethasone (Figure 6a). When Anx-A1^{-/-} cells were examined we found that the basal release of arachidonic acid was 80 % higher than in the Anx-A1^{+/+} cells and that addition of 10 % FCS elevated this release to 133 %. However, FCS-stimulated arachidonic acid release was not significantly inhibited by 0.1 μM dexamethasone in Anx-A1^{-/-} cells (Figure 6a). We also measured the release of prostaglandin E₂ from serum-starved Anx-A1^{+/+} cells and found that the addition of 10 % FCS stimulated the release of this eicosanoid by 380 % and this release was inhibited by 0.1 μM dexamethasone (Figure 6b). In Anx-A1^{-/-} cells we found that FCS stimulated the release of prostaglandin E₂ by over 800 % in serum-starved cells but that this release was not

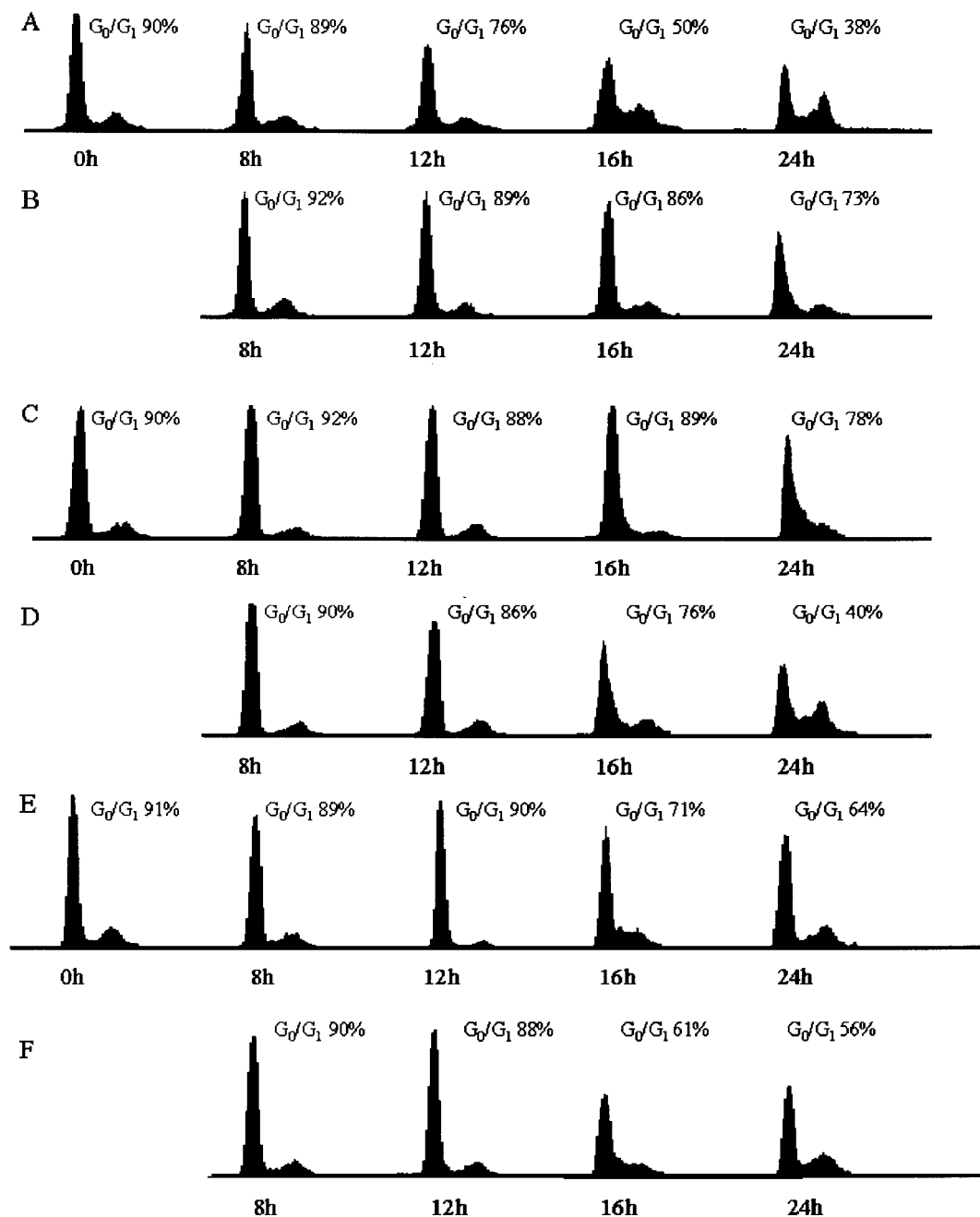


Figure 4 Cell cycle progression in *Anx-A1*^{-/-} cells is resistant to dexamethasone

(A) Cells were serum starved for 24 h prior to stimulation with 10 % FCS to initiate cell-cycle progression; *Anx-A1*^{+/+} cells move into S phase at 16 h. (B) Cycle progression in *Anx-A1*^{+/+} cells is reversed in the presence of 0.1 μ M dexamethasone. (C) Cells were serum-starved for 24 h prior to stimulation with 10 % FCS to initiate cell-cycle progression; *Anx-A1*^{-/-} cells move into S phase at 24 h. (D) Cycle progression in *Anx-A1*^{-/-} cells is unaffected by 0.1 μ M dexamethasone. (E) Cells were serum-starved for 24 h prior to stimulation with 10 % FCS to initiate cell-cycle progression; *Anx-A1*^{+/-} cells move into S phase at 16 h. (F) Cycle progression in *Anx-A1*^{+/-} cells is unaffected by 0.1 μ M dexamethasone. Each panel is representative of three independent experiments.

significantly inhibited by 0.1 μ M dexamethasone (Figure 6b). Again, *Anx-A1*^{+/-} cells exhibited an intermediate response.

DISCUSSION

Anx-A1 has been implicated in many aspects of cell physiology including the regulation of cell growth [1] and differentiation [4], signal transduction and arachidonic acid release [5,6], as well as intracellular vesicle trafficking [7,8]. In pathology *Anx-A1* has

been implicated directly or indirectly in tumour development [18] and could be a diagnostic marker for certain types of tumour [19]. Evidence that *Anx-A1* mediates the growth-inhibitory properties of dexamethasone in A549 cells was furnished by immunoneutralization [3,3a] and antisense oligonucleotide strategies [20] and the protein may play a similar role in lymphocytes [21]. However, the effects of overexpression of *Anx-A1* in both rapidly proliferating hepatocytes [22] and human foreskin fibroblasts [23] suggest that the situation is more complex and other studies suggest a different interpretation [24]. Here we

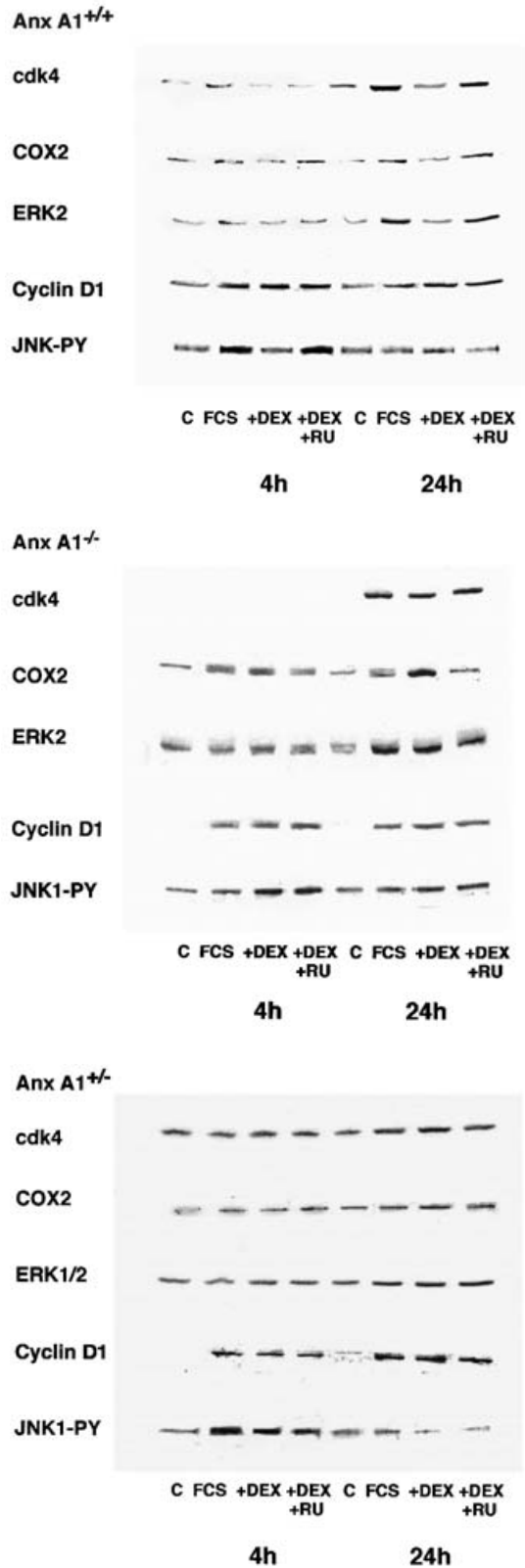


Figure 5 Expression of cell-cycle-associated intermediates in Anx-A1^{-/-} cells is unaffected by dexamethasone

Top panel: in serum-starved Anx-A1^{+/+} cells the induction, by addition of 10% FCS, of COX 2, ERK2, cdk4 and JNK phosphorylation (PY), but not cyclin D1, was reversed by 0.1 μM dexamethasone (DEX) to basal levels at both 4 and 24 h. The effects of dexamethasone were blocked in the presence of 1 μM RU486 (RU). Middle panel: in serum-starved Anx-A1^{-/-}

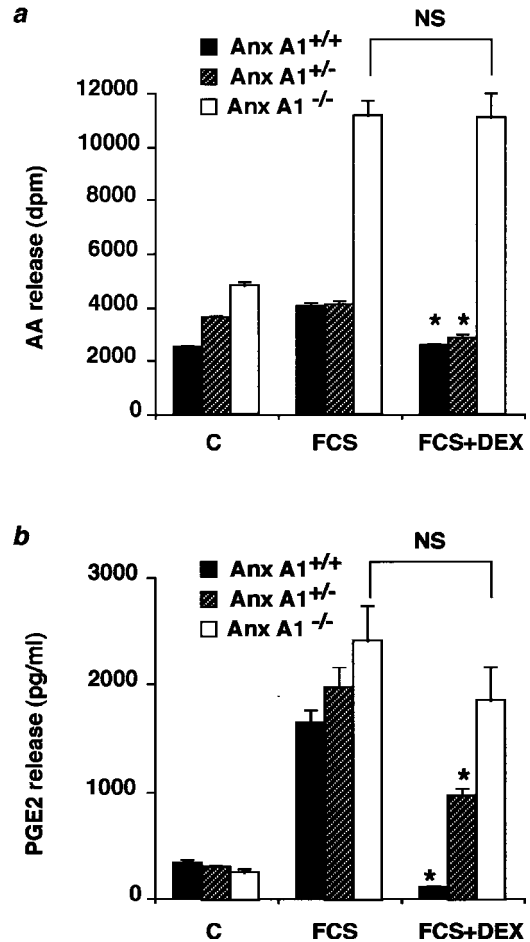


Figure 6 Exaggerated arachidonic acid (AA) and prostaglandin E₂ (PGE₂) release in Anx-A1^{-/-} cells

(a) Release of [³H]arachidonic acid from pre-labelled cells that had been serum-starved for 24 h. Serum-starved Anx-A1^{+/+} cells treated with 10% FCS increased release of arachidonic acid by 60%. This was significantly reduced (**P* < 0.001; Student's *t* test) by 0.1 μM dexamethasone (DEX) to 3%. Serum-starved Anx-A1^{-/-} cells treated with 10% FCS increased release of arachidonic acid by 133% and this was resistant to 0.1 μM dexamethasone. Anx-A1^{+/-} cells showed an intermediate response. (b) Serum-starved (24 h) Anx-A1^{+/+} cells treated with 10% FCS increased prostaglandin E₂ release by 380%. This was significantly reduced (**P* < 0.001) by 0.1 μM dexamethasone to below control levels. Serum-starved Anx-A1^{-/-} cells treated with 10% FCS increased prostaglandin E₂ release by 830% and this was not significantly (NS) affected by 0.1 μM dexamethasone. Anx-A1^{+/-} cells showed an intermediate response. Data are representative of three separate experiments.

show that a lung cell line derived from Anx-A1^{-/-} mice and characterized as fibroblastic in origin are completely refractory to growth inhibition by dexamethasone compared with Anx-A1^{+/+} cells, whereas drugs that inhibit the generation of growth-stimulatory eicosanoids such as indomethacin inhibit the growth of both cell lines. Paradoxically, unstimulated Anx-A1^{-/-} cells proliferate at a slower rate and this is mirrored by a slower cell-cycle progression in these cells. Thus it appears that Anx-A1

cells the induction by addition of 10% FCS of COX 2, ERK2, cdk4, cyclin D1 and JNK phosphorylation was unaffected by 0.1 μM dexamethasone at both 4 and 24 h. Basal levels of COX 2 were higher (by 32%) in serum-starved Anx-A1^{-/-} cells whereas cdk4 levels were reduced by 90%. The effects of dexamethasone were unchanged in the presence of 1 μM RU486. Bottom panel: in serum-starved Anx-A1^{+/-} cells the induction by addition of 10% FCS of COX 2, ERK2, cdk4, and cyclin D1 was unaffected by 0.1 μM dexamethasone at both 4 and 24 h. However, JNK phosphorylation was reduced by 39% at 4 h.

is necessary for normal cell cycling but, following exposure of cells to glucocorticoids, intracellular Anx-A1 can function as a negative growth modulator, an interpretation that could resolve several apparently contradictory observations in the field. In fact, Anx-A1 is rapidly phosphorylated on serine residues in A549 cells following dexamethasone treatment [6], and it is this species that blocks epidermal growth factor signal-transduction pathways in these cells. A similar mechanism has been proposed by other authors to account for the blocking action of dexamethasone on signal transduction initiated by rat liver insulin receptor kinase (which preferentially phosphorylates Anx-A1 *in vivo* following dexamethasone treatment), perhaps through simple competition with other signalling intermediates [25]. We have recently described a novel rapid receptor-dependent, genome-independent, signalling effect of glucocorticoids in the A549 cell line mediated by *src*-induced phosphorylation of Anx-A1 that is utilized preferentially by some glucocorticoids (including dexamethasone), but not by others [26].

Considering the fact that Anx-A1 has been reported to participate in so many intracellular processes [27] it is perhaps surprising that there is little difference in the viability of the Anx-A1^{-/-} and Anx-A1^{+/+} JACRO cells. One possibility is that up-regulation of annexins 2, 4 and 6 in Anx-A1^{-/-} cells partially compensates for the absence of Anx-A1 in this respect. The N-terminal sequence Anx-A1-(13–25) blocks recruitment of Grb2 to activated epidermal-growth-factor receptors and thus downstream activation of ERK-dependent pathways [6]. Despite sharing considerable identity within their phospholipid-binding core domains [27], other annexins cannot substitute signalling functions mediated through the Anx-A1 N-terminal region and the up-regulation of related species cannot restore glucocorticoid sensitivity. However, the increased expression of other proteins such as cPLA₂, sPLA₂ and COX 2 that we saw in Anx-A1^{-/-} cells may be more significant. Intestinal cells overexpressing COX 2 exhibit a delay in entering G₁ [28], which is correlated with reduced expression of cyclin D1 and cdk 4. An inverse correlation between Anx-A1 levels and the regulation of cPLA₂ and COX 2 expression has been described in the A549 cell line [29] and also in the adrenalectomized rat [30].

The expression of intermediates during cell-cycle progression in the JACRO cell lines fits with the cell-proliferation data. In serum-starved Anx-A1^{+/+} cells, FCS induction of JNK phosphorylation, COX 2, ERK2 and cdk4 expression (but not cyclin D1) is apparent at both 4 and 24 h and is blocked by dexamethasone at both time points. These effects of dexamethasone are reversed by RU486, which suggests that occupancy of GR is required. Overexpression of cyclin D1 is known to shorten the time cells spend in the G₁ interval [31], although cyclin D1 levels are independent of ERK signalling pathways in human airway smooth muscle cells [32]. It would appear therefore that dexamethasone inhibition of cell proliferation is not mediated through cyclin D1 *per se*, but rather by blocking the induction of ERK-dependent pathways and cdk4. Anx-A1^{-/-} cells express higher basal levels of COX 2 but lower levels of cdk4, thus explaining the longer duration spent by these cells in the G₁ phase. More importantly, the induction of these intermediates in Anx-A1^{-/-} cells is unaffected by dexamethasone treatment, a finding congruent with the insensitivity of these cells to growth inhibition by dexamethasone. Rather, it seems that dexamethasone treatment enhances expression of COX 2 in these cells and similarly enhances entry into the S phase. Such a mechanism would also help to explain the paradoxical role of Anx-A1 as a mediator of cell proliferation, but an inhibitor of cell-signalling processes. Again the heterozygote Anx-A1^{+/-} cells exhibit an intermediate

response with S-phase entry similar to wild-type cells and a response to dexamethasone similar to knockout cells.

The reciprocal relationship between Anx-A1 expression and that of COX 2, cPLA₂ and sPLA₂ in Anx-A1^{-/-} cells suggests that these cells may express an altered profile of eicosanoid release. Indeed, Anx-A1^{-/-} cells exhibit an elevated basal level, and an exaggerated FCS-induced release, of arachidonic acid compared with Anx-A1^{+/+} cells and both parameters are insensitive to dexamethasone. Disturbances in arachidonic acid release secondary to an up-regulation in activated cPLA₂ could also account for the differences in morphology observed between the Anx-A1^{+/+} and Anx-A1^{-/-} cells. Arachidonic acid metabolites such as leukotrienes acting through the small GTPase Rho can induce actin stress fibre formation and cytoskeletal remodelling in some cells [33]. Whereas it is currently unclear why there is such a marked difference in the accumulation of intracellular vesicles in the Anx-A1^{-/-} cells, several authors have commented on the role of Anx-A1 in intracellular vesicle trafficking and the disposition of phagosomes and endosomes [8]. Additionally, lipid-rich cytoplasmic bodies are increased in some cell types as a result of increased eicosanoid synthesis [34].

Glucocorticoids such as dexamethasone control a plethora of cellular processes including nuclear factor- κ B activation, direct genomic effects mediated through glucocorticoid response elements as well as other signalling effects [35], often making it difficult to identify key mechanisms involved in any one particular response. The complete attenuation of dexamethasone inhibition of cell growth, cell cycling, cell signalling and eicosanoid release in Anx-A1^{-/-} cells supplies persuasive evidence for a prime role for Anx-A1 in these effects.

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