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17β-Estradiol promotes the synthesis and the secretion of annexin I in the CCRF-CEM human cell line

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Aims: Annexin I (ANXA1), a 37 kDa member of the annexin family of Ca^{2+}-binding and phospholipid-binding proteins, is particularly abundant in various populations of peripheral blood leukocytes. Since this protein modulates the anti-inflammatory actions of the steroid hormones, the purpose of this study was to investigate the effects of the female sex steroid hormone, 17β-estradiol ($E_2\beta$), on the synthesis and secretion of ANXA1 in the human CCRF-CEM acute lymphoblastic leukemia cell line.

Methods: **Complementary reverse transcription-polymerase chain reaction and Western blot assays were performed to study the effect of $E_2\beta$ on the expression of mRNA and protein ANXA1, respectively.**

Results and discussion: **Treatment of CCRF-CEM cells with $E_2\beta$, for 30 min, stimulated the synthesis of ANXA1 mRNA molecules, and increased the cellular level of ANXA1 protein. Moreover, when the cells were incubated with $E_2\beta$, under the same experimental conditions, a significant increase in the amount of ANXA1 secreted from the cells was also detected. ICI 182,780, a selective inhibitor of the intracellular estrogen receptor, had no effect on the $E_2\beta$-stimulated expression and externalisation of ANXA1. Taken together, these results indicate that $E_2\beta$ induces *de novo* synthesis of ANXA1 and stimulates its secretion in the CCRF-CEM cell line, apparently through a mechanism independent of the intracellular estrogen receptor.**

Key words: Annexin I, 17β-Estradiol, Lymphoblastic cell line, ICI 182,780

Introduction

Annexin I (ANXA1) belongs to the annexin family of Ca^{2+}-binding and phospholipid-binding proteins that lack the classic 'EF-hand' motif for Ca^{2+} binding.[1] A growing body of evidence indicates that ANXA1 acts as an endogenous anti-inflammatory mediator. ANXA1 has been implicated in several biological processes, including cell growth and differentiation, inhibition of chemotaxis, and release of inflammatory mediators, such as eicosanoids[2,3] and inflammatory cytokines.[4]

Human monocytes and polymorphonuclear neutrophils constitutively express the protein ANXA1,[1,5] as well as plasma membrane, specific and Ca^{2+}-dependent binding sites for this protein.[1,2] In contrast to what has been described for those cells, little is known about the capacity of lymphocytes to synthesise and secrete ANXA1.[6–8] Interestingly, the steroid hormone 17β-estradiol ($E_2\beta$) has been reported to have anti-inflammatory properties, which result, at least in part, from a direct action of the hormone on lymphocytes.[9] Among lymphoid cells, T lymphocytes appear to be the most sensitive to the effects of $E_2\beta$.[10,11] Furthermore, some of the anti-inflammatory effects of $E_2\beta$ are identical to those exerted by ANXA1.[12,13] However, the ability of $E_2\beta$ to induce ANXA1 synthesis either in lymphocytes or other cell types has not been reported to date. Therefore, the objective of this study was to investigate the synthesis and secretion of ANXA1 in lymphocytes, in response to $E_2\beta$. For this purpose, we used the human acute lymphoblastic leukaemia cell line CCRF-CEM, which expresses characteristics of T lymphocytes.[14]

Materials and methods

Chemicals

Mouse monoclonal antibodies against ANXA1 and actin were purchased, respectively, from BAbCO (CA, USA) and Boehringer Mannheim (Carnaxide, Portugal), and the horseradish peroxidase-conjugated goat anti-mouse immunoglobulin was from Pierce (IL, USA). ICI 182,780 was kindly supplied by AstraZeneca Biociência (Barcarena, Portugal). All other reagents were from Sigma Chemical Co.

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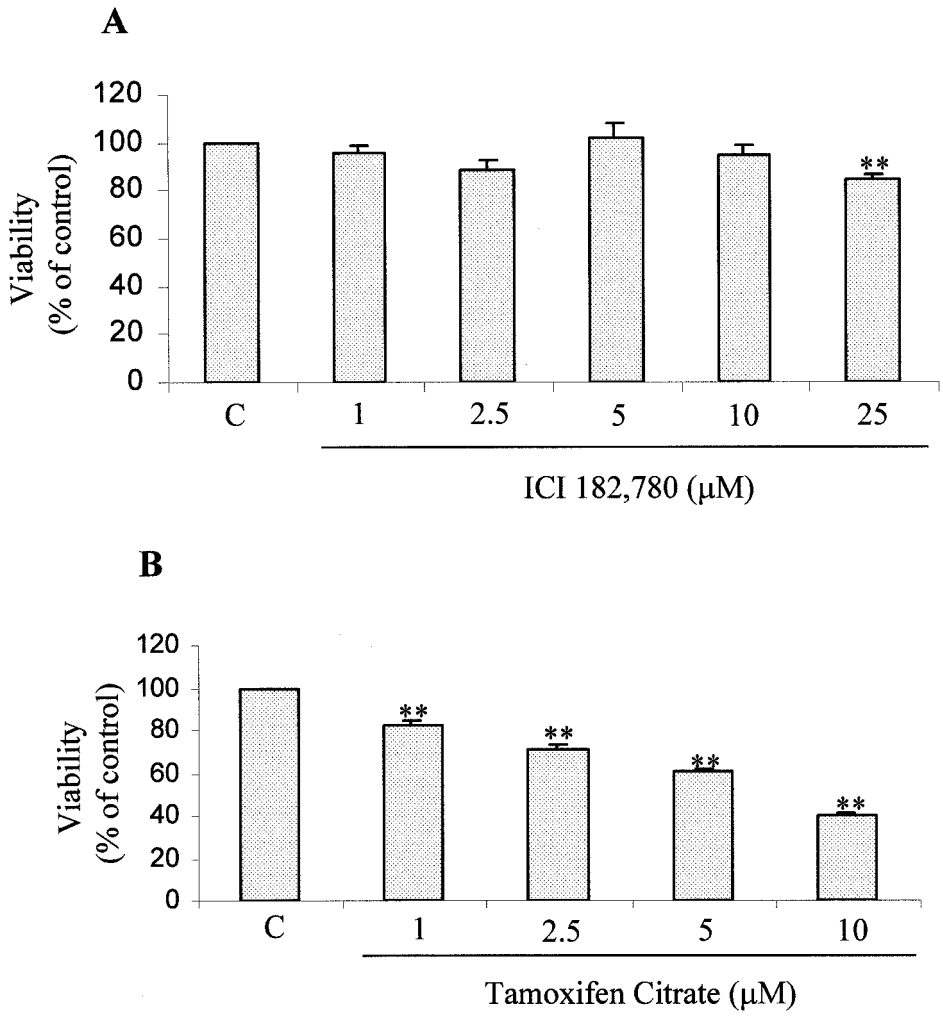

FIG. 1. Cell viability of CCRF-CEM cells when incubated in the presence of estrogen antagonists. Cells were incubated in the absence (control, C) or in the presence of ICI 182,780 (1–25 μM) (A), or with tamoxifen citrate (1–10 μM) (B). Cell viability was assessed by the MTT reduction test. The data shown are an average of three independent experiments. Values are mean±SD, ** $p < 0.01$ as determined by one-way ANOVA with Dunnett's post test.

Cell culture

The human T-cell CCRF-CEM acute lymphoblastic leukemia cell line (ATCC, Rockville, MD, USA) was maintained in RPMI medium supplemented with 10% fetal calf serum (FCS), 100 μg/ml of streptomycin and 100 U/ml of penicillin, at 37°C, in an atmosphere of 5% CO_2/95% air. CCRF-CEM cells (1×10^6 cells/ml) were plated in six-well culture dishes with RPMI supplemented with 2.5% FCS and antibiotics, for 14 h prior to all the experiments described.

Cell viability

Viability of the CCRF-CEM cells was always checked by the trypan blue exclusion and MTT reduction tests prior to the experiments. Assessment of MTT reduction was made by the colorimetric assay, using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), as previously described.[15]

Reverse transcription-polymerase chain reaction assays

For the reverse transcription-polymerase chain reaction (RT-PCR) assays, CCRF-CEM cells were incubated in the presence of or in the absence of $E_2\beta$ (0.01–1 μM), or in $E_2\beta$ (1 μM) + the estrogen antagonist ICI 182,780 (10 μM), for 30 min–4 h. After incubation, the cells were washed twice in phosphate-buffered saline (PBS) with 0.1% diethyl pyrocarbonate, and the total RNA was isolated with TRIzol. RT-PCR was standardised by amplifying a 410 base pair (bp) sequence of ANXA1 mRNA with the primers 5′CAAACTGTGAAGTCATCCAAAG3′ and 5′TCCTCTCTGTAGACCCTG3′, and a 540 bp sequence of the β-actin mRNA (as an internal control) with the primers 5′GTGGGGCGCCCCAGGCACCA3′ and 5′CTCCTTAATGTCACGCACGATTTC3′. The reactions were performed with Ready-to-go RT-PCR beads using 150 ng of total RNA and the

following program: 42°C for 30 min, 95°C for 5 min, 20 cycles (95°C for 1 min, 55°C for 1 min, 72°C for 1 min), and 72°C for 5 min. The RT-PCR products were separated in a 1% agarose gel electrophoresis, and visualised by ethidium bromide staining.

Western blot analysis

For immunodetection of ANXA1, CCRF-CEM cells were incubated with or without $E_2\beta$ (0.01–1 μM), or with $E_2\beta$ (1 μM) + ICI 182,780 (10 μM), for 30 min-4 h. After incubation, the cells were washed by centrifugation in PBS. The secreted ANXA1 bound to plasma membrane receptors was extracted by washing the cells, for 10 sec, in 1 mM of ethylenediaminetetraacetic acid (EDTA), as previously described.[16] The supernatant was collected for secreted plasma membrane-bound ANXA1 detection, and the pelleted cells were washed once again in PBS to remove all secreted protein contaminant. Cells were then lysed in lysis buffer (10 mM of Tris–HCl (pH 7.6), 10% glycerol, 5 mM of β-mercaptoethanol, and the protease inhibitor cocktail) and sonicated. Samples of cellular lysates (30 μg) and the cell supernatant containing the secreted proteins (2 μg) were added (1 : 1) to denaturating buffer (200 mM of Tris, 200 mM of bicine, 8 M of urea, 4% sodium dodecyl sulfate (SDS), 10% β-mercaptoethanol and 0.2% bromophenol blue), and subjected to 12% SDS-polyacrylamide gel electrophoresis. After electrotransferration to a PVDF membrane, ANXA1 was detected by incubation with a mouse anti-ANXA1 monoclonal antibody (2 μg/ml), followed by incubation with a horseradish peroxidase-conjugated goat anti-mouse antibody. The immunocomplexes were visualised by the enhanced chemiluminescence (ECL) method. To demonstrate equivalent total protein loading, the stripping of the membrane was performed with stripping buffer (100 mM of β-mercaptoethanol, 2% SDS, 62.5 mM of Tris–HCl (pH 6.7) at 55°C, and the membrane was treated as already indicated using the anti-actin mouse monoclonal antibody (0.1 μg/ml). The Ponceau S staining of the PVDF membrane was used to show equivalent secreted protein loading.

Statistical analysis

Assay data were expressed as mean±SD. Comparison of data from two treatment groups was made by the Student's two-tailed unpaired *t*-test. When comparison of data from more than two treatment groups was required, one-way analysis of variance (ANOVA) with Dunnett's post test was used. A probability of less than 5% (i.e. $p < 0.05$) was taken as statistically significant.

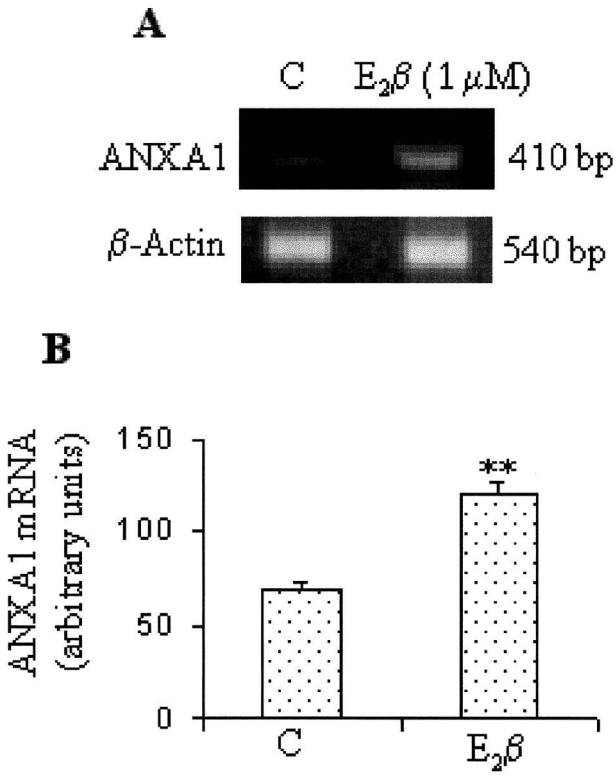

FIG. 2. Effect of E2β on ANXA1 mRNA synthesis in CCRF-CEM cells. Cells were incubated in the absence (control, C) and in the presence of $E_2\beta$ (1 μM) for 30 min (A). The bands were quantified with an image analyser (B). ANXA1 mRNA was detected by RT-PCR with a pair of primers specific for a fragment of ANXA1 mRNA. The data shown are an average of three independent experiments. Values are mean±SD, ** $p < 0.01$ as determined by Student's two-tailed unpaired *t*-test.

Results

Cell viability

The cell viability was previously assessed prior to each the experiment. The viability of CCRF-CEM cells incubated in the presence of $E_2\beta$ was always >95% (data not shown). When the cells were incubated with the pure anti-estrogen ICI 182,780 at the concentrations used in the present work (10 μM), the viability was also > 95%. Concentrations higher than 10 μM of ICI 182,780 caused cell death (Fig. 1A). The estrogen antagonist tamoxifen citrate, at the concentration of 1 μM, decreased cell viability by more than 18% (Fig. 1B). Therefore, tamoxifen citrate was not used in the present work.

Effect of $E_2\beta$ on ANXA1 mRNA synthesis in CCRF-CEM cells

The ability of $E_2\beta$ to induce ANXA1 mRNA synthesis in CCRF-CEM cells was studied by RT-PCR, using a specific pair of primers that amplify a 410 bp sequence of ANXA1 mRNA (Fig. 2).

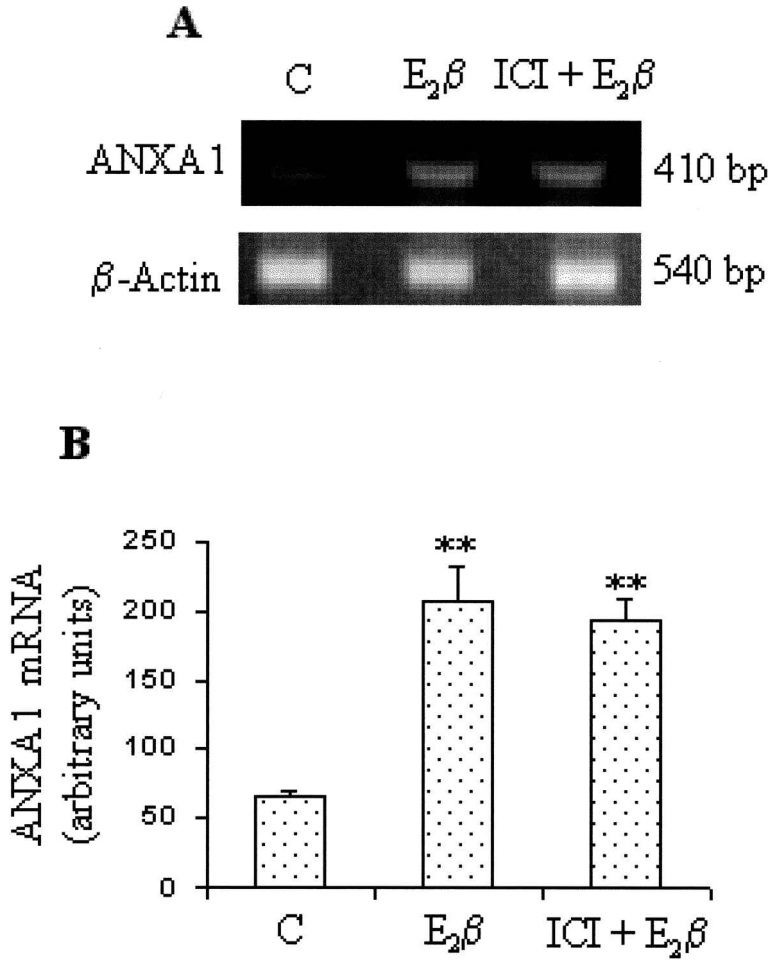

FIG. 3. Effect of E2β and ICI 182,780 on ANXA1 mRNA synthesis in CCRF-CEM cells. Cells were incubated in the absence (control, C) and in the presence of $E_2\beta$ (1 μM), and in E2β (1 μM) + ICI 182,780 (10 μM) for 30 min (A). The bands were quantified with an image analyser (B). ANXA1 mRNA was detected by RT-PCR with a pair of primers specific for a fragment of ANXA1 mRNA. The data shown are an average of three independent experiments. Values are mean±SD, ** $p < 0.01$ as determined by one-way ANOVA with Dunnett's post test.

CCRF-CEM cells incubated in the presence of $E_2\beta$ at a concentration ranging from 0.01 to 1 μM, for 30 min, showed an increase in ANXA1 mRNA expression (data not shown). However, the increase was constant and reached statistical significance only when the cells were treated with 1 μM of $E_2\beta$ (120.2 ± 6.7 arbitrary units (AU) versus 69.6 ± 2.6 AU, in control; $p < 0.01$) (Fig. 2). Longer incubation in the presence of $E_2\beta$ did not change these results (data not shown).

Co-incubation of CCRF-CEM cells with $E_2\beta$ (1 μM) and the estrogen antagonist ICI 182,780 (10 μM) was performed to investigate the role of the estrogen receptor (ER) in the effect of $E_2\beta$ on the ANXA1 mRNA expression (Fig. 3). The rise in the ANXA1 mRNA level induced by $E_2\beta$ (1 μM), in the presence or in the absence of ICI 182,780 (10 μM) was not significantly different ($p > 0.05$). However, confirming the previous results, both $E_2\beta$ (1 μM) (207.7 ± 25.2 AU) and $E_2\beta$ (1 μM) + ICI 182,780 (10 μM) (194.1 ± 14.1 AU) induced a significant increase of the control level of ANXA1 mRNA (74.25 ± 2.2 AU) ($p < 0.01$) (Fig. 3B).

Effect of $E_2\beta$ on ANXA1 protein expression and secretion in CCRF-CEM cells

To test the effect of $E_2\beta$ on ANXA1 protein synthesis and secretion, CCRF-CEM cells were incubated in the presence or in the absence of different concentrations of $E_2\beta$ (0.01–1 μM). A significant, measurable and constant effect was only observed with 1 μM of $E_2\beta$ (data not shown). As a consequence, 1 μM of $E_2\beta$ was the concentration used in all the experiments performed to study ANXA1 protein expression and secretion.

As shown in Fig. 4, the basal level of cellular ANXA1 was significantly decreased after 30 min (359.1 ± 16.1 AU) of incubation with $E_2\beta$ relative to control untreated cells (488.8 ± 33.6 AU; $p < 0.01$). After 2 h (518.9 ± 35.1 AU) and 4 h (568.2 ± 13.8 AU) of incubation with $E_2\beta$, the cellular con-

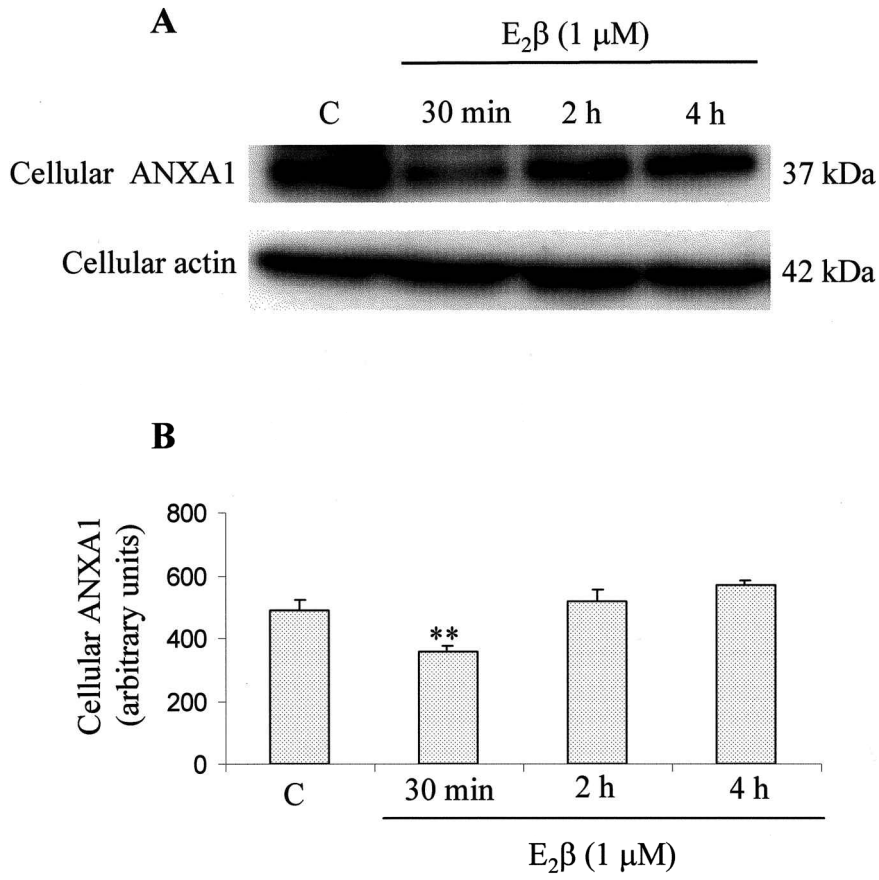

FIG. 4. Effect of E2β on cellular ANXA1 protein expression in CCRF-CEM cells. Cells were incubated in the absence (control, C) or in the presence of $E_2\beta$ (1 μM), for 30 min, 2 h and 4 h. The cellular content of ANXA1 was determined by Western blot analysis (A), and the bands were quantified with an image analyser (B). Detection of cellular ANXA1 protein was achieved using a mouse monoclonal anti-ANXA1 antibody. The data shown are an average of three independent experiments. Values are mean±SD, ** $p < 0.01$ as determined by one-way ANOVA with Dunnett's post test.

tent of the protein was increased, reaching levels statistically equivalent to the control (488.8 ± 33.6 AU; $p > 0.05$). Since cell viability was well maintained throughout the experiments (data not shown), the loss in cellular ANXA1 after 30 min of exposure to $E_2\beta$ is certainly due to the specific secretion of the protein. In fact, the level of the plasma membrane-bound EDTA-extractable ANXA1 increased from 184.1 ± 29.2 AU (control) to 378.8 ± 29.9, 584.3 ± 79.3 and 607.3 ± 47.1 AU in cells treated with $E_2\beta$ for 30 min, 2 h and 4 h, respectively ($p < 0.05$, $p < 0.01$ and $p < 0.01$) (Fig. 5). Taken together, these results indicate that $E_2\beta$ induces *de novo* synthesis of ANXA1 and stimulates its secretion in CCRF-CEM cells.

Co-incubation of CCRF-CEM cells with $E_2\beta$ (1 μM) and the estrogen antagonist ICI 182,780 (10 μM) was performed to investigate the role of the ER in the effect of $E_2\beta$ on the synthesis and secretion of ANXA1. ICI 182,780 neither affected the amount of plasma membrane-bound ANXA1 in control cells or reversed the $E_2\beta$-dependent secretion of ANXA1 in CCRF-CEM cells (data not shown).

Discussion

Several studies reported in the literature have shown that glucocorticoid hormones have a remarkable effect on ANXA1 protein expression and secretion in a variety of *in vivo* and *in vitro* systems.[1,4,5] But, to our knowledge, this is the first study demonstrating that the sex steroid hormone $E_2\beta$ stimulates the synthesis and the secretion of ANXA1.

Incubating the lymphoblastic CCRF-CEM cells with $E_2\beta$ (1 μM) for 30 min resulted in an increased expression of ANXA1 mRNA (Fig. 2). When the cells were incubated with this hormone for 2 h, the synthesis of ANXA1 mRNA molecules was followed by an increase in the cellular content of ANXA1 protein (Fig. 4). Moreover, treatment of CCRF-CEM cells with $E_2\beta$ (1 μM) for 30 min caused a significant increase in the amount of ANXA1 bound to specific ANXA1 binding receptors localised in the outer surface of the plasma membrane of these cells (Fig. 5). These results are in agreement with those published by Philip *et al.*[16] They reported that washing the cells in a Ca^{2+}-free medium removes the ANXA1 from the outer

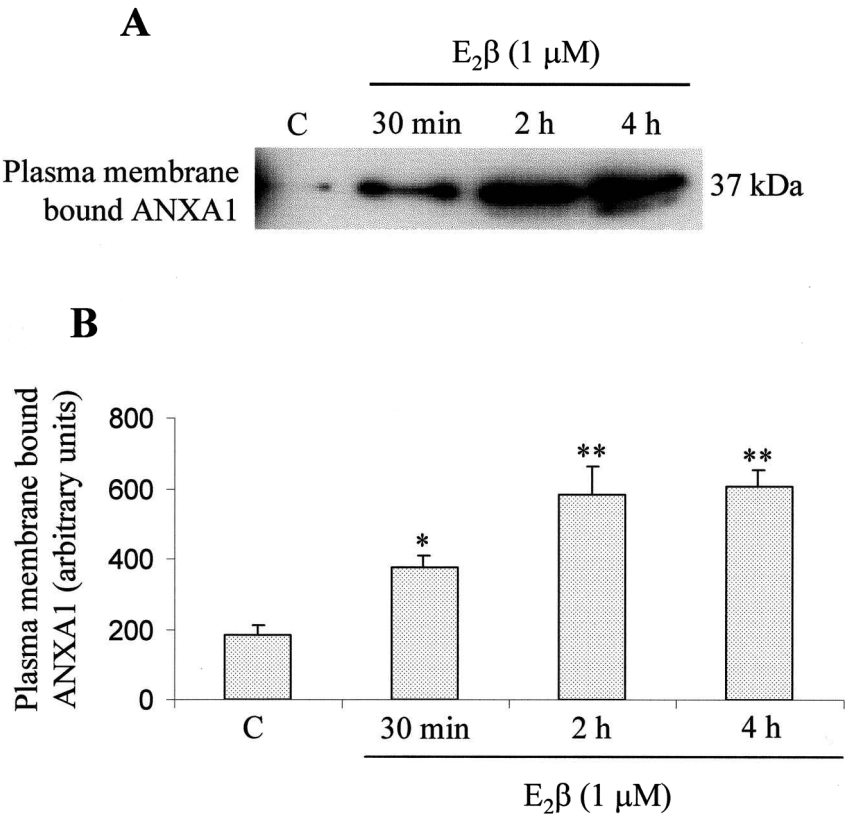

FIG. 5. Effect of E2β on ANXA1 secretion in CCRF-CEM cells. Cells were incubated in the absence (control, C) or in the presence of $E_2\beta$ (1 μM) for 30 min, 2 h and 4 h (A). The bands were quantified with an image analyser (B). Plasma membrane bound ANXA1 was obtained after washing the cells with 1 mM EDTA buffer, as described in 'Materials and methods'. Detection of plasma membrane bound ANXA1 was achieved using a mouse monoclonal anti-ANXA1 antibody. The data shown are an average of three independent experiments. values are mean±SD, * $p < 0.05$ and ** $p < 0.01$ as determined by one-way ANOVA with Dunnett's post test.

surface of the plasma membrane of the cells, and that this protein corresponds to the externalised ANXA1. Based on these findings, our results indicate that $E_2\beta$ efficiently stimulates ANXA1 secretion.

The mechanisms by which $E_2\beta$ stimulates the synthesis and the secretion of ANXA1 are still unknown. It is known that $E_2\beta$ exerts its major effects on cell growth, differentiation and function by specifically interacting with intracellular ER.[17] The complex $E_2\beta$-ER migrates to the nucleus of the cells, where it binds to estrogen-responsive elements present in the genomic DNA. The anti-estrogens compete with the hormone ($E_2\beta$) for the estrogen receptor, giving rise to inactive ligand–receptor complexes.[18] For example, ICI 182,780 binds with high affinity to intracellular ER, inhibiting its dimerisation, and functions as a pure $E_2\beta$ antagonist.[19,20]

In this work, we used ICI 182,780 (10 μM) to investigate the involvement of the intracellular ER on ANXA1 expression and secretion induced by $E_2\beta$ (1 μM). Our results show that ICI 182,780 did not inhibit the $E_2\beta$-induced stimulation of ANXA1 mRNA expression (Fig. 3). We also observed that ICI 182,780 was without effect on the synthesis and secretion of ANXA1 protein induced by $E_2\beta$ (data not shown). Taken together, these results suggest that the intracellular ERs are probably not involved in mediating the $E_2\beta$ effects reported here. Receptor-independent mechanisms have been suggested to mediate the response of estrogen in different cell types, in a manner dependent on the culture conditions and estrogen concentrations.[21] Nanomolar concentrations of estrogen seem to exert their effects by a direct genomic action of the complex estrogen-ER on DNA, activating specific estrogen-responsive genes,[17] whereas micromolar concentrations of this hormone appear to also activate other intracellular signaling pathways, like the mitogen-activated protein kinase cascade.[21–23] It is worth noticing that, in our study, the concentrations of $E_2\beta$ that reproducibly induced ANXA1 expression and secretion are in the micromolar range, thus further supporting that the $E_2\beta$ effects observed are independent of the ER. Nevertheless, it could be argued that other ER antagonists, such as tamoxifen citrate, as well as higher concentrations of ICI 182,780 should be used to fully characterise the involvement of ER in the $E_2\beta$-induced responses. Unfortunately, we found that tamoxifen citrate and ICI 182,780 are toxic to CCRF-CEM cells in concentrations higher than 1 and 10 μM, respectively

(Fig. 1), which precluded the performance of those studies. Thus, we cannot fully exclude the involvement of the ER in mediating $E_2\beta$-induced ANXA1 expression and secretion, although it seems highly unlikely.

In summary, the synthesis and secretion of ANXA1 in CCRF-CEM cells in response to $E_2\beta$ (1 μM) is consistent with *in vivo* studies showing that $E_2\beta$ in physiological concentrations (1 pM–100 μM) participates in the regulation of anti-inflammatory events.[9,24,25] Although the results from our studies suggest that $E_2\beta$ acts by an ER-independent pathway, further studies are being performed to better understand the mechanisms by which this hormone regulates the expression of ANXA1 in the lymphoid lineage.

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